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Calpain Methods and Protocols

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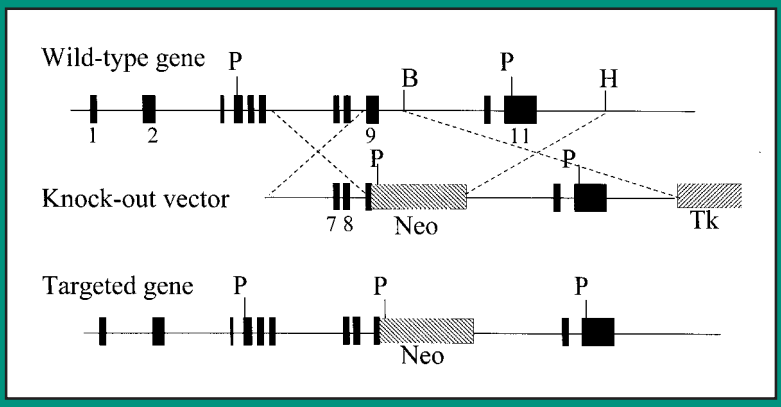

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Glossary of Abbreviations

BSA	bovine serum albumin
DEAE	diethylaminoethyl-
DMSO	dimethyl sulfoxide
DTE	dithioerythritol
DTT	dithiothreitol
E-64	trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetra-acetic acid
EGTA	ethylene glycol-bis[β-aminoethylether] *N,N,N',N'*-tetra-acetic acid
FITC	fluorescein isothiocyanate
FPLC	fast protein liquid chromatography
HEPES	*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethane sulfonic acid]
IPTG	isopropyl-D-thiogalactoside
2-ME	2-mercaptoethanol
MES	2-[*N*-morpholino]ethane sulfonic acid
MOPS	3-[*N*-morpholino]propane sulfonic acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PIPES	piperazine-*N,N'*-bis[2-ethane sulfonic acid]
PMSF	phenylmethane sulfonyl fluoride
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid

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Purification of μ-Calpain, m-Calpain, and Calpastatin from Animal Tissues

Valery F. Thompson and Darrel E. Goll

1. Introduction

This chapter describes procedures for purification of μ-calpain, m-calpain, and calpastatin simultaneously from a single tissue sample. The procedures and reagents described apply equally well to purification of any one or two of these three proteins from an appropriate tissue with a corresponding decrease in effort. The series of steps and columns that can be employed permits many variations, but at the outset there are some important initial choices to be made concerning tissue source, means of homogenization, and initial steps of fractionation.

A particular tissue may be a poor source of μ-or m-calpain, or of calpastatin, so the choice of tissue is important. In some tissues and cells the calpains seem to be associated with subcellular structures, probably the cytoskeleton or the plasma membrane or both, and vigorous homogenization is required to extract them in high yield. In the presence of Ca^{2+}, the calpains autolyze quickly, even at low temperatures, and the homogenization buffer must include a Ca^{2+} chelator such as EDTA or EGTA at concentrations of 1 to 10 m*M* to prevent autolysis. Also, calpastatin seems to be very susceptible to proteolytic degradation, and it is necessary to include a complete cocktail of protease inhibitors in the homogenization buffer to avoid calpastatin degradation.

The initial fractionation method depends to a large extent on the scale of the purification. For small-scale purifications (~100–200 mL of extract), it is most convenient to load the extract directly onto the first column. For larger scale purifications (~200 to 6,000–9,000 mL), it may be more efficient to start with ammonium sulfate precipitation. For an even larger scale, when ammonium

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sulfate precipitation can become tedious, the extract may be stirred with an ion-exchange resin to allow the calpains and calpastatin in the extract to bind to the resin. The resin with the bound calpains and calpastatin is then washed, filtered, resuspended in buffer, and packed into a column.

Neither the calpains nor calpastatin are easy to purify, and several column chromatographic steps are required. The first step is nearly always an anion-exchange column, usually with a diethylaminoethyl (DEAE) functional group. Occasionally, a hydrophobic column (phenyl functional group) has been used as the first step, but it is less convenient to work with large hydrophobic columns than with large ion-exchange columns, and hydrophobic chromatography media are generally more expensive than ion-exchange media (with the exception of some of the new synthetic organic ion-exchange matrices, which are quite expensive).

Anion-exchange chromatography separates m-calpain from μ-calpain and calpastatin and, because of the occurrence of different forms of calpastatin, may or may not separate μ-calpain and calpastatin. Following the ion-exchange step, phenyl-Sepharose chromatography is most appropriate because samples eluted from the anion-exchange column can be loaded directly onto phenyl-Sepharose columns with little or no further manipulation. If phenyl-Sepharose chromatography has been used as the first step, it should be followed by anion-exchange chromatography, again because no additional sample manipulation is required. Phenyl-Sepharose chromatography separates calpastatin cleanly from μ- and m-calpain, but does not cleanly separate the two calpains.

Even after anion-exchange and phenyl-Sepharose chromatography, the calpain and calpastatin are very impure, and two to three additional chromatographic steps are needed to purify these proteins to 85–95% homogeneity. We present here step-by-step protocols for purification of each of these three proteins, but the choice and order of the many possible steps can vary, and some options and alternatives are mentioned along the way. The protocols were designed to minimize the number of salting-out or concentration steps and hence decrease the time required to purify the calpains and calpastatin and the losses that accompany these extra steps. The possibilities for affinity purification of calpains are described elsewhere in Chapters 4 and 5.

2. Materials

The choice of tissue or cells depends on the proteins required. The approximate quantities of μ-calpain, m-calpain, and calpastatin in several different tissues and cells are listed in **Table 1**. Kidney and skeletal muscle from several different species contain reasonable amounts of both μ-and m-calpain. Cardiac muscle contains significant quantities of m-calpain and calpastatin *(2,5,7)*, and smooth muscle contains m-calpain, but very little μ-calpain. Human placenta

Table 1
Approximate Relative Proportions of μ-Calpain, m-Calpain and Calpastatin in Different Tissues[a]

Tissue	μ-Calpain	m-Calpain	Calpastatin	Ref.
Ovine skeletal muscle	3–5	3–5	0.6–0.9	*1,2*
Chicken skeletal muscle	1–4	4–7	ND	*1,2*
Bovine cardiac muscle	0.15–0.30	3–6	7–12	*1,2*
Turkey gizzard smooth muscle	0–0.2	10–15	10–14	*1,2*
Human platelets	60–100	0–0.1	40–100	*1,2*
Bovine platelets	0–0.1	60–100	ND	*1,2*
Bovine kidney	0–2	8–16	ND	*1,2*
Bovine brain	0–1	2–5	ND	*1,2*
Ovine skeletal muscle	1.07	1.06	3.56	*3,4*
Rat liver	2.14	13	39.6	*5,6*
Rat kidney	11.1	21.4	ND	*5,6*
Rat brain	0.12	8.35	3.30	*5,6*
Rat lung	0.88	20.1	23.9	*5,6*
Rat submandibular gland	59.4	27	ND	*5,6*
Rat adipose tissue	1.97	5.42	6.93	*5,6*
Rat skeletal muscle	3.61	2.89	9.35	*5,6*
Rat cardiac muscle	3.37	10.5	30.7	*5,6*
Rat erythrocytes	11.3	0	8.06	*5,6*
Rat spleen cells	2.63	2.54	3.01	*5,6*
Rat thymus cells	1.35	1.02	2.96	*5,6*

[(a)] ND, not determinded. The units in the table vary. For results from references *1* and *2,* the units are milligrams of purified protein per kilogram of wet weight of tissue. For results from references *3* and *4,* the units are activity per gram of wet weight of tissue; and for results from references *5* and *6*, the units are units of activity per 5 g wet weight of tissue. The differences in assays and procedures make it extremely difficult to compare absolute values between laboratories, although the ratios reported from different laboratories between different components are often similar.

is the only readily accessible tissue from which milligram quantities of both human μ-and m-calpain can be obtained ***(8)***. Human platelets and erythrocytes contain μ-calpain and calpastatin but little or no m-calpain. The calpastatin in human erythrocytes is a truncated 46-kDa form that differs from the larger 70–85 kDa form present in most other tissues ***(9,10)***; the calpastatin in human platelets has not yet been well characterized. The calpastatin in human placenta also has not been well characterized but seems to be a mixture consisting largely of the smaller 46-kDa erythrocyte type, which may originate partly from the blood in placental tissue, and of several other types. Tissues can be stored at –20 to –85°C before the calpains are

extracted with no evident effect on calpain extractability or activity but freezing purified calpain results in loss of activity ***(4)***.

1. Stock solutions of inhibitors: 2.5 m*M* E-64 in water, stored at –20°C; 0.4 *M* PMSF in ethanol. These are added to buffers immediately before use (*see* **Notes 1** and **2**).
2. Homogenization buffer ***(2,13)***: 20 m*M* Tris-HCl, pH 8.0, 5 m*M* EDTA, 0.1% 2-ME, 0.1 mg/mL ovomucoid containing ovoinhibitor (Sigma Type II-0, partly purified) 2.5 μ*M* E-64, 0.2 m*M* PMSF (*see* **Note 3**).
3. A coarse, water-hardened filter paper: We use Whatman 541.
4. TEM: 20 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, 0.1% 2-ME (*see* **Note 4**).
5. TEMA: 20 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, 0.1 % 2-ME, 1 m*M* sodium azide (NaN_3.)
6. 20 m*M* Tris-MES, pH ≤ 7.0 (*see* **Note 5**).
7. DEAE-cellulose (Whatman, Inc., Fairfield, NJ) stored at 4°C with 0.02% benzalkonium chloride, and recycled after each use in accordance with the manufacturer's instructions. Sodium azide is not used here, since it binds to anion exchange resins.
8. Phenyl–Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) stored at 4°C with 5 m*M* NaN_3 (*see* **Note 6**).
9. Butyl–Sepharose (Amersham Pharmacia Biotech) or butyl-TSK (Supelco, Inc., Bellefonte, PA) (*see* **Note 6**).
10. Sephacryl S-300 (Amersham Pharmacia Biotech) (*see* **Note 7**).
11. TMAE 650(S) tentacle (EM Science, Gibbstown, NJ) stored at 4°C with 0.02% benzalkonium chloride and recycled according to the manufacturer's instructions (*see* **Note 8**).
12. DEAE-TSK (ToyoPearl) 650S (Supelco, Bellefonte, PA) (*see* **Note 9**).
13. Reactive Red agarose 120 (Sigma) or TSK AF-Red ToyoPearl 650 resin (Supelco) (*see* **Note 10**).
14. Ultrapure ammonium sulfate.
15. Ultrapure Tris base (*see* **Note 11**).

3. Methods

All homogenization and subsequent column chromatography steps are done in the cold room at 2–4°C using precooled buffers and instruments to decrease the extent of proteolysis either by calpain itself or by contaminating non-calpain proteases.

3.1. Homogenization

1. Chop and rinse large pieces of tissue in ice-cold buffer to remove as much blood as possible.
2. Grind muscle or other fibrous or tough tissues in a precooled meat grinder before homogenization. Soft tissues such as liver can be cut into slices and placed

directly in the homogenizer.

3. Homogenize chopped soft tissue or minced tissue in 6 mL of homogenization buffer per gram wet weight of tissue in a Waring blender at top speed (18,000 rpm) using two 20-s bursts separated by a 20-s cooling period. Other blenders or homogenizers such as Polytron or Virtis are equally effective, but vigorous homogenization is essential to achieve high recoveries of activity ***(16)*** (*see* **Note 12**).
4. Recover cells such as platelets or erythrocytes by centrifugation in ice-cold PBS or appropriate buffer.
5. Lyse the cells by suspension in homogenization buffer or similar low ionic strength buffer containing protease inhibitors, and centrifuge to remove cell debris.

3.2. Preparation of the Initial Extract and Application to DEAE-Cellulose

1. Centrifuge the homogenate at 15,000-20,000*g* (max) for 15–20 min, or longer if necessary, to form a firm pellet so that the supernatant can be decanted.
2. Filter the supernatant through glass wool to remove floating lipid and other small particles to obtain a clarified extract.
3. For supernatant volumes less than 200–300 mL, apply the clarified extract directly onto a DEAE-cellulose column (~200 mL of packed DEAE-cellulose), making sure that the ionic strength is sufficiently low, so that calpastatin, which begins eluting at 40–80 m*M* salt at pH 7.5, can bind to the column (*see* **Note 13**).
4. For supernatant volumes of 200 mL to a few liters, precipitate the calpastatin and calpain at 50% ammonium sulfate saturation.
5. Collect the precipitated protein by centrifugation at 15,000–20,000*g* for 10 min, redissolve it in TEM, dialyze against several changes of TEM, and clarify by centrifugation at 100,000*g* for 90 min.
6. Apply this solution to a DEAE-cellulose column (~500–600 mL of packed DEAE-cellulose).
7. For very large preparations (greater than 10 L of supernatant), stir the extract at very low ionic strength with DEAE cellulose (~3 L of packed DEAE-cellulose; in general, use 1 mL of packed DEAE-cellulose for every 10 mg of protein loaded) with an overhead stirrer at high speed for 2–3 h before filtering the resin/extract suspension through Whatman No. 541 filter paper. The filtrate is saved until it is confirmed that calpastatin and both calpains have been eluted from the first column (i.e., that all three proteins bound to the resin).
8. Wash the DEAE-cellulose cake with two volumes of TEM to remove all unbound protein, suspend it in TEM, and pour the suspension into a column (5×60 cm or larger) (*see* **Note 14**).

3.3. The First Two Columns

This section describes the first and second column chromatographic steps on DEAE-cellulose and phenyl-Sepharose which separate calpastatin, μ-calpain,

and m-calpain. The following sections describe further purification of each of these proteins. The size of the columns is influenced by the scale of the purification as described in **Subheading 3.2.** In all cases of gradient elution, the combined volume of the elution gradient buffers is 4–6 times the volume of the packed resin in the column. Flow rates vary with column size: 20–40 mL/h for a column that is 1.6 cm in diameter; 35–60 mL/h for a column that is 2.6 cm in diameter, and 120–180 mL/h for a column that is 5.0 cm in diameter. These flow rates are slower than often recommended by manufacturers because equilibration is slower at cold-room temperatures.

3.3.1. First Column: DEAE-Cellulose

1. After applying the clarified extract to the DEAE-cellulose column, elute the bound proteins with a linear gradient from 0 to 500 m*M* KCl in TEM. Calpastatin elutes first at 40–110 m*M* KCl, depending on the tissue or cell source; μ-calpain elutes between 90 and 180 m*M* KCl, and m-calpain elutes at ~180–320 m*M* KCl (*see* **Note 15**).
2. As an alternative, it is possible to use a phenyl-Sepharose column as the first step (*see* **Note 16**).

3.3.2. Second Column: Phenyl-Sepharose

The calpastatin, μ-calpain, and m-calpain fractions eluted from the initial anion-exchange column are loaded onto three different phenyl-Sepharose columns (or two different columns if the calpastatin/μ-calpain fractions are collected together).

3.3.3. Calpastatin

1. Establish which fractions from the DEAE-cellulose column contain calpastatin either by an immuno dot blot assay with anti-calpastatin antibodies, by assay of inhibition of a standard calpain preparation, or by an estimate on the basis of previous experience.
2. Pool these fractions and adjust to 1 *M* ammonium sulfate (23% saturation) by adding solid ammonium sulfate: add the ammonium sulfate slowly to prevent precipitation of μ-calpain.
3. Centrifuge at 15,000 - 20,000*g* for 10 min to remove any protein that may have precipitated. This pellet should not contain either calpastatin or μ-calpain.
4. Apply the supernatant to a phenyl-Sepharose column (~1.6 × 20–30 cm for samples smaller than 60 g, initial weight; ~2.6 × 20–30 cm for 60 to 300 g samples; and ~5.0 × 20–60 cm for 300 to 3000 g samples) equilibrated with 1 *M* ammonium sulfate, TEMA.
5. Elute the column with a descending linear gradient from 1 *M* to 0 ammonium sulfate in TEMA, followed by washing with 1 m*M* EDTA, 0.1% 2-ME. The large calpastatin isoforms (70–85 kDa) bind to phenyl-Sepharose in 1 *M* ammonium

sulfate and are eluted at 0.7– 0.5 *M* ammonium sulfate (*see* **Note 17**). Any μ-calpain that had coeluted with calpastatin from DEAE-cellulose is eluted from phenyl-Sepharose in 1 m*M* EDTA, 0.1% 2-ME. This μ-calpain should be combined with the μ-calpain recovered from the "μ-calpain" phenyl-Sepharose column (**Subheading 3.3.4.**).

3.3.4. μ-*Calpain (See* **Note 18***.)*

1. Establish which fractions from the DEAE-cellulose column contain μ-calpain, either by immunodetection, by assay of calpain activity, or by casein zymogram, or simply pool all fractions in the appropriate section of the gradient.
2. Load fractions containing μ-calpain activity eluted from DEAE-cellulose onto a phenyl-Sepharose column (size depends on size of original sample; ~1.6 x 25-30 cm for samples smaller than 60 g tissue; 2.6 × 25–30 cm for 60–300 g samples; and 5 × 30–60 cm for 300–3,000 g samples) that has been equilibrated in 125 m*M* KCl, TEMA.
3. Wash the column with 125 m*M* KCl, TEMA until OD 280 nm of the effluent returns to baseline, and elute μ-calpain with 1 m*M* EDTA, 0.1% 2-ME. Any calpastatin in the μ-calpain fractions after the DEAE step is not retained by phenyl-Sepharose in 125 m*M* KCl, and this calpastatin can be collected and combined with other calpastatin samples.

3.3.5. m-*Calpain*

1. Load fractions containing m-calpain activity eluted from DEAE-cellulose onto a phenyl-Sepharose column (size depends on size of the original sample; columns slightly smaller than those used for μ-calpain can be used here) that has been equilibrated in 125 m*M* KCl, TEMA.
2. Wash the column with 125 m*M* KCl, TEMA until OD 280 nm returns to baseline, and elute m-calpain with 1 m*M* EDTA, 0.1% 2-ME.

3.4. Further Purification of Calpastatin

1. Precipitate calpastatin (from **Subheading 3.3.3.5.** and/or **3.3.4.3.**) at 65% ammonium sulfate saturation, redissolve the precipitate in a minimum volume of TEM required to dissolve the precipitate and clarify the solution by centrifugation at 100,000*g*.
2. Load the clarified supernatant onto a Sephacryl S-300 column (size of column should be such that the volume loaded is less than 10% of the total column volume) and elute the column with TEMA. Calpastatin elutes earlier from this column than most of the remaining impurities because it has an unordered conformation and therefore a large Stokes radius ***(21)***.
3. Assay the fractions to determine the position of the eluted calpastatin. Calpastatin contains no Trp residues, so that purity of the calpastatin can be estimated by the presence of high calpastatin activity in fractions having very low absorbance at 280 nm.

4. Collect the fractions containing calpastatin activity, and load them directly onto an anion-exchange column (use a TMAE-tentacle or a DEAE-TSK column to obtain maximum resolution; a 1.6 × 20–30 cm column for initial samples up to 4–6 kg; smaller columns for smaller initial samples).
5. Elute calpastatin with a shallow gradient from 20 to 125 m*M* KCl in TEM.
6. Various attempts at immunoaffinity purification of calpastatin have not succeeded, even though the resistance of calpastatin to denaturing agents suggests that this should be a useful method.

3.5. Further Purification of μ-Calpain *(See* **Note 19.***)*

1. Add solid ammonium sulfate to 0.8 M to the μ-calpain fraction following anion-exchange and phenyl-Sepharose chromatography (**Subheading 3.3.4.3.**), and centrifuge the solution at 15,000–20,000*g* for 10 min to remove any protein that may have precipitated.
2. Load the solution onto a butyl-Sepharose column that has been equilibrated with 0.8 *M* ammonium sulfate in TEMA, and wash the column with 0.8 *M* ammonium sulfate, TEMA until OD 280 nm of the eluate returns to baseline (*see* **Notes 20** and **21**).
3. Elute the column with a descending linear gradient from 0.8 *M* to zero ammonium sulfate in TEMA. μ-Calpain elutes between 0.7 and 0.2 *M* ammonium sulfate.
4. Pool the fractions containing μ-calpain and dialyze against 20 m*M* Tris-HCl, pH 8.5, 1 m*M* EDTA, 0.1% 2-ME.
5. Load the dialyzed μ-calpain solution onto a TMAE tentacle column (a 1.6 × 25–30 cm column or smaller if the initial sample was small) that has been equilibrated with 20 m*M* Tris-HCl, pH 8.5, 95 m*M* KCl, 1 m*M* EDTA, 0.1 % 2-ME *(see* **Note 22**).
6. Wash the column with this buffer until OD 280 nm has returned to baseline.
7. Elute the column with a linear gradient from 95 to 200 m*M* KCl in 20 m*M* Tris-HCl, pH 8.5, 1 m*M* EDTA, 0.1% 2-ME. μ-Calpain elutes early in this gradient at 100–140 m*M* KCl.
8. Pool the fractions containing μ-calpain activity and dialyze briefly against 20 m*M* Tris-MES, pH 6.5, 1 m*M* EDTA, 0.1% 2-ME to reduce the KCl concentration to less than 20 m*M*.
9. Load this solution onto a DEAE-TSK column (a 1.6 × 15–30 cm column or smaller columns for smaller initial sample sizes) that has been equilibrated with 20 m*M* Tris-MES, pH 6.5, 40 m*M* KCl, 1 m*M* EDTA, 0.1% 2-ME.
10. Wash the column with the same buffer until OD 280 nm of the eluant returns to baseline.
11. Elute the column with a linear gradient from 40 to 135 m*M* KCl in 20 m*M* Tris-MES, pH 6.5, 1 m*M* EDTA, 0.1 % 2-ME. μ-Calpain elutes as two or three closely spaced peaks of activity between 45 to 85 m*M* KCl. Some (50–60 %) but not all of the μ-calpain recovered from the DEAE-TSK column is pure (>90 %), and recycling the impure fractions back through the TMAE tentacle and DEAE-TSK columns eventually results in purification of most of the μ-calpain.

3.6. Further Purification of m-Calpain

1. Add solid NaCl to 0.5 *M* to the m-calpain fraction following anion-exchange and phenyl-Sepharose chromatography (**Subheading 3.3.5.2.**).
2. Load this solution on to a Reactive Red column (2.6 × 25–30 cm column for initial tissue samples smaller than 300 g; 5.0 × 10–30 cm column for samples larger than 300 g) that has been equilibrated in 0.5 *M* NaCl, TEMA (*see* **Note 23**).
3. Wash the column with 0.5 M NaCl, TEMA until OD 280 nm of the eluant returns to baseline. Elute m-calpain with 20 m*M* Tris-HCl, pH 8.5, 1 mM EDTA, 0.1% 2-ME (*see* **Note 24**).
4. Pool the m-calpain-containing fractions and load them onto a DEAE-TSK column (1.6 × 20–30 cm or smaller for initial tissue samples less than 300 g) that has been equilibrated with 20 m*M* Tris-MES, pH 6.5, 135 m*M* KCl, 1 mM EDTA, 0.1% 2-ME.
5. Wash the column with the same buffer, and elute with a linear gradient from 135 to 275 m*M* KCl in 20 m*M* Tris-MES, pH 6.5, 1 m*M* EDTA, 0.1% 2-ME. Approximately 80% of the m-calpain eluted from the DEAE-TSK column is pure (>90%), and much of the remaining 20% can be purified by rechromatography on DEAE-TSK.
6. Approximately 2–3 mg of reasonably pure μ-calpain or m-calpain and approximately 1 mg of reasonably pure calpastatin can be obtained from 1 kg of bovine skeletal muscle by using the procedures described here (**Fig. 1**).

The purified calpains are stable at 20–25°C ***(11)*** for 2–8 h if they are in 5–10 m*M* EDTA or EGTA. They are denatured by freezing in the absence of cryoprotectant, but can be stored at –20°C with 30% glycerol or 10% sucrose. Calpastatin is stable over a wide range of pH values (1–13), temperatures (not affected by freezing, stable up to 95°C), and denaturing solvents (full activity can be recovered after exposure to 8 *M* urea or 6 *M* guanidine).

SDS-PAGE of calpastatin, μ-calpain, and m-calpain purified from bovine skeletal muscle shows that calpastatin and m-calpain are 90–95% pure after the procedures described. However, μ-calpain still contains some contaminating polypeptides that can be removed by recycling through the TMAE and DEAE-TSK columns and selecting the purest fractions.

4. Notes

1. E-64 is an irreversible inhibitor of cysteine proteases including the calpains, but does not react with calpain in the presence of EDTA ***(11)***. E-64 is a particularly effective inhibitor in calpain preparations because it inhibits all cysteine proteases except the calpains.
2. PMSF has a half-life in aqueous solution at 25°C and pH 8.0 of 35 min ***(12)***, and a stock solution of 0.4 *M* PMSF in 100% ethanol must be prepared. PMSF is toxic and can be fatal if inhaled, if ingested, or if it comes in contact with the

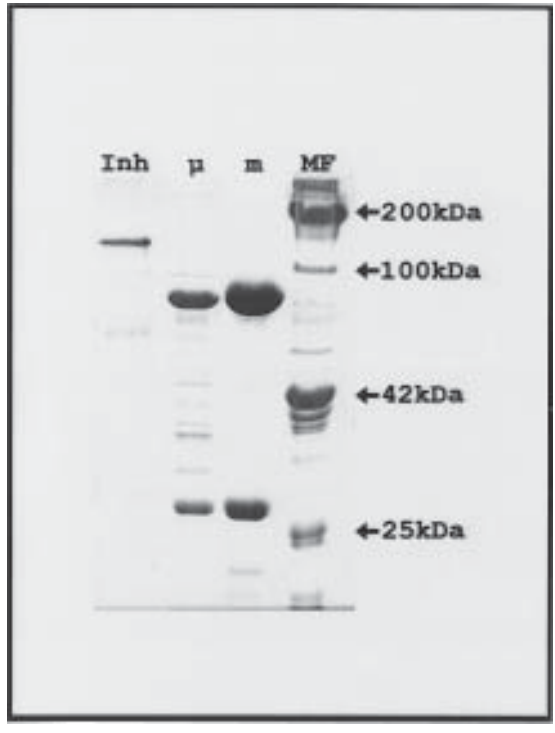

Fig. 1. SDS-polyacrylamide gel showing calpastatin (*lane 1*, Inh), μ-calpain (*lane 2*, μ), and m-calpain (*lane 3*, m). All three proteins were purified from bovine skeletal muscle. *Lane 4* contains myofibrils purified from bovine cardiac muscle and used as molecular weight markers. Lanes were loaded with 5 μg (Inh and m-calpain), 2.5 μg (μ-calpain), or 10 μg (myofibrils) of protein. Only the ~125 kDa (in SDS-PAGE) isoform of calpastatin is obtained from bovine skeletal muscle. The m-calpain sample seems pure even at the very large amounts of protein loaded on this gel.

skin; use gloves and take the proper precautions.

3. These inhibitors and conditions combine to inhibit aspartate proteases (high pH), serine proteases (PMSF and ovomucoid inhibitors), cysteine proteases (E-64), and metalloproteases (EDTA).
4. We use the free acid form of EDTA and adjust pH of the EDTA with Tris base to avoid the inclusion of Na^+ or K^+. DTT or DTE or other sulfhydryl reducing agents such as cysteine will work as well as 2-ME. DTT is more expensive but less offensive in odor than 2-ME.
5. Tris alone is a poor buffer below pH 7.5. MES has a pK_a of 6.15 at 20°C. The 20 m*M* Tris-MES buffer is made by titrating Tris to a selected pH with MES, so the solution is 20 m*M* Tris, but the concentration of MES depends on the pH.
6. Phenyl-Sepharose and butyl-Sepharose are stable for several years if stored in the cold room at 4°C in the presence of 5 m*M* NaN_3. They are regenerated in the

column by washing successively with: 1.5 column vol of 5% SDS; 1 vol of H_2O; 2 vol of 100% ethanol; 2 vol of *n*-butanol; 1.5 vol of 100% ethanol; and 10 vol H_2O. Flushing with ethanol and butanol removes water from the Sepharose beads, so we flush the column with 2 vol H_2O and then repack it to remove all the "air pockets" before flushing with the remaining 8 vol H_2O. We have reused phenyl-Sepharose over 50 times after such regeneration with no detectable loss in performance.

7. Other size exclusion matrices may be effective, but it is important to use a matrix with large pore size because of the large Stokes radius of calpastatin.
8. This is a small-diameter (25–40 μm) polymethyl acrylate matrix bead with a quaternary amine functional group attached via a long polymer chain averaging ~30 methyl acrylate units.
9. This is a small-diameter (20–50 μm) polyvinyl matrix bead with the tertiary amine DEAE functional group. DEAE-TSK columns are regenerated simply by washing with 2 column vol of 0.5 *M* NaOH followed by 3 vol of equilibration buffer.
10. Reactive Red agarose can be prepared in house to reduce expense ***(14)***. The cost to make 200 mL of Reactive Red agarose is $3 for the dye and $85 for the agarose (Sepharose CL-4B) compared with $300 for an equal amount prepared commercially. Reactive Red agarose is regenerated in the column using the same protocol as described for phenyl-Sepharose (*see* **Note 6**). We have reused this column over 20 times without loss of performance. The dye linkage is stable ***(15)***, and the columns can also be cleaned with 0.5 *N* NaOH after the usual regeneration. We have been unsuccessful in regenerating Reactive Red columns with solutions containing only 2–5 *M* urea or 2–4 *M* NaCl or any combination of these. It appears that the red color eluted from Reactive Red columns is due to breakage of glycosidic bonds in the agarose matrix, and not to leakage of the dye from the matrix.
11. The pH of Tris buffers is routinely adjusted at room temperature, but is ~0.4 pH unit higher at 4°C.
12. Homogenization must be carried out at low ionic strength if the extract will be loaded directly onto an anion exchange column ***(13)***. If the extract is to be concentrated by ammonium sulfate precipitation, or if the extract will be loaded onto phenyl-Sepharose, the homogenization may include 100 m*M* KCl. This may increase extraction of calpains and calpastatin, but also solubilizes other cellular proteins, which will make purification more difficult (this is true especially for muscle tissue).
13. E-64 does not bind to the DEAE-cellulose, and will inhibit assays of calpain activity in the nonretained fractions.
14. Batch absorption onto DEAE-cellulose typically provides less resolution than application of the extract to a prepacked column.
15. These KCl values depend on the pH of the eluting buffer, but are nearly constant for calpains from different tissues. The different calpastatin isoforms from different tissues (9,17) vary slightly in elution position, but calpastatin elution usually overlaps m-calpain elution to some extent.
16. If a phenyl-Sepharose column is used as the first chromatographic step, adjust the clarified extract to ~150 m*M* KCl by adding solid KCl, and load this solution onto a phenyl-Sepharose column previously equilibrated in 150 m*M* KCl in

TEMA. If the protein has been precipitated with ammonium sulfate, dissolve the pellet in 150 m*M* KCl in TEM, clarify the solution by centrifugation, and apply it to the phenyl-Sepharose column. Calpastatin does not bind under these conditions and can be recovered from the nonretained fractions by 65% ammonium sulfate precipitation for further purification. The calpains are eluted from phenyl-Sepharose in 1 m*M* EDTA, 0.1% 2-ME, and would then normally be applied to an anion exchange column.

17. Calpastatins that are smaller than 90 kDa (on SDS-PAGE; less than 50 kDa actual molecular weight) such as the 70 kDa (SDS-PAGE; 46 kDa actual molecular weight) form found in mammalian erythrocytes do not bind to phenyl-Sepharose columns, even in the presence of 1 *M* ammonium sulfate, and pass straight through this column. To purify these smaller calpastatin isoforms, it is necessary to collect the eluant that does not bind to the phenyl-Sepharose, salt it out at 65% ammonium sulfate saturation, and solubilize the precipitated protein. Clarify the dissolved protein at 100,000*g* for 30 min, and load the clarified supernatant onto a Sephacryl S-300 column. Collect the tubes containing calpastatin activity from this column, heat the pooled fractions at 95°C for 5 min (it may be necessary to concentrate the calpastatin before heating), and sediment the undesired heat-precipitated protein at 20,000–30,000g for 20 min. The supernatant is loaded directly onto a DEAE-TSK anion-exchange column and eluted with a shallow 20–125 m*M* KCl gradient.
18. Elution of the calpains from phenyl-Sepharose and butyl-Sepharose is less consistent and predictable than it is from anion-exchange columns ***(18)***. One factor seems to be the ratio of the total quantity of protein loaded to the quantity of phenyl-Sepharose. If this ratio is small or if the calpains have been partly purified before being loaded onto the phenyl-Sepharose column, the calpains bind more tightly, and it is necessary to include 10–30% ethylene glycol in the eluting buffer. If a large amount of protein is loaded onto a small phenyl-Sepharose column, the calpains may elute at 50 m*M* KCl ***(19,20)***. Autolysis of the calpains increases their hydrophobicity considerably, and autolyzed μ-calpain can be eluted only with SDS or some similar detergent (autolyzed m-calpain can be eluted off phenyl-Sepharose columns with ethyleneglycol). Hence phenyl-Sepharose chromatography removes all autolyzed μ-calpain. Autolyzed μ-calpain can be eluted from butyl-Sepharose columns.
19. μ-Calpain is the most difficult of the three proteins to purify, and if μ-calpain is the only protein needed, it is advisable to start with a tissue rich in μ-calpain, such as human platelets or erythrocytes (**Table 1**). Size exclusion chromatography has not been useful in purifying μ-calpain. Binding of μ-calpain to a variety of dye matrices including Reactive Red agarose is unpredictable, and also has not been useful as a means of purification. Thiopropyl-Sepharose can be used successfully, but is no more effective than the two TMAE and DEAE-TSK steps described here.
20. If purification is monitored with SDS-PAGE, butyl-Sepharose chromatography seems to provide little purification, but it does remove some smaller polypeptides (<25 kDa) that are very difficult to remove otherwise. If a tissue/cell rich in μ-calpain

is the starting material, it may be possible to omit this butyl-Sepharose step.

21. This wash is important because most of the impurities removed by the butyl-Sepharose elute just before μ-calpain.
22. High resolution anion-exchange chromatography at pH 8.5 is useful because μ-calpain elutes as a sharper peak at pH 8.5, for reasons which are not entirely clear, whereas it elutes as two and sometimes three closely spaced peaks at lower pH values. Loading this column at an ionic strength close to the ionic strength required to elute μ-calpain facilitates purification of μ-calpain because most of the impurities in the μ-calpain fraction at this stage elute just before the μ-calpain. Use a quaternary amino functional group to retain high ion-exchange capacity at high pH.
23. Potassium salts should be avoided when working with Reactive Red dye affinity columns because the K^+ ion causes precipitation of the triazine dye.
24. Increasing the pH of the eluting buffer from 7.5 to 8.5 increases the rate at which m-calpain elutes off Reactive Red columns; when the eluting buffer pH is 7.5, it sometimes requires several liters to elute m-calpain activity completely. The forces involved in binding m-calpain to Reactive Red are not entirely clear, but for whatever reasons, Reactive Red is very effective in purifying m-calpain; m-calpain typically is 60–75% pure after Reactive Red chromatography. These factors make it considerably easier to purify m-calpain than μ-calpain.

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References

1. Goll, D. E., Edmunds, T., Kleese, W. C., Sathe, S. K., and Shannon, J. D. (1985) Some properties of the Ca^{2+}-dependent proteinase, in *Intracellular Protein Catabolism* (Khairallah, E. A., Bond, J. S., and Bird, J. W. C., eds.), Alan R. Liss, New York, pp.151–164.
2. Goll, D. E., Kleese, W. C., Sloan, D. A., Shannon, J. D., and Edmunds, T. (1986) Properties of the Ca^{2+}-dependent proteinases and their protein inhibitor. *Cienc. Biol. (Portugal)* **11,** 75–83.
3. Wheeler, T. L. and Koohmaraie, M. (1991) A modified procedure for simultaneous extraction and subsequent assay of calcium-dependent and lysosomal protease systems from a skeletal muscle biopsy. *J. Anim. Sci.* **69,** 1559–1565.
4. Koohmaraie, M. (1990) Quantification of Ca^{2+}-dependent protease activities by hydrophobic and ion-exchange chromatography. *J. Anim. Sci*. **68,** 659–665.
5. Murachi, T. (1983) Intracellular Ca^{2+} protease and its inhibitor: Calpain and calpastatin, in *Calcium and Cell Function, Vol. IV* (Cheung, W.Y, ed.), Academic Press, New York, pp. 377–410.

6. Murachi, T. (1983) Calpain and calpastatin. *Trends Biochem. Sci*. **8,** 167–169.
7. Clark, A. F., DeMartino, G. N., and Croall, D. E. (1986) Fractionation and quantification of calcium-dependent proteinase activity from small tissue samples. *Biochem. J*. **235,** 279–282.
8. Thompson, V. F., Cong, J., Luedke, D. W., and Goll, D. E. (1999) Purification of μ-calpain, m-calpain, and calpastatin from human placenta. *Protein Expression Purification* (in press).
9. Cong, M., Thompson, V. F., Goll, D. E., and Antin, P. (1998) The bovine calpastatin gene promoter and a new N-terminal region of the protein are targets for cAMP-dependent protein kinase activity. *J. Biol. Chem*. **273,** 660–666.
10. Geesink, G. H., Nonneman, D., and Koohmaraie, M. (1998) An improved purification protocol for heart and skeletal muscle calpastatin reveals two isoforms resulting from alternative splicing. *Arch. Biochem. Biophys.* **356,** 19–24.
11. Thompson, V. F., Goll, D. E., and Kleese, W. C. (1990) Effects of autolysis on the catalytic properties of the calpains. *Biol. Chem. Hoppe-Seyler* **371,** 177–185.
12. James, G. T. (1978) Inactivation of the protease inhibitor phenylmethylsulfonyl fluoride in buffers. *Anal. Biochem*. **86,** 574–579.
13. Edmunds, T., Nagainis, P. A., Sathe, S. K., Thompson, V. F., and Goll, D. E. (1991) Comparison of the autolyzed and unautolyzed forms of μ- and m-calpain from bovine skeletal muscle. *Biochim. Biophys. Acta* **1077,** 197–208.
14. Atkinson, T., Hammond, P. M., Hartwell, R. D., Hughes, P., Scawen, M. D., Sherwood, R. F., Small, D. A., Bruton, C. J., Harvey, M. J., and Lowe, C. R. (1981) Triazine-dye affinity chromatography. *Biochem. Soc. Trans.* **9,** 290–293.
15. Stellwagen, E. (1990) Chromatography on immobilized reactive dyes. *Methods Enzymol*. **182,** 343–357.
16. Reville, W. J., Goll, D. E., Stromer, M. H., Robson, R. M., and Dayton, W. R. 1976. A Ca^{2+}- activated protease possibly involved in myofibrillar protein turnover: Subcellular localization of the protease in porcine skeletal muscle. *J. Cell Biol.* **70,** 1–8.
17. Goll, D. E., Thompson, V. F., Taylor, R. G., Ouali, A., and Chou, R. R. (1999). The calpain system in muscle tissue, in *Calpain: Pharmacology and Toxicology of a Calcium-Dependent Cellular Protease* (Wang, K. K. W. and Yuen, P-W., eds.) Taylor and Francis, Bristol, PA, pp. 127–160.
18. Wolfe, F. H., Sathe, S. K., Goll, D. E., Kleese, W. C., Edmunds, T., and Duperret, S. M. (1989) Chicken skeletal muscle has three Ca^{2+}-dependent proteinases. *Biochim. Biophys. Acta* **998,** 236–250.
19. Karlsson, J-O., Gustavsson, S., Hall, C., and Nilsson, E. (1985) A simple one-step procedure for the separation of calpain I, calpain II and calpastatin. *Biochem. J.* **231,** 201–204.
20. Gopalakrishna, R. and Barsky, S. H. (1986) Hydrophobic association of calpains with subcellular organelles. Compartmentalization of calpains and the endogenous inhibitor calpastatin in tissues. *J. Biol. Chem.* **261,** 13,936–13,942.
21. Otsuka, Y. and Goll, D. E. (1987) Purification of the Ca^{2+}-dependent proteinase inhibitor from bovine muscle and its interaction with the millimolar Ca^{2+}-dependent proteinase. *J. Biol. Chem*. **262,** 5839–5851.

2

A Simple Protocol for Separation and Assay of μ-Calpain, m-Calpain, and Calpastatin From Small Tissue Samples

Jan-Olof Karlsson

1. Introduction

In many cases separation of calpains and calpastatin has to be performed with a relatively small amount of tissue, so that large-scale and complicated multiple-column procedures are not appropriate. In designing a method suitable for small amounts of tissue, it is important that the extraction and separation methods should be rapid in order to avoid postmortem changes in the subcellular distribution of the enzymes, and also to avoid autolysis or degradation of the enzymes themselves. The method presented here uses relatively simple and inexpensive equipment and reagents, although the assay of calpain activity is greatly simplified by access to a fluorometer with the ability to read microtiter plates.

The procedure includes rapid perfusion of the tissue with EDTA before removal of the sample, homogenization, and centrifugation, followed by a single step of hydrophobic interaction chromatography on a column of phenyl-Sepharose ***(1)***. The method separates μ-and m-calpain from each other and from the endogenous inhibitor, calpastatin, and requires only 1 d for a small sample. At this stage, μ-calpain, m-calpain, and calpastatin activity may be assayed separately. Both the μ-calpain-containing and the calpastatin-containing fractions still contain many other proteins, while the m-calpain is relatively pure. If necessary, further purification of μ-calpain and m-calpain is performed by means of ion-exchange chromatography on a Mono Q column ***(2)***. This procedure results in electrophoretically pure preparations of μ-calpain and m-calpain.

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2. Materials

1. Casein (Hammersten preparation) (Merck, Darmstadt, Germany).
2. DTT, (Boehringer-Mannheim, Mannheim, Germany): 100 m*M* in water, stored at –20°C in small tightly capped aliquots; after thawing, the DTT solution is used only for 1 d.
3. Phenyl-Sepharose CL-4B, Sephadex G-25, and the FPLC anion column (Mono Q; HR 5/5) were obtained from Pharmacia (Uppsala, Sweden). Other chemicals were of analytical grade.
4. The synthetic calpain substrate succinyl-Leu-Tyr-7-amino-4-methylcoumarin (Succ-Leu-Tyr- AMC) was obtained from Bachem (Bubendorf, Switzerland) AG ***(3)***. A 1 m*M* solution is prepared in 20 m*M* Tris-HCl, pH 7.5, 3% (v/v) DMSO, 1 m*M* DTT, 3 m*M* sodium azide (NaN_3).
5. White microtiter plates (Microfluor) were obtained from Dynatech (Chantilly, VA).
6. Homogenization buffer: 10 m*M* sodium borate, pH 8.0, m*M* EDTA, 3 m*M* NaN_3 (*see* **Note 1**).
7. Equilibration buffer for the phenyl-Sepharose column: 10 m*M* sodium borate, pH 7.5, 500 m*M* NaCl, 1 m*M* EDTA, 3 m*M* NaN_3.
8. Starting buffer for the phenyl-Sepharose column: 10 m*M* borate, pH 7.5, 100 m*M* NaCl, 1 m*M* EDTA, 3 m*M* NaN_3.
9. Final buffer for the phenyl-Sepharose column: 10 m*M* sodium borate, pH 8.0, 1 m*M* EDTA, 3 m*M* NaN_3.
10. Buffer for the Mono Q column: 20 m*M* sodium borate, pH 8.0, 0.1 m*M* EDTA, 3 m*M* NaN_3.
11. Perkin-Elmer (Norwalk, CT) LS 50B luminescence spectrometer with a microtiter plate attachment.

3. Method

3.1. Homogenization

1. If possible, it is an advantage to perfuse the experimental animal with ice-cold 150 m*M* NaCl, 5 m*M* EDTA (pH adjusted to 8.0 with NaOH) via the heart ventricle before dissection of tissue (*see* **Note 2**).
2. Take tissue samples of approximately 0.5 g (wet weight) (*see* **Note 3**).
3. Homogenize the tissue in a loose-fitting Teflon-to-glass homogenizer (1500 rpm, ~6 strokes) with 5 mL ice-cold homogenization buffer. For larger samples, use 10 vol of homogenization buffer.
4. Centrifuge the homogenate at 100,000*g* at 4°C for 1 h.
5. Decant the supernatant and add solid NaCl to a final concentration of 500 m*M*.
6. Permit the sample to warm to room temperature, and adjust to pH 7.0 with NaOH or HCl. All subsequent work is carried out room temperature with minimum delay.

3.2. Separation on Phenyl-Sepharose *(See* **Note 4.***)*

1. Apply the sample at a rate of 50 mL/h to a column (0.8 × 13 cm, **Note 5**) of phenyl-Sepharose (~26 mL) that has been equilibrated at room temperature in the equilibration buffer. If calpastatin is to be purified or assayed, the flow-through

fractions containing protein not bound to the phenyl-Sepharose column should be frozen immediately.
2. Wash the column thoroughly with equilibrating buffer until a stable $A_{280\ nm}$ baseline is obtained.
3. Elute m-calpain and μ-calpain with a linear gradient of increasing pH and decreasing salt concentration in borate buffer ***(1)***. Use 150 mL of starting buffer and 150 mL of final buffer.
4. Collect fractions of 3–6 mL and analyze aliquots for proteolytic activity.

The enzymes are isolated by virtue of their different hydrophobic characteristics. Using a phenyl-Sepharose column with the conditions described, m-calpain is eluted first, at 50–80 m*M* NaCl, pH 7.5 (**Fig. 1**), as a single broad peak, with very little contaminating protein. μ-Calpain is eluted at 10–25 m*M* NaCl, pH 7.9, as one or two peaks, just before and partly overlapping a major peak of hydrophobic protein at the end of the gradient. A further major peak of more firmly bound protein may be eluted with distilled water or 1 m*M* NaOH, but no additional calcium-activated proteolytic activity is detected.

3.3. Further Purification on Mono Q

1. Equilibrate the Mono Q column (HR 5/5) in Mono Q buffer at a rate of 1 mL/min.
2. Apply the μ- or m-calpain sample (3–20mL) from the phenyl-Sepharose separation step.
3. Wash the column with buffer and elute at 1 mL/min until a stable $A_{280\ nm}$ baseline is obtained.
4. Elute the calpains with an appropriate linear gradient of NaCl concentration with a total volume of 40 mL. For μ-calpain the gradient should be from 0 to ~300 m*M* NaCl; for m-calpain the gradient should be from 0 to 500 m*M* NaCl. Under these conditions, μ-calpain is eluted at 185–200 m*M* NaCl and m-calpain at 380 m*M* NaCl (**Figs. 2** and **3**). Calpastatin (always applied separately) elutes at ~220 m*M* NaCl ***(2)***.
5. Collect fractions of 1 mL and assay aliquots for proteolytic activity (*see* **Note 6**).
6. For Western blotting, the enzyme-containing fractions can be desalted on a small Sephadex G-25 column (PD-10, Pharmacia) according to the manufacturer's instructions. The columns are equilibrated in 0.1% (w/v) SDS containing 0.01 m*M* EDTA (allowing for the subsequent concentration by lyophilization). The desalted proteins are immediately lyophilized and subjected to SDS-PAGE.

3.4. Assay of Proteolytic Activity with Synthetic Peptide Substrate (*See* **Note 7.**)

1. Mix 25-μL samples of enzyme solution (tissue extract supernatants or fractions from the phenyl-Sepharose or Mono Q columns), with 25 μL of 20 m*M* Tris-HCl, pH 7.5, 3% (v/v) DMSO, 3 m*M* NaN_3 in a white microtiter plate.
2. Preincubate for 10 min at room temperature.
3. Add 100 μL of 1 m*M* Succ-Leu-Tyr-AMC, 1 m*M* DTT, 3%DMSO and final net

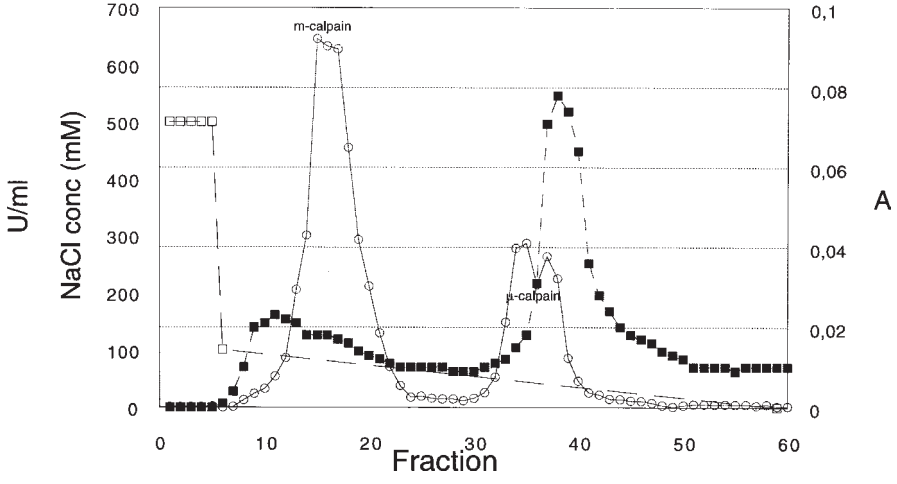

Fig. 1. Separation of calpains on phenyl-Sepharose. The supernatant from 30 g (wet weight) of rabbit lungs was applied to the column and the nonbinding proteins were saved and frozen for assay of calpastatin activity. The gradient of decreasing NaCl concentration was applied at fraction 1 and ended at fraction 60. ■, absorbance at 280 nm; ○, proteolytic activity; □, NaCl concentration.

concentrations of 5 m*M* EDTA or 1 m*M* $CaCl_2$ ***(3)***.

4. Ca^{2+}-dependent proteolysis is determined with 1 m*M* Ca^{2+} in excess of the EDTA concentration. Ca^{2+}-independent proteolysis is measured as a control with 5 m*M* EDTA in the incubation mixture instead of Ca^{2+}.
5. Set the spectrometer with an excitation wavelength of 380 nm (slit 5 nm) and an emission wavelength of 460 nm (slit 10 nm).
6. Observe hydrolysis of the substrate at intervals of 2–3 min at a rate that is linear for at least 2 h (*see* **Note 8**).

3.5. Microtitre Plate Calpain Assay with Casein and Fluorescamine

(See **Note 9.)**

1. Preparation of substrate ***(1)***. Suspend 1.25 g of casein in ~500 mL of water and heat to 80°C while stirring. Adjust pH to 9.5 with 1 *M* KOH, and allow to cool. Add 1 mL of 100 m*M* EDTA, 10 mL of 300 m*M* NaN_3, 7.5 g of KCl and adjust pH to 7.5 with 0.5 *M* boric acid. Dilute to 1000 mL, filter and freeze at –20°C in 10-mL portions.
2. Add DTT to 1 m*M* and $CaCl_2$ to 2 m*M* to an aliquot of casein substrate solution.
3. Add 80 µL of enzyme sample to a 96-well microtiter plate.
4. Add 200 µL of casein substrate solution, 1 m*M* DTT, 2 m*M* Ca^{2+}, or 5 m*M* EDTA, and incubate for 1–2 hours or overnight at room temperature.

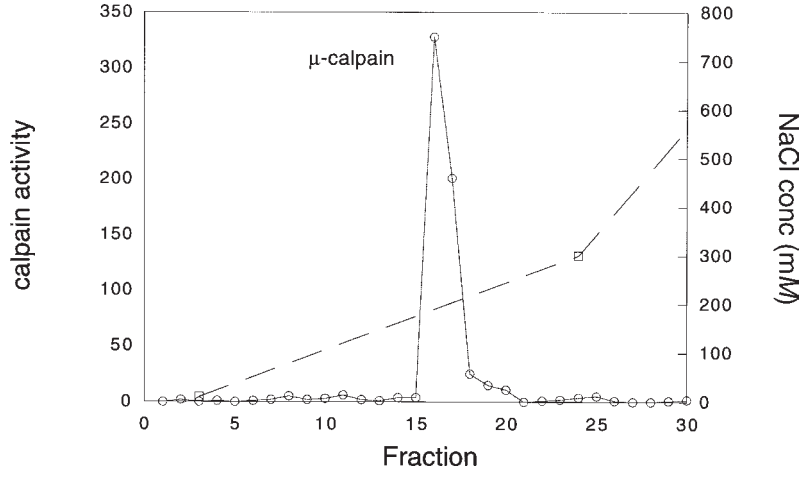

Fig. 2. Purification of μ-calpain on Mono Q. The peak of μ-calpain activity from a phenyl-Sepharose column was applied to the Mono Q column. A gradient from 10 to 300 m*M* NaCl was applied to elute μ-calpain activity at ~200 m*M* NaCl. ○, proteolytic activity; □, NaCl concentration.

5. Add 40 μL of 40% TCA to stop the proteolysis. Centrifuge the microtiter plates at 750*g* for 20 min.
6. Transfer 30 μL of the TCA supernatants (*see* **Note 10**) to a white microtiter plate, and add 225 μL of 0.5 *M* sodium borate, pH 10.
7. Add 30 μL fluorescamine (1 mg/mL in acetone) and mix rapidly ***(4)*** with the pipette.
8. Measure the fluorescence within 40 min at an excitation wavelength at 390 nm (slit 15 nm) and at an emission wavelength of 480 nm (slit 20 nm). Subtract appropriate zero time values (TCA added before the sample; controls with 5 m*M* EDTA). Use a standard of 0–2 m*M* glutamate.

3.6. Assay of Calpastatin

1. Heat samples (0.2–0.5 mL) of homogenates, supernatants, or fractions containing protein not bound to the phenyl-Sepharose column at 96°C for 10 min to destroy endogenous proteolytic activity ***(5)***.
2. Centrifuge at 10,000*g* for 5 min and freeze at –20°C.
3. Thaw the heated samples and centrifuge again (*see* **Note 11**).
4. Mix aliquots of the heated samples (0.5, 1, 2, 5, 10, 20, and 50 μL) with a fixed amount of μ- calpain (*see* **Note 12**) and incubate with substrate (synthetic peptide or casein) and 1 m*M* Ca^{2+} as described above.
5. Plot the observed residual proteolytic activity against the amount of added calpastatin sample. Calculate the inhibitory capacity from the straight portion of the titration curve ***(5)***. 1 unit of calpastatin is defined as that which inhibits 1 unit of μ-calpain under the described conditions (*see* **Note 13**).

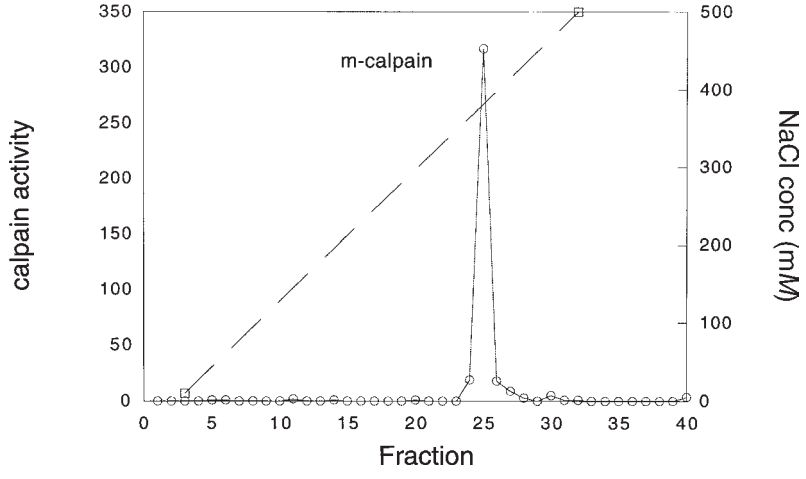

Fig. 3. Purification of m-calpain on Mono Q. The peak of m-calpain activity from a phenyl-Sepharose column was applied to the Mono Q column, followed by a gradient from 10 to 500 m*M* NaCl, eluting m-calpain activity at ~400 m*M* NaCl. ○, proteolytic activity; □, NaCl concentration.

4. Notes

1. No additional salt is included in the homogenization buffer, in order to decrease possible binding of calpains to membranes. 1 m*M* DTT and 0.1 m*M PMSF* may be added to the homogenization solution, although in our hands no improvement of the separation has been seen with these additions. By contrast, it may be an advantage to delay addition of DTT until assay of the partially purified enzyme, since this may reduce the activity of other thiol proteases in the crude extracts.
2. Fresh tissue from nonperfused animals may be used, but perfusion of the animal removes most of the contaminating blood and Ca^{2+} from the tissue and also chills the samples.
3. The protocol works with relatively small amounts of tissue, normally from 0.5 to 30 g wet weight. Fresh tissue is preferable but samples may be stored in sealed vials at –80°C. We have successfully analyzed brain samples stored frozen for 2 yr or more.
4. It is essential that the hydrophobic interaction chromatography is carried out between 20 and 25°C. At lower temperatures the hydrophobic interaction with the phenyl-Sepharose is significantly decreased, which will affect the elution pattern.
5. For larger amounts of sample (up to 30 g wet weight) the column size should be increased to 0.8 × 30 cm. In this case the column may be washed overnight with a flow rate of 10–30 mL/h to obtain a stable baseline before starting the gradient.
6. Fractions obtained from the phenyl-Sepharose column containing μ-calpain or m-calpain may be stored for long periods (several months) at 4°C in tightly

capped tubes with no loss of activity. Fractions containing calpastatin should be frozen immediately. Pure fractions of μ-calpain or m-calpain obtained after the separation on the Mono Q column are less stable and they should be used within a week when stored at 4°C. They can be stored indefinitely in the presence of 50% glycerol at –70°C. Freezing of enriched or pure calpain fractions in the buffer results in the loss of most (>75%) of the enzymatic activity.

7. Both this assay, and the following assay with casein substrate, can be scaled up for use with a conventional fluorometer, without the use of a microtiter plate attachment.
8. Zero time values are subtracted from each individual well. Standard curves with AMC in the appropriate buffer are used to express the data in picomoles of AMC formed per minute and per milliliter of enzyme solution (unit per milliliter). Addition of a known amount of AMC to the complete incubation mixture gives a fluorescence recovery better than 95%.
9. Do not use Tris or other buffers containing amino groups in this assay, as these groups react with fluorescamine.
10. For higher signals, more of the TCA supernatant may be used, but the pH of the final mixture should be checked (pH 10).
11. For samples containing a substantial amount of low molecular weight material, which may disturb the assay, it is an advantage to perform a buffer exchange on a small gel filtration column. A 2.5-mL portion of the supernatant is applied to a small Sephadex G-25 column (PD-10, Pharmacia) equilibrated in 3 m*M* sodium borate buffer, pH 8.0, 1 m*M* NaN_3, 0.1 m*M* EDTA, 1 m*M* DTT.
12. Make a preliminary titration first to determine a suitable amount of μ-calpain for the particular assay.
13. It is usual to determine the volume of calpastatin which gives 50% inhibition of the enzyme and then to make the calculations.

References

1. Karlsson, J.-O., Gustavsson, S., Hall, C., and Nilsson, E. (1985) A simple one-step procedure for the separation of calpain I, calpain II and calpastatin. *Biochem. J.* **231,** 201–204.
2. Nilsson, E. and Karlsson, J.-O. (1990) Slow anterograde axonal transport of calpain I and II. *Neurochem. Int.* **17,** 487–494.
3. Sasaki, T., Kikuchi,T., Yumoto, N., Yoshimura, N., and Murachi, T. (1984). Comparative specificity and kinetic studies on porcine calpain I and calpain II with naturally occurring peptides and synthetic fluorogenic substrates. *J. Cell Biol.* **259,** 12,489–12,494.
4. Nakai, N., Lai, C. Y., and Horecker, B. L. (1974) Use of fluorescamine in the chromatographic analysis of peptides from proteins. *Anal. Biochem.* **58,** 563–570.
5. Blomgren, K., Nilsson, E., and Karlsson, J-O. (1989) Calpain and calpastatin levels in different organs of the rabbit. *Comp. Biochem. Physiol.* **93B,** 403–407.

3

Purification and Quantification of Calcium-Activated Neutral Proteases I and II and Novel Isoforms from Cultured Osteoblastic Cells by Ion-Exchange Fast Protein Liquid Chromatography

Elsa J. B. Murray, Keyvan Behnam, Mario S. Grisanti, and Samuel S. Murray

1. Introduction

The methods described in this chapter are designed for rapid and convenient separation and quantitation of calpain isozymes in cultured osteoblastic cells by modern semiautomated strong-anion exchange FPLC. The isozymes of the calpain–calpastatin system are abundant in osteoblastic or bone-forming cells, where they are regulated by parathyroid hormone ***(1)*** and bone morphogenetic protein ***(2)***. High levels of calpain II immunoreactivity are present in other mineralizing tissues, including calcifying cartilage ***(3)*** and fracture callus ***(4)***. Extracellular calpain II is present in terminally differentiated chondrocyte cultures, where it degrades large proteoglycan monomers and contributes to the initiation of biomineralization ***(5)***. Calpain protein and activity are present in osteoarthritic synovial fluid and synoviocytes ***(6)***, where calpain catalyzes degradation of major proteoglycan core proteins, abolishing hyaluronic acid binding and damaging cartilage ***(7)***.

Although it is clear that the calpain–calpastatin system is very important in the development and pathophysiology of bone and cartilage, it is difficult to study calcium-activated neutral protease activities in bones and teeth because mineralized tissues are highly acellular and consist largely of calcium phosphate salts, such as hydroxyapatite ***(8)***. The biochemistry and physiological regulation of calcium-dependent enzyme systems in osteoblasts are routinely studied

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in vitro in clonal monolayer cell cultures initially prepared by sequential collagenase/trypsin digestion of fetal or neonatal rat, mouse, or human bone or by cellular outgrowth from bone chips, which limits the amount of protein available for study ***(1,2)***. The direct determination of calpain activities in osteoblastic cells is further confounded by high levels of calpastatin, the endogenous inhibitor ***(2)***. We describe a method for extracting protein from cultured osteoblasts, separating the calpains by anion-exchange chromatography ***(9),*** and quantitating protease activities on the basis of the rate of fluorescein-tagged casein hydrolysis in the absence and presence of calcium. The method is written so that researchers with experience in cell culture and basic biochemistry can successfully separate and determine calpain activities in osteoblastic cells.

2. Materials

1. Soluble osteoblastic cell protein extract: Prepare as indicated in **Subheading 3.1.**
2. Hypotonic lysis buffer: 20 m*M* Tris-HCl, pH 7.5, 6.25 m*M* 2-ME, 1 m*M* EDTA, 1 m*M* EGTA, 10 m*M* NaCl, 0.1% (w/v) octyl-β-D-glucopyranoside (OBG), 5 μ*M* lactacystin, (a specific proteasome inhibitor). Make 100-mL quantities using freshly autoclaved reagent-grade (double-distilled deionized) water, and store 10-mL aliquots at –20°C (*see* **Notes 1** and **2**).
3. FPLC buffer A: 20 m*M* Tris-HCl, pH 7.5, 1 m*M* EGTA, 1 m*M* EDTA, 6.25 m*M* 2-ME, 10 m*M* NaCl. Prepare 1 L fresh daily.
4. FPLC buffer B: 20 m*M* Tris-HCl, pH 7.5, 1 m*M* EGTA, 1 m*M* EDTA, 6.25 m*M* 2-ME, 1 *M* NaCl.
5. Filter both FPLC buffers A and B through a 0.22 -μm membrane, degas, and keep both solutions at room temperature.
6. FITC-labeled casein substrate solution: 5 mg/mL FITC-casein in 50 m*M* Tris-HCl, pH 7.2 (*see* **Notes 3** and **4**). Make up in 100-mL quantities and store 4-mL aliquots at –20°C for up to 1 yr.
7. Calcium-free calpain assay buffer: 30 m*M* Tris-HCl, pH 7.5, 9.4 m*M* 2-ME, 15 m*M* NaCl, 1.5 m*M* EGTA, 1.5 m*M* EDTA. Prepare 500 mL and store at 4°C for up to 2 mo.
8. Calpain I (low-calcium) assay buffer: 30 m*M* Tris-HCl, pH 7.5, 9.4 m*M* 2-ME, 15 m*M* NaCl, 1.5 m*M* EGTA, 1.5 m*M* EDTA, 5.2 m*M* $CaCl_2$. Make 100 mL and store at 4°C for up to 2 mo.
9. Calpain II (high-calcium) assay buffer: 30 m*M* Tris-HCl, pH 7.5, 9.4 m*M* 2-ME, 15 m*M* NaCl, 1.5 m*M* EGTA, 1.5 m*M* EDTA, 10.2 m*M* $CaCl_2$. Make 100 mL and store at 4°C for up to 2 mo.
10. Stop solution for calpain assays: 5 % (w/v) TCA. Make 1.0 L and store at 4°C for up to 2 yr.
11. Diluent for fluorescent readings: 500 m*M* Tris-HCl, pH 8.5. Make 2.0 L and store at 4°C for up to 6 mo.

3. Methods

3.1. Preparation of Osteoblastic Cell Protein

1. Plate five T-175 cm^2 flasks of osteoblastic cells as usual and culture to near confluence (*see* **Note 5**).
2. Gently scrape the cells from each flask into 10 mL of Ca^{2+}, Mg^{2+}-free phosphate-buffered saline at 4°C.
3. Centrifuge at 250*g* for 15 min at 4°C.
4. Gently resuspend the cells in 3.0 mL of hypotonic lysis buffer and incubate at 4°C for 20 min.
5. Homogenize with 20 strokes of a type "B" pestle in a 15-mL glass Dounce homogenizer (Kontes, Vineland, NJ) on ice.
6. Sonicate twice for 15 sec at 30% power at 4°C with a microbore probe.
7. Centrifuge the homogenate at 100,000*g* for 30 min at 4°C to clarify (*see* **Note 6**).
8. Determine the protein content of the clear supernatant by the Bradford method, using a commercial test kit (BioRad, Hercules, CA or Pierce, Rockford, IL) and a protein standard diluted in hypotonic lysis buffer. The lysis buffer also serves as the blank. The clear supernatant should contain at least 5 mg protein/mL. The preparation should be timed so that the FPLC separation can be run immediately after determining the protein content. If a delay occurs, hold the supernatant at 4°C.

3.2. Strong Anion-Exchange FPLC

1. Program a BioLogic FPLC controller (BioRad) as outlined in **Table 1** or modify the program as necessary for existing equipment.
2. Purge pumps A and B with their respective buffers.
3. Pre-equilibrate a Bio-Scale Q5 [$-N^+ (CH_3)_3$, BioRad], 5 mL quaternary anion exchange column by washing with at least 25 mL of buffer A at 1 mL/min, until a stable UV baseline is established, and the conductivity is about 2–3 mS/cm.
4. Load 2.0 mL of 0.22 µm-filtered hypotonic lysis buffer in the sample loop and execute a blank run to verify the performance of the FPLC system.
5. Reequilibrate the column with FPLC buffer A.
6. Load 2.0 mL of the clear supernatant obtained by ultracentrifugation (at least 10 mg protein) in a 2-mL sample loop.
7. Execute the separatory program (**Table 1**) and collect 1.0-mL fractions into 13 × 100-mm polypropylene tubes (Sarstedt, Newtown, NC). A sample separation of 12 mg of MC3T3-E1 osteoblastic cell protein is shown in **Fig. 1** (*see* **Notes 7** and **8**).
8. Transfer the tubes containing the FPLC fractions to a 4°C ice bath.
9. Reequilibrate the column and assay calpain activities and protein in the fractions.

3.3. Protease Assay

1. Label 3 sets (Ca^{2+}-free, low-calcium, and high-calcium) of 12 × 75-mm polystyrene tubes (Falcon 2008) in duplicate for each fraction, the initial homogenate, and the buffer blank.

Table 1
BioLogic FPLC Program for Calpain Separations

Step no.	Start (mL)	Step
1	0.0	Collect fractions of size 1.00 mL during entire run
2	0.0	Turn chart recorder ON
3	0.0	Turn UV lamp ON
4	0.0	Set UV baseline to 0.0
5	0.0	Isocratic flow with 100% A at 1 mL/min for 5 mL[a]
6	5.0	Static loop:Inject 2.0 mL sample at 1 mL/min for 2 mL
7	7.0	Isocratic flow with 100% A at 1 mL/min for 10 mL
8	17.0	Linear gradient with 0–80% B at 1 mL/min for 30 mL[b]
9	48.0	Isocratic flow with 20% A at 1 mL/min for 3 mL
10	50.0	End of protocol

[a] Buffer A: 20 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, 1 m*M* EGTA, 6.25 m*M* 2-ME, 10 m*M* NaCl.
[b] Buffer B: 20 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, 1 m*M* EGTA, 6.25 m*M* 2-ME, 1 *M* NaCl.

2. Pipette 100 µL of Ca^{2+}-free, low-calcium or high-calcium assay buffer into each set of duplicate assay tubes.Transfer the tubes to a 4°C ice bath.
3. Pipette 100 µL aliquots of each fraction into each of its six respective tubes. Pipette 100 µL aliquots of the homogenate and FPLC buffer A into their respective sets of tubes (*see* **Note 9**).
4. Start the reaction by adding 50 µL of FITC-labeled casein substrate solution to each tube.
5. Vortex the tubes gently and incubate overnight (18 h) at 22°C.
6. Add 250 µL of 5% trichloroacetic acid (TCA) to each tube.
7. Centrifuge at 500*g* at 4°C for 30 min to pellet undegraded protein.
8. Remove 300 µL of clear supernatant to a clean 12 × 75-mm polystyrene tube. Add 3.0 mL of 500 m*M* Tris-HCl, pH 8.5, to each tube.
9. Read the fluorescence of the samples at an excitation wavelength of 365 nm and an emission wavelength of 525 nm after blanking the instrument against reagent-grade water at a sensitivity of 1.0.
10. Express the enzymatic activity as fluorescence units per fraction, after correcting for dilution, and subtracting the Ca^{2+}-free value from the value obtained in the presence of low-or high-calcium buffer.
11. Determine the protein content of each fraction by the Bradford method.
12. Plot enzyme activity (fluorescence units per fraction) vs fraction number.
13. Calculate the specific activity of active fractions by dividing the enzymatic activity by the protein content of the fraction (*see* **Note 10**).

4. Notes

1. All solutions should be prepared using sterile, double-distilled deionized water. Glassware should be washed as usual, then soaked in 3 *M* HCl for 2 h and rinsed at least three times with reagent-grade water to remove residual calcium.

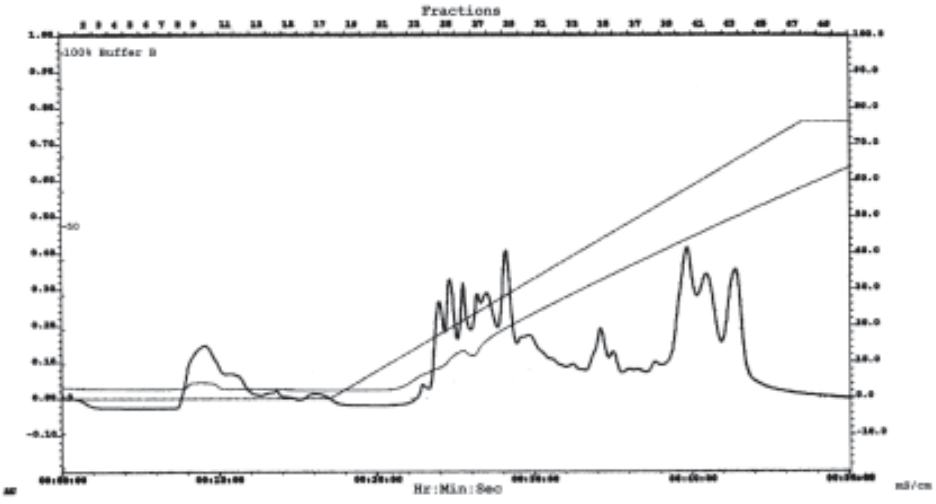

Fig. 1. Chromatogram of 12 mg of murine MC3T3-E1 osteoblastic cell protein separated by anion-exchange chromatography on a Q5 column (BioRad).

2. Octyl-β-D-glucopyranoside (OBG) is included in the hypotonic lysis buffer to solubilize proteins. We determined that commercially available calpain I and II (Calbiochem, San Diego, CA) can be incubated with up to 1% OBG at 4ºC for up to 36 h without loss of specific activity. Lactacystin is included in the lysis buffer to inhibit the high levels of endogenous proteasome activities in the osteoblast ***(10)***.
3. FITC-casein can be purchased from commercial vendors, such as Sigma (St. Louis, MO [catalog numbers C- 0403, -3777, or -0528]). However, we prepare our own FITC-casein about once a year by the simple method of Twining ***(11)***. This is much cheaper and results in a product with lower background fluorescence and a higher degree of FITC-conjugation.
4. This solution is light-sensitive and should be kept in foil-wrapped tubes or boxes. Do not subject the substrate to repeated cycles of freezing and thawing. If colloidal materials form in the substrate solution during defrosting, centrifuge at 250*g* for 15 min at 22°C to pellet them, and use the clear amber supernatant in the assay.
5. Do not culture osteoblastic cells past confluence in order to avoid formation of mineralized nodules. Five T-175-cm^2 flasks will provide at least 10 mg protein for FPLC. If there is less protein, it is not easy to detect novel calcium-activated neutral protease activities in the fractions.
6. It is not a simple matter to prepare significant amounts of particulate-free osteoblastic cell protein for FPLC. We have used physical means of disrupting the cells, followed by 0.22 µm filtration to clarify the solution for FPLC. It is extremely difficult to filter the cell extracts, and protein recovery after filtration can be as low as 1%, even with low protein-binding membranes. The cell lysate

can be clarified by ultracentrifugation, and the clear supernatant can be loaded directly into the sample loop for FPLC if OBG is present in the lysis buffer at 0.1–1% (w/v). Recovery of soluble proteins is very high (>90%) if this approach is used, without impairing the long-term performance of the column. It should be noted that FPLC separation of hypotonic lysis buffer containing OBG gives rise to chromatograms with UV-absorbing peaks at 20 and 21% buffer B which are not observed in the absence of OBG. This ultraviolet (UV) artifact is highly reproducible and does not impair the FPLC separation of osteoblastic cell proteins.

7. The back-pressure of the Q5 column routinely runs below 200 psi; if it increases, simply reverse the flow and purge the column according to manufacturer's instructions. If the column is not used on a daily basis, store it in 20% ethanol (v/v) in water, and wash it extensively with water before its next use to avoid precipitation of buffer salts. This column has a recommended loading capacity of 50 mg and gives good separations with 10–20 mg of soluble protein. However, Q2 columns or their equivalents, with a volume of 2.0 mL and a recommended maximum protein loading capacity of 20 mg, also give good results. Careful preequilibration is essential for high reproducibility.
8. Porcine erythrocyte calpain I (Calbiochem) elutes at 36 % B (tube 31), while porcine kidney calpain II (Calbiochem) elutes at 47% B (tube 35), with total protein and enzymatic activity recoveries approaching 100%. Similar elution patterns for calpain I and II are observed in osteoblastic cell preparations (**Table 2**). The identity of calpain I and II isozymes in fractions obtained by FPLC separation of osteoblastic cell proteins can be confirmed by Western blotting ***(2)***. The calcium-activated neutral proteases eluted at 22% B (tube 26) were subjected to SDS-PAGE and Western blotting with antibodies to calpains I and II. However, no immunopositive bands were observed, suggesting that this is a novel activity.
9. Note the EDTA, EGTA, and $CaCl_2$ concentrations in the FPLC buffers and in the assay buffers. The free calcium ion concentrations in the assay mixtures are calculated to be 0 μ*M* (calcium-free), 80 μ*M* (low-calcium), and 2 m*M* (high-calcium).
10. The protease assay can also be conducted in microtiter plates using as little as 10 μL of sample, 10 μL of calcium buffer, and 5 μL of FITC-casein. After addition of 25 μL of 5% TCA, the microtiter plates can be centrifuged, and clear supernatant can be removed for dilution. The fluorescence can then be measured using a fluorometric microtiter plate reader. The advantage of this approach is that very small amounts of reagent are used. Most of the volume of the active fractions will be available for subsequent analysis or purification. However, great care and precision are required to achieve results that are as reproducible as those obtained with larger volumes.

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Table 2
Elution Patterns for Calcium-Activated Neutral Proteases Separated by Q5 FPLC

	Isozyme Elution Conditions		
Source	Isozyme Identification and Specific Activity	% B	Tube No.
Porcine red cell	Calpain I	36	31
Porcine kidney	Calpain II	47	35
Osteoblast	Noncalpain I/II (8.4 AFU/μg protein)	22	26
Osteoblast	Calpain I (9.1 AFU/μg protein)	38	32
Osteoblast	Calpain II (92.3 AFU/μg protein)	49	36

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References

1. Tram, K. K.-T., Spencer, M. J., Murray, S. S., Lee, D. B. H., Tidball, J. G., and Murray, E. J. B. (1993) Identification of calcium activated neutral protease activity and regulation by parathyroid hormone in mouse osteoblastic cells. *Biochem. Mol. Biol. Intern.* **29,** 961–967.
2. Murray, S. S., Grisanti, M. S., Bentley, G. V., Kahn, A. J., Urist, M. R., and Murray, E. J. B. (1997) The calpain-calpastatin system and cellular proliferation and differentiation in rodent osteoblastic cells. *Exp. Cell Res.* **233,** 297–309.
3. Shimizu, K., Hamamoto, T., Hamakubo, T., Lee, W. J., Suzuki, K., Nakagawa, Y., Murachi, T, and Yamamuro, T. (1991) Immunohistochemical and biochemical demonstration of calcium-dependent cysteine proteinase (calpain) in calcifying cartilage of rats. *J. Orthop. Res.* **9,** 26–36.
4. Nakagawa, Y., Shimizu, K., Hamamoto, T., Suzuki, K, Ueda, M., and Yamamuro, T. (1994) Calcium-dependent neutral proteinase (calpain) in fracture healing in rats. *J. Orthop. Res.* **12,** 58–69.
5. Yasuda, T., Shimizu, K., Nakagawa, Y., Yamamoto, S., Niibayashi, H., and Yamamuro, T. (1995) m-Calpain in rat growth plate chondrocyte cultures: its involvement in the matrix mineralization process. *Dev. Biol.* **170,** 159–168.
6. Suzuki, K., Shimizu, K., Hamamoto, T., Nakagawa, Y., Hamakubo, T., and Yamamuro T. (1990) Biochemical demonstration of calpains and calpastatin in osteoarthritic synovial fluid. *Arthritis Rheum.* **33,** 728–732.
7. Suzuki, K., Shimizu, K., Hamamoto, T., Nakagawa, Y., Murachi, T., and Yamamuro, T. (1992) Characterization of proteoglycan degradation by calpain. *Biochem. J.* **285,** 857–862.
8. Neuman, W. F. (1980) Bone material and calcification mechanisms, in *Fundamental and Clinical Bone Physiology* (Urist, M. R., ed.), Lippincott, Philadelphia, PA, pp. 83–107.

9. Spencer, M. J., Croall, D. E., and Tidball, J. G. (1995) Calpains are activated in necrotic fibers from *mdx* dystrophic mice. *J. Biol. Chem.* **270,** 10,909–10,914.
10. Murray, E. J. B., Bentley, G. V., Grisanti, M. S., and Murray, S. S. (1998) The ubiquitin–proteasome system and cellular proliferation and regulation in osteoblastic cells. *Exp. Cell Res.* **242,** 460–469.
11. Twining, S. S. (1984) Fluorescein isothiocyanate-labeled casein assay for proteolytic enzymes. *Anal. Biochem.* **143,** 30–34.

4

Purification of Calpain by Affinity Chromatography on Reactive Red-Agarose or on Casein-Sepharose

Dorothy E. Croall

1. Introduction

There is no single affinity chromatography method that is universally effective for the purification of calpains. Dye affinity chromatography and calcium-dependent binding to immobilized substrates or inhibitors are the most commonly attempted affinity methods. Our laboratory has used Reactive Red-agarose in the purification of m-and μ-calpain from bovine heart and other tissues for many years ***(1,2)***. However, this method has not achieved widespread use due to reported variability in outcomes. The procedure provided below has proven to be highly reproducible. It is particularly effective for the purification of m-calpain, although it is also useful for μ-calpain. Others have described the use of Blue Sepharose-CL-6B for the purification of both m-and μ-calpain, requiring the use of 1 *M* urea in the elution buffers ***(3)***. Our unpublished attempts to use this type of resin were not reproducibly successful, and this method will not be discussed further here. Calcium-dependent binding of calpain either to immobilized substrates, like casein-Sepharose ***(4,5)***, or to the inhibitor calpastatin ***(6)***, is highly selective but has the inherent risk that autoproteolysis may alter the calpain while it is being purified. Inactivation of calpain through mutation ***(7)*** or with covalent inhibitors ***(8)*** can be used to avoid autoproteolysis, although the uses for the purified inactive calpain are more limited.

2. Materials

1. Reactive Red-agarose, Type 3000-CL (Sigma, St. Louis, MO) either as a slurry or from the lyophilized powder (*see* **Note 1**).
2. Binding buffer-1: 20 m*M* MOPS, pH 7.0, 2 m*M* EGTA, 2m*M* EDTA, 5 m*M* 2-ME, 0.5 *M* NaCl.

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3. α-Casein from bovine milk (Sigma C7891).
4. Casein/Ca^{2+} binding buffer: 25 m*M* Tris-HCl (or MOPS), pH 7–7.5, 0.1 *M* NaCl, 5 m*M* $CaCl_2$, 5 m*M* 2-ME.
5. CNBr-Sepharose (Sigma or Pharmacia, Piscataway, NJ).

3. Metμhods

3.1. Dye Affinity Methods

3.1.1. Purification of m-Calpain Using Reactive Red-Agarose (See **Note 2.)**

1. Preparation of the column: pre-swell lyophilized Reactive Red-agarose powder in 0.5 *M* NaCl to generate a slurry.
2. Pour slurry into a column. A bed volume of 25 mL in a 2.5 × 10 cm column is sufficient for purification of at least 15 mg of m-calpain (*see* **Note 3**).
3. Preparation and care of resin (*see* **Note 4**). Before the first application of sample, and after each subsequent use, the resin is subjected to extensive washing. Wash the resin at room temperature (at a flow rate of 1–2 mL/min) with 2 column volumes each of:
   a. 4 *M* urea;
   b. distilled deionized water;
   c. 4 *M* NaC1;
   d. distilled deionized water.

   Subsequent steps are carried out at 4°C or on ice, unless otherwise stated.
4. Equilibrate the resin with binding buffer-1 for at least 8–12 h before use. Do not allow the resin to go dry during equilibration. Repeat **Step 3** after each use of the resin (*see* **Note 5**).
5. For long-term storage, store the resin at 4°C in binding buffer-1 supplemented with sodium azide (0.05%) in a brown glass or opaque container.
6. Preparation of m-calpain sample: Typically the sample is the pooled fractions from a DEAE ion exchange column, a common first step in calpain purification. Gradient or step elution from the DEAE column will separate μ-calpain/calpastatin (eluted together at approximately 0.1 *M* NaCl) from m-calpain (eluted at approximately 0.25 *M* NaCl).
   a. If the DEAE column was not developed in MOPS buffer, dialyze the pooled m-calpain fractions against binding buffer-1 (*see* **Note 6**);
   b. If the DEAE column was developed in MOPS buffer, estimate the NaCl concentration of the pooled fractions (e.g., by conductivity measurements) and add solid NaCl to increase [NaCl] to 0.5 *M*;
   c. For expressed recombinant m-calpain, or for cells predominantly expressing m-calpain, a third option exists: Sonicate or homogenize cells in 50 m*M* MOPS, pH 7.5, 5 m*M* EDTA, 5 m*M* EGTA, 5 m*M* 2-ME, (with added inhibitors such as PMSF if desired), and centrifuge at 20,000–50,000*g* for 15-30 min to isolate the soluble fraction. Add solid NaCl to 0.5 *M* to this fraction (*see* **Note 7**).
7. Apply the sample immediately to the Reactive Red column at a flow rate of 1–2 mL/min or less.

8. Wash the column with at least 1–2 column volumes of binding buffer-1 to remove unbound proteins. When it is clear that the bulk of unbound protein has washed through the column (as monitored by a Bradford assay, or $A_{280\ nm}$) prepare to elute the enzyme by allowing the 0.5 *M* NaCl buffer to run flush with the upper surface of the resin before applying elution buffer.
9. Apply the elution buffer by first layering it cautiously onto the surface of the column, and then with a pump at a flow rate of 0.5 mL/min or less (*see* **Note 8**).
10. For m-calpain from natural sources (80 + 28 kDa) (mammalian heart, liver, or cultured cells), elute the column with 20 m*M* MOPS, pH 7.0, 2 m*M* EDTA, 2 m*M* EGTA, 5 m*M* 2-ME. The eluted enzyme peak is typically sharp with a trailing edge **(Fig. 1A)** (*see* **Note 9**).
11. For recombinant rat m-calpain containing a truncated small subunit (80 + 21 kDa) ***(7,10)***, elute the column with distilled, deionized water. To minimize exposure of the enzyme to water and to maintain the integrity of the eluted enzyme, a 0.1 fraction volume of a 10× buffer containing reducing agent and chelators is prealiquoted into each collecting tube before enzyme elution (*see* **Notes 10–12**).

3.1.2. Purification of μ-Calpain With Reactive Red-Agarose ***(Note 13)****:*

1. Preparation of the column is identical to that for m-calpain.
2. Apply sample (*see* **Note 14)** and begin chromatography as described for m-calpain.
3. Monitor column outflow for protein to detect when most of the unbound proteins have washed through the resin.
4. Change the buffer to 20 m*M* MOPS, pH 7.0, 1 *M* NaCl, 2 m*M* EDTA, 2 m*M* EGTA, 5 m*M* 2-ME. This sharpens the eluted peak of μ-calpain and decreases the volume in which it is recovered **(Fig. 1B)**.
5. Pass the eluted μ-calpain through a phenyl-Sepharose column and elute with low ionic strength buffer, such as 5 m*M* Tris-HCl, pH 7.5, 2 m*M* EDTA, 2 m*M* EGTA, 5 m*M* 2-ME ***(1)*****.** This exchanges the buffer and concentrates the enzyme.

3.2. Immobilized Substrates as Affinity Resins

Methods of affinity chromatography exploiting the calcium-dependent binding of calpain to the immobilized substrate casein were used early on in attempts to purify calpain ***(4,5)***. The major drawbacks to this approach are autoproteolysis of calpain, and copurification of other proteins that show calcium-dependent binding to casein, such as transglutaminase ***(11)***. This method is useful, for purification of inactive calpain mutants or covalently inhibited calpains, in which autoproteolysis cannot occur, or if some degree of autoproteolysis is not an objection.

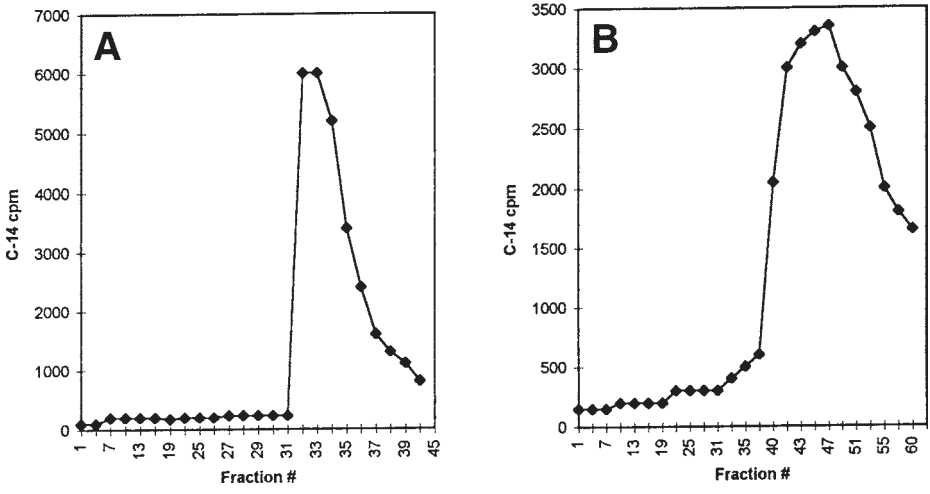

Fig. 1. Chromatography of m-calpain (A) and μ-calpain (B) on Reactive Red-agarose. Calpains from an extract of rat liver (75 g wet weight) were subjected to chromatography on Sephacryl S-300 to remove calpastatin and then separated by gradient elution (0–0.4 *M* KCl) on DEAE-cellulose (DE52, Whatman). The crude calpains were dialyzed separately against 20 m*M* MOPS, pH 7.0, 0.5 *M* NaCl, 1 m*M* EDTA, 1 m*M* EGTA, 5 m*M* 2-ME, before loading onto a column of Reactive Red-agarose (2.5 × 8 cm packed resin) that was pre-equilibrated as described in this chapter. The fraction volumes were 5 mL. After loading the samples, the columns were washed with binding buffer-1 until fraction 26 (A) or fraction 34 (B). m-Calpain was eluted with 20 mM MOPS, pH 7.0, 1 mM EDTA, 1 mM EGTA, 5 mM 2-ME. μ-Calpain was eluted with 20 m*M* MOPS, pH 7.0, 1 *M* NaCl, 1 m*M* EDTA, 1 m*M* EGTA, 5 m*M* 2-ME. Activity was measured by hydrolysis of ^{14}C-labeled casein and the results are plotted as acid-soluble cpm of ^{14}C-released in 15 min (A) or 30 min (B) at 25°C.

3.2.1. Preparation of Casein-Sepharose

Couple casein to commercially available CNBr-Sepharose using the conditions recommended by the manufacturer.

1. Soak and prewash dry resin (normally 1–2 g) with 1 m*M* HCl, to give a resin bed volume of 5–10 mL.
2. Wash quickly with 1–1.5 resin volumes of coupling buffer (0.1 *M* $NaHCO_3$, 0.5 *M* NaCl, pH 8.2) and mix immediately with 2 vol of 20 mg/mL casein in coupling buffer.
3. Check the pH of the reaction mix which should be at least 8.0. Adjust if necessary.
4. Allow the activated resin to mix gently with the casein solution for 2 h at room temperature, or overnight at 4°C.
5. Filter the resin on a sintered glass funnel to remove unbound casein (measure unbound protein in the filtrate to establish that most has become bound) and wash the resin in 50 mL of coupling buffer.
6. Resuspend resin in 10–20 mL of 0.2 *M* glycine, pH 8.0, and incubate for an addi-

tional 2 h to terminate coupling and block residual reactive groups.
7. Collect resin and wash with 250 mL of 0.1 *M* sodium acetate, pH 3.8, then with 250 mL of coupling buffer, and repeat the alternating washes twice more.
8. Store in coupling buffer with 0.05% sodium azide.

3.2.2. Chromatography of m- or μ-calpain on casein-Sepharose:

1. Equilibrate the casein-Sepharose with casein/Ca^{2+} buffer (*see* **Note 15**).
2. Place the equilibrated resin in a beaker to allow very rapid mixing.
3. Add the enzyme sample to the casein resin and mix well for 2–5 min (*see* **Note 16**).
4. Transfer the mixture to an appropriately sized column and wash out unbound proteins with casein/Ca^{2+} buffer.
5. Elute bound calpain with 50 m*M* Tris-HCl, pH 7.5, 5 m*M* EGTA, 2 m*M* EDTA, 5 m*M* 2-ME.
6. Dialyze the eluted enzyme extensively against an appropriate buffer to remove excess chelator and Ca^{2+}-chelator complexes, and casein fragments, especially if active enzyme has been purified.

The use of an immobilized peptide from the Ca^{2+}-ATPase for affinity purification of μ-calpain ***(8)*** is described in Chapter 5.

4. Notes

1. We have no experience with other sources or variants of Reactive Red resins. Other types are expected to be suitable but might require modification of the method described.
2. This method was first described for purification of m-calpain from smooth muscle ***(9)***. The Reactive Red dye matrix was originally designed to bind NADH/NADPH-dependent enzymes that bind to the procion red dye at low ionic strength and can be eluted by increasing the salt concentration. By contrast, the binding of m-calpain appears to be predominantly through hydrophobic interactions because binding occurs at 0.5 *M* NaCl and elution is achieved by reducing ionic strength.
3. The amount of resin required needs to be determined empirically as it is highly dependent on the sample applied. From experiments with partially purified recombinant calpain it is clear that more than 15–20 mg can bind to 25 mL resin; however if the applied sample contains many proteins that also bind to the resin, possibly even more tightly than calpain, the useful capacity may be reduced. In experiments where the binding capacity of the resin is exceeded, calpain activity is detectable in the wash fractions and may be recovered by reapplication to fresh, or recycled, resin in a separate experiment.
4. Purchased slurries may contain thimerosal, a mercurial antibacterial agent. If these are present, add 10 m*M* 2-ME and 2 m*M* EDTA to the solutions used to prepare the column.
5. Many other proteins are retained on the resin after the standard calpain chromatography

procedure and must be removed before reuse. Regeneration of the resin can be done at room temperature, but chromatography is carried out at 4°C.

6. m-Calpain fractions eluted from DEAE chromatography are stable for a 4°C overnight dialysis into binding buffer-1. It seems to be important to use MOPS buffer, or at least to avoid Tris buffers. Although we normally use pH 7, similar results are obtained at pH 7.5.
7. For extracts from *E. coli*, chromatography must proceed quickly after addition of salt, because the solution becomes increasingly turbid and will form a precipitate. If the cloudy supernatant is viscous it may need further sonication to fragment DNA, or it may be diluted with binding buffer-1. For large-scale preparations from any source it is advisable to use DEAE chromatography, with either gradient or batchwise elution, before applying samples to Reactive Red-agarose.
8. The abrupt fall in ionic strength produced by draining the buffer from the top of the column before applying the next eluant, appears to be significant for achieving a sharp elution peak. Some red dye leaks from the column during elution, particularly if resin has not been used for long periods of time. In our hands the resin has a useful lifetime of several years. The leaked dye is readily separated from the enzyme by dialysis or subsequent chromatography steps.
9. Eluted enzyme activity appears to be quite stable at 4°C as long as the enzyme concentration is greater than 0.2 mg/mL protein.
10. The composition of the 10× buffer may be adapted to suit a subsequent chromatography step. Routinely we use 0.2 *M* MOPS, pH 7.5, 10 m*M* EGTA, 10 m*M* EDTA, and 50 m*M* 2-ME.
11. The truncated small subunit is overexpressed in *E. coli* BL21(DE3) cells relative to the 80 kDa catalytic subunit ***(7)***. Excess small subunit can be removed by gradient elution from DEAE before chromatography on Reactive Red-agarose. If the DEAE column is omitted, the 21 kDa protein will also bind to the Reactive Red-agarose and can be eluted with low ionic strength MOPS buffer before elution of the heterodimer with water.
12. The difference in elution conditions required for natural (80 + 28 kDa) and recombinant m-calpain (80 + 21 kDa) suggests that autoproteolyzed calpain within a tissue or tissue homogenate may be separated from the intact enzyme by this resin. This is advantageous, as the elution product is stable and more homogeneous for further study. It also suggests a potential method for studying physiological alterations of calpain that has not yet been exploited, if autoproteolysis is indicative of activation, as suggested by some authors.
13. Unlike m-calpain, μ-calpain does not bind tightly to Reactive Red-agarose. When fractions containing this isoform of calpain from bovine heart are passed through the resin under identical binding conditions to those described above, the enzyme is retarded; that is, it is not in the unbound fraction but it appears to wash out of the column in the 0.5 *M* NaCl buffer. Increasing the NaCl to 1 *M* allows a sharper elution and a significant purification of the enzyme from bovine heart ***(1)***.
14. μ-Calpain elutes from DEAE columns together with calpastatin and the bulk of

cellular proteins in extracts of mammalian tissues such as bovine heart, so that some additional purification is recommended before the Reactive Red-agarose step. Typically this crude enzyme fraction is further purified by either gel filtration, after ammonium sulfate precipitation to decrease its volume; or by hydrophobic interaction chromatography on phenyl-Sepharose (***1***). We prefer phenyl-Sepharose because it takes significantly less time. Significant purification of μ-calpain on phenyl-Sepharose can be achieved by binding at an intermediate ionic strength (50 m*M* Tris-HCl, pH 7.5, 50 m*M* KCl, 2 m*M* EDTA, 2 m*M* EGTA, 5 m*M* 2-ME) and eluting in 5 m*M* MOPS, pH 7.0, 2 m*M* EGTA, 2 m*M* EDTA, 5 m*M* 2-ME. This requires only dilution of the sample with MOPS and addition of NaCl for application to the Reactive Red-agarose column.

15. This concentration of calcium is larger than is strictly necessary but allows for the presence of other calcium binding proteins, including casein, and chelators in the enzyme sample. If the enzyme volume is large, and includes significant amounts of chelators, additional calcium may be added to ensure an excess of 2–3 m*M* Ca^{2+} during binding. Note that reaction of Ca^{2+} with EDTA generates H^+, and the pH must be maintained at 7–7.5.
16. Rapid mixing is important to promote the rapid binding between calpain and the immobilized casein. This aims to minimize autoproteolysis when working with active enzyme and to minimize aggregation, because the enzyme is exposed to calcium only in the presence of excess substrate.

References

1. Croall, D. E. and DeMartino, G. N. (1984) Comparison of two calcium dependent proteinases from bovine heart. *Biochim. Biophys. Acta* **788,** 348–355.
2. DeMartino, G. N. and Croall, D. E. (1983) Purification and characterization of a calcium dependent protease from rat liver. *Biochemistry* **22,** 6287–6291.
3. Yoshimura, N., Kikuchi, T., Sasaki, T., Kitahara, A., Hatanaka, M., and Murachi, T. (1983) Two distinct Ca^{2+} dependent proteases (calpain I and calpain II) purified concurrently by the same method from rat kidney. *J. Biol. Chem*. **25,**, 8883–8889.
4. Kubota, S., Suzuki, K.,and Imahori, K. (1981) A new method for the preparation of a calcium activated neutral protease highly sensitive to calcium ions. *Biochem. Biophys. Res. Commun.* **100,** 1189–1194.
5. Croall, D. E. and DeMartino, G. N. (1983) Purification and characterization of calcium dependent proteases from rat heart. *J. Biol. Chem*. **258,** 5660–5665.
6. Anagli, J., Vilei, E. M., Molinari, M., Calderara, S., and Carafoli, E. (1996) Purification of active calpain by affinity chromatography on an immobilized peptide inhibitor. *Eur. J. Biochem*. **241,** 948–954.
7. Elce, J. S., Hegadorn, C., Gauthier, S., Vince, J. W., and Davies, P. L. (1995) Recombinant calpain II: Improved expression systems and production of a C105A active-site mutant for crystallography. *Protein Eng.* **8,** 843–848.
8. Molinari, M., Maki, M., and Carafoli, E. (1995) Purification of μ-calpain by a novel affinity chromatography approach. *J. Biol. Chem*. **270,** 14576–14581.

9. Hathaway, D. R., Werth, D. K., and Haeberle, J. R. (1982) Limited autoproteolysis reduces the Ca^{2+} requirement of a smooth muscle calcium activated neutral protease. *J. Biol. Chem.* **257,** 9072–9077.
10. Graham-Siegenthaler, K., Gauthier, S., Davies, P. L., and Elce, J. S. (1994) Active recombinant calpain. *J. Biol. Chem.* **26,** 30457–30460.
11. Croall, D. E. and DeMartino, G. N. (1986) Calcium dependent affinity purification of transglutaminase from rat liver. *Cell Calcium* **7**, 29–39.

5

Affinity Purification of μ-Calpain from Erythrocytes on an Immobilized Peptide from the Plasma Membrane Calcium Pump

Some Studies on Erythrocyte μ-Calpain

Maurizio Molinari and Ernesto Carafoli

1. Introduction

The plasma membrane Ca^{2+}-ATPase (Ca^{2+} pump) ***(1)*** is a ubiquitously distributed P-type ATPase ***(2)***, consisting of a single polypeptide chain of about 134 kDa, which spans the cellular membrane 10 times. A large cytosolic loop (between transmembrane segments 4/5) contains the catalytic region of the enzyme. A peculiarity of the Ca^{2+} pump among P-type ATPases is an extended C-terminal tail of about 160 residues, which is the target of many regulators of the pump's activity, such as calmodulin, some kinases, and calpain. Under resting conditions, the C-terminal tail acts as an autoinhibitory domain covering the active site. Energy from ATP hydrolysis allows the extrusion of cytosolic Ca^{2+} by the pump against a 10,000-fold concentration gradient. Since the maintenance of a submicromolar Ca^{2+} concentration in the cytosol is vital to its second messenger function, the role played by the Ca^{2+}-ATPase in cellular regulation is obvious.

Calmodulin and calpain are important regulators of Ca^{2+}-ATPase activity. Calmodulin, an acidic protein that changes conformation and interacts with target proteins upon Ca^{2+}-binding, is the canonical activator of the pump ***(3,4)***. Under conditions of increased cytosolic Ca^{2+}, calmodulin interacts with the C-terminal portion of the Ca^{2+} pump and removes it from its binding site near the active site. This exposes the active site and increases the affinity of

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the enzyme for Ca^{2+}, which is then efficiently extruded to the extracellular milieu. The activation by calmodulin is reversible, and the activity of the pump returns to its low resting level when a fall in cytosolic Ca^{2+} concentration promotes calmodulin dissociation.

Calpain cleaves the pump within the C-terminal calmodulin-binding domain ***(5)***. It may be noted that the C-terminal domains of the two calpain subunits are themselves called "calmodulin-like," which may partly explain why both calmodulin and calpain interact with the same portion of the Ca^{2+}-ATPase. At least in erythrocytes, Ca^{2+}-ATPase has been shown to be the preferred calpain substrate ***(6)***. This observation has prompted us to use the Ca^{2+}-ATPase as a model substrate for calpain. Thus we have used the native Ca^{2+}-ATPase embedded in the erythrocyte membrane, as well as the isolated pump, or recombinant portions of it, including the calpain cleavage and recognition sites, to investigate debated topics of the calpain field, such as the autoproteolysis requirement for calpain activity and the importance of PEST sequences in promoting the interaction of calpain with substrates ***(7–9)***. We present here a method for purification of μ-calpain, which depends on its Ca^{2+} dependent binding to its target peptide in the Ca^{2+}-ATPase.

2. Materials

1. Pall™ filter (Pall Schweiz AG, Muttenz, Switzerland)
2. Synthetic polypeptide of 49 amino acids, C49, EEIPEEELAE DVEEIDHAER ELRRGQILWF RGLNRIQTQI RVVNAFRSS ***(9)***, comprising the C-terminal calmodulin binding region of isoform 1CI of the plasma membrane Ca^{2+}-ATPase (hPMCA1CI) ***(10)***.
3. Wash buffer: 100 m*M* $NaHCO_3$, pH 8.3, 500 m*M* NaCl, 0.05 m*M* $CaCl_2$.
4. Buffer A: 50 m*M* sodium acetate, pH 6.7, 1 m*M* EDTA, 1 m*M* EGTA, 0.5 m*M* 2-ME.
5. Buffer B: 50 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, 1 m*M* EGTA, 0.5 m*M* 2-ME.

3. Methods

3.1. Coupling of Peptide C49 to CNBr-Activated Sepharose 4B

1. Swell 1 g of CNBr-activated Sepharose 4B for 15 min in 25 mL of 1 m*M* HCl, and wash the resin on a sintered glass funnel with 500 mL of 1 mM HCl.
2. Dissolve 10 mg of peptide C49 (1.7 μmoles) in 7.8 mL of 100 m*M* $NaHCO_3$, 500 m*M* NaCl, 0.05 m*M* $CaCl_2$, pH 8.3, mix with the wet resin and incubate overnight at 4°C.
3. Wash the resin with 100 m*M* $NaHCO_3$, 500 m*M* NaCl, 0.05 m*M* $CaCl_2$, pH 8.3.
4. Incubate overnight in 10 mL of 1 *M* ethanolamine.
5. The C49-resin is ready for use after an additional washing step with 50 m*M* Tris-HCl, pH 7.5, 0.5 m*M* $CaCl_2$, 0.5 m*M* 2-ME.

3.2. Purification of Calpain From Human Erythrocytes

1. Filter 1 unit (450 mL) of freshly drawn venous human blood in citrate buffer through a Pall™ filter to eliminate the white cells (*see* **Notes 1** and **2**).
2. Centrifuge the filtrate at 800*g* for 15 min to obtain the erythrocytes. All subsequent steps are performed at 4°C.
3. Wash the erythrocytes three times in 500 mL of ice-cold PBS, pH 7.2, 1 m*M* EDTA, by gentle resuspension and centrifugation at 800*g* for 15 min.
4. Lyse the packed erythrocytes (250 mL) in 5 vol of 10 m*M* sodium acetate, pH 7.2, 2.5 m*M* EDTA, 0.1 m*M* PMSF.
5. Centrifuge the hemolysate at 13,000*g* for 20 min to remove the cell membranes.
6. Incubate the membrane-free lysate with 165 g of DEAE-Sepharose CL-6B (Pharmacia, Uppsala, Sweden) previously equilibrated with buffer A.
7. Adjust the pH to 6.7 and stir the suspension for 30 min.
8. Wash the lysate-loaded DEAE-Sepharose in a Buchner funnel with 10 L of buffer A containing 50 m*M* NaCl, and pack the resin in a glass column (5 × 10 cm).
9. Elute the peak (400 mL) containing μ-calpain in a single step with 0.2 *M* NaCl in buffer A.
10. Precipitate protein with 45% saturated ammonium sulfate.
11. Resuspend the protein pellet in 30 mL of buffer B containing 50 m*M* NaCl, and dialyze overnight against the same buffer.
12. Centrifuge the dialyzed sample at 20,000*g* and rechromatograph the supernatant on a DEAE-Sepharose column (~50 mL of equilibrated DEAE-Sepharose CL-6B) with a linear gradient of 0.05–0.2 *M* NaCl in buffer B. μ-Calpain is eluted between 0.15 and 0.17 *M* NaCl.
13. Locate the μ-calpain by SDS-PAGE of fractions from this column (*see* **Note 3**).
14. Pool the relevant fractions and concentrate the μ-calpain with an Amicon (Wallisellen, Switzerland) concentrator (membrane cutoff: 30 kDa), using three rapid steps of dilution with buffer B and recentrifugation in place of overnight dialysis.
15. Calpastatin can be eliminated by chromatography on butyl-agarose, since it does not bind to this resin, but calpain binds at low salt concentration and is eluted with a linear NaCl gradient (0–1 *M* NaCl in buffer B). The μ-calpain is eluted at ~0.7 *M* NaCl (*see* **Note 4**).

3.3. Affinity Purification of Calpain

Starting from the partially purified μ-calpain obtained in **Subheading 3.2. step 12**, an efficient purification of calpain is achieved by affinity chromatography on the C49 peptide derived from the calmodulin-binding domain of the plasma membrane Ca^{2+}-ATPase (*see* **Note 5**).

1. Inactivate the partially purified μ-calpain by incubation with 100 μ*M* (final concentration) of the irreversible, active site-directed calpain inhibitor Cbz-Leu-Leu-Tyr-CHN_2 for 30 min in the absence of Ca^{2+} with gentle mixing at 4°C.
2. Add $CaCl_2$ stepwise to a final concentration of 500 μ*M* and incubate on ice for an additional 3 h (*see* **Note 6**).

3. Load the inhibitor-modified crude enzyme preparation onto the C49-Sepharose affinity column (0.8 × 6 cm) previously equilibrated with wash buffer.
4. Wash the column with 100 mL of wash buffer containing 1 *M* NaCl and then with 100 mL of wash buffer to remove contaminating proteins.
5. Elute the bound calpain with 50 m*M* Tris-HCl, pH 7.5, 2 m*M* EDTA, 0.5 m*M* 2-ME.

3.4. Study of Calpain Activity in Erythrocytes In Vivo

Erythrocyte preparation and washing is performed at room temperature.

1. Filter 20 mL of freshly collected human blood through a Pall™ filter into clean centrifugation tubes to eliminate the white cells *(see* **Notes 1** and **7**).
2. Wash the erythrocytes by centrifugation at 800*g* for 15 min and resuspend them in an isotonic buffer (10 m*M* HEPES, pH 7.3, 140 m*M* NaCl, 5 m*M* KCl, 2 m*M* EDTA).
3. Wash three times in the same buffer without EDTA.
4. Finally resuspend the erythrocytes from 20 mL of blood in 20 mL of 10 m*M* HEPES, pH 7.3, 140 m*M* NaCl, 5 m*M* KCl, 3 m*M* $MgCl_2$. Modulation of the calcium concentration in the erythrocyte cytosol is obtained by selective permeabilization of the cell membrane with a calcium ionophore (A23187) in the presence of Ca^{2+}.
5. Preincubate 1 mL aliquots of the cells with 1–3 µM A23187 for 15 min at 37°C (*see* **Note 8**).
6. Add 100 µ*M* Ca^{2+} (*see* **Note 9**).
7. At times from 0 to 3 h, centrifuge the erythrocytes at 800*g* for 15 min and remove the supernatant buffer.
8. Lyse the cells in 10 vol of ice-cold 10 m*M* HEPES, pH 7.3.
9. Separate the membranes by centrifugation at 13,000*g* for 20 min, mix samples of the diluted cytosolic supernatant with SDS gel sample buffer, and analyze the soluble proteins by gel electrophoresis and immunoblotting.
10. Wash the membranes three times with 10 m*M* HEPES, pH 7.3, resuspend and heat in SDS gel sample buffer, and analyze membrane-bound proteins by immunoblotting.
11. Perform control experiments including incubation of erythrocytes with the ionophore in the presence of 1 m*M* EDTA, and incubation of erythrocytes with a specific, cell-permeable calpain inhibitor such as Cbz-Leu-Leu-Tyr-CHN_2, which is added to the cells before the addition of Ca^{2+}.

3.5. Study of Calpain Activity in Isolated Erythrocyte Membranes

1. Mix erythrocytes with 30 µ*M* Ca^{2+} and 1 µ*M* A23187 to induce calpain migration to the membrane, and incubate for 15 min at room temperature.
2. Lyse erythrocytes in 10 vol of 10 m*M* Hepes, pH 7.5, 1.75 µ*M* Ca^{2+}.
3. Recover and wash the membranes three times with 10 m*M* HEPES, pH 7.3, 1.75 µ*M* Ca^{2+}, to remove cytosolic contaminants (especially cytosolic calpain), and continue the incubation at room temperature in the presence of 1.75 µ*M* total Ca^{2+}.

4. Resuspend and heat the membranes in SDS gel sample buffer and analyze the content of calpain, Ca^{2+}-ATPase, band 3 and band 4.2 by immunoblotting with specific antibodies.

4. Notes

1. In all our studies we have used human-derived material such as calpain and the Ca^{2+}-ATPase. For optimal results, the blood used to isolate calpain must be freshly drawn. It is therefore impossible to test the blood for the presence of viruses such as human immunodeficiency virus (HIV) or hepatitis. In our case one of the authors (M.M.) was the donor, and the blood was processed only a few minutes after collection.
2. The stability of the purified μ-calpain depends strongly on the rapidity of processing the raw material. The purification procedure should be accomplished in 2 days; the protein stored at 4°C is then stable for months.
3. Calpain cannot be assayed by conventional assay in the fractions collected from the second DEAE-chromatography step, because the fractions still contain calpastatin. These fractions are therefore analyzed by SDS-PAGE (or by casein zymography). The 80-and 30-kDa bands corresponding to the catalytic and regulatory subunits of μ-calpain are easily visible by Coomassie Brilliant Blue staining.
4. Calpain can be detected in the fractions by SDS-PAGE or by activity measurement. Further purification by gel filtration on Sephadex G-200 is possible, but greatly dilutes the enzyme.
5. Essentially similar affinity purification schemes for calpain have been described using Ca^{2+}- dependent binding of calpain to peptides derived from calpastatin ***(11,12)***.
6. Under these conditions the inhibitor alkylates the essential cysteine residue at the active site of calpain, preventing autolysis and blocking the enzyme in the native 80/30 kDa heterodimeric form. It is possible to run the affinity columns with active calpain in the presence of reversible calpain inhibitors such as leupeptin, but some degree of autolysis cannot then be avoided.
7. Human erythrocytes represent a simple cell model to study events linked to the increased Ca^{2+} concentration in cytosol, including calpain activity. The cells are easy to prepare and 10 mL of freshly drawn blood is an adequate amount. Filtering the blood through Pall™ filter is the easiest procedure to eliminate the white cells completely. Alternatively, white cells, which are visible during the low speed washes above the erythrocytes, can be carefully aspirated with a Pasteur pipette. Erythrocytes are rich in calpain, and its preferred substrates (the Ca^{2+}-ATPase and spectrin) are well characterized. This represents a simple and fast protocol to prepare human erythrocytes to be used as a model to study the translocation of calpain into the membrane fraction in vivo, as well as the proteolysis of substrates.
8. It is important that the ionophore concentration should not exceed these values. Higher concentrations (20 μ*M* is a concentration often found in the literature) are deleterious to the stability of the cell membrane. In the case of erythrocytes, membrane damage is indicated by the release of hemoglobin in the buffer. Check this by pelletting the erythrocytes in Eppendorf tubes: if membrane leakage has occurred in control cells the supernatant buffer will become red.

9. The Ca^{2+} added to the medium equilibrates rapidly with the erythrocyte cytosol and leads to calpain association with the cell membrane as well as to the activation of its proteolytic activity toward the Ca^{2+}-ATPase. In this model system, at calcium concentration in the low micromolar range, the proteolysis of the Ca^{2+}-ATPase has been shown to precede the autolytic cleavage of calpain, which dissociates from membranes in its uncleaved form upon removal of calcium. At higher concentrations of calcium, calpain eventually undergoes proteolytic cleavage and attacks a wider range of structural proteins (spectrin, band 3, band 4.2).

References

1. Schatzmann, H. J. (1966) ATP–dependent calcium extrusion from human red cells. *Experientia* **22,** 364–368.
2. Pedersen, P. L. and Carafoli, E. (1987) Ion motive ATPases: Ubiquity, properties and significance to cell function. *Trends Biochem. Sci.* **12,** 146–150.
3. Gopinath, R. M. and Vincenzi, F. F. (1977) Phosphodiesterase protein activator mimics red blood cell cytoplasmic activator of the (Ca^{2+}, Mg^{2+})-ATPase. *Biochem. Biophys. Res. Commun.* **77,** 1203–1209.
4. Jarrett, H. W. and Penniston, J. T. (1977) Partial purification of the (Ca^{2+}, Mg^{2+})-ATPase activator from human erythrocytes: Its similarity to the activator of 3'–5' cyclic nucleotide phosphodiesterase. *Biochem. Biophys. Res. Commun.* **77,** 1210–1216.
5. James, P., Vorherr, T., Krebs, J., Morelli, A., Castello, G., McCormick, D. J., Penniston, J. T., De Flora, A., and Carafoli, E. (1989) Modulation of erythrocyte Ca^{2+}-ATPase by selective calpain cleavage of the calmodulin–binding domain. *J. Biol. Chem.* **264,** 8289–8296.
6. Salamino, F., Sparatore, B., Melloni, E., Michetti, M., Viotti, P. L., Pontremoli, S., and Carafoli, E. (1994) The plasma membrane calcium pump is the preferred calpain substrate within the erythrocyte. *Cell Calcium* **15,** 28–35.
7. Molinari, M., Anagli, J., and Carafoli, E. (1994) Ca^{2+}–activated neutral proteinase is active in the erythrocyte membrane in its non–autolyzed 80–kDa form. *J. Biol. Chem.* **269,** 27,992– 27,995.
8. Molinari, M., Anagli, J., and Carafoli, E. (1995) PEST sequences do not influence substrate susceptibility to calpain proteolysis. *J. Biol. Chem.* **270,** 2032–2035.
9. Molinari, M., Maki, M., and Carafoli, E. (1995) Purification of μ–calpain by a novel affinity chromatography approach. *J. Biol. Chem.* **270,** 14,576–14,581.
10. Carafoli, E. (1994) Plasma membrane calcium ATPase: 15 years of work on the purified enzyme. *FASEB J.* **13,** 993–1002.
11. Asada, K., Ishino, Y., Shimada, M., Shimojo, T., Endo, M., Kimizuka, F., Kato, I., Maki, M., Hatanaka, M., and Murachi, T. (1989) cDNA cloning of human calpastatin: Sequence homology among human, pig, and rabbit calpastatins. *J. Enzyme Inhib.* **3,** 49–56.
12. Anagli, J., Vilei, E. M., Molinari, M., Calderara, S., and Carafoli, E. (1996) Purification of active calpain by affinity chromatography on an immobilized peptide inhibitor *Eur. J. Biochem.* **241,** 948–954.

6

Expression of m-Calpain in *Escherichia coli*

John S. Elce

1. Introduction

The purification of μ-and m-calpain on a medium to large scale from animal tissues such as bovine heart or skeletal muscle, and on a microscale from less than 0.5 g of tissue, is described in Chapters 1 and 2. These preparations are excellent for many purposes, but for some other purposes, for example for crystallization of calpain, and for structure–function studies which require mutations, it is clear that artificial expression of the cloned enzymes is required. The cloning also provides the opportunity to introduce a His-tag, which fortunately in m-calpain does not affect activity; the His-tag is not obligatory, but it is found to increase the expression yield markedly, and its presence enormously simplifies the purification, making it possible to achieve a higher final degree of purity with a reasonable yield.

Methods of expression of foreign proteins in systems such as *E. coli*, yeast, and insect cells, have improved greatly over the last few years, with continuous introduction of modifications both to the expression vectors and to the host cells. Nonetheless, success in expressing a new protein remains highly unpredictable. In the calpain field, it is found that m-calpain is readily expressed in *E. coli* in very useful yields ***(1)***, but the yield of μ-calpain from *E. coli* expression is very poor, for reasons which remain obscure, and for μ-calpain it seems to be necessary to use the baculovirus system and insect cells ***(2)***. We have confirmed the success of the baculovirus system in expressing rat μ-calpain, but have little experience in that system. This chapter is confined to bacterial expression of m-calpain.

Calpain offers the further complication that two different subunits must be expressed. While in principle the two subunits could be separately expressed and later combined, in practice reassociation of calpain subunits that have been dissociated by denaturation is not efficient ***(3)***. In addition, while some small

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subunit constructs expressed alone in *E. coli* give high yields of soluble protein, the m-calpain 80-kDa large subunit expressed alone gives only low yields of soluble (and inactive) large subunit protein ***(3)***. It is therefore necessary to engineer compatible plasmids for coexpression of the large and small subunits ***(1)***.

Most of our work on expression of m-calpain has used a fully active combination of an 80 kDa large subunit with a C-terminal His-tag, and a 21 kDa small subunit construct, which contains only the C-terminal 184 amino acid residues of the natural 270 residue subunit, the Ca^{2+}-binding domain, of the small subunit. This construct is referred to as m-80k-$CHis_6$/21k. The presence and location of the His-tag can be varied without impairing activity, but the 80 kDa subunit with a C-terminal His-tag gives the highest yields of soluble active calpain ***(4)***. The full-length 28-kDa small subunit can also be expressed, but the yields of activity are smaller, and we have not been able to prevent considerable proteolysis of domain V of this small subunit during extraction from *E. coli* ***(5)***. The resulting calpain is heterogeneous with respect to its small subunit, and contains only a small proportion of small-subunit-intact calpain.

Purification of the bacterially expressed m-80k-$CHis_6$/21k differs very little in principle from that of natural calpain, except for two factors. The inclusion of the His-tag provides a simple and highly specific step in purification; and the recombinant m-calpain represents a much higher percentage of the protein in the initial extract than in an extract of animal tissue. Purification of active enzyme is followed by normal casein-based assays. For inactive mutants of m-calpain, a Western blot probed with a large subunit antibody may be desirable to locate the product in the eluate of the first column, particularly if a given mutant is less well expressed, but in general the large subunit is sufficiently enriched that it may be detected simply by Coomassie brilliant blue staining of SDS gels.

We describe here in brief form a protocol for expression and purification of rat m-calpain as a heterodimer of 80 kDa and 21 kDa subunits. The yields of expression depend on cell densities and on the individual construct, but in terms of the caseinolytic activity unit used in this laboratory ***(1)***, the initial yield of m-calpain activity from 4 L of *E. coli* is ~36,000 units (the specific activity of the purified enzyme is ~1800 U/mg). The yield of pure protein from 8 L, after 3 or 4 column steps, is commonly between 15 and 20 mg.

2. Materials

1. cDNA clones for calpain subunits:
   a. pET-24-m-80k-$CHis_6$ (cDNA encoding the rat m-calpain 80 kDa large subunit with a C- terminal His-tag with a total of 714 amino acid residues) ***(1,6)***; the expression vector pET-24 (Novagen Inc., Madison, WI) carries the gene for kanamycin resistance, the ColE1 origin of replication, and the T7 promoter sequence;

b. pACpET-21k (cDNA encoding the C-terminal 184 amino acid residues of the rat calpain small subunit) ***(1,3)***; this vector contains the gene for ampicillin resistance, the A15 origin of replication, and the T7 promoter.

2. *E. coli* strain BL21(DE3), without pLysS, (Novagen) cotransformed with both of the above plasmids, and maintained on media containing 100 µg/mL ampicillin and 50 µg/mL kanamycin. Frozen stocks of the transformant are kept at –70°C.
3. Growth medium (LB): premixed powder for bacterial culture (Difco Labs, Detroit, MI), dissolved at the rate of 25 g/L in water, and autoclaved.
4. Antifoam 289 (Sigma, St. Louis, MO).
5. STE: 10 m*M* Tris-HCl, pH 7.8, 1 m*M* EDTA, 0.1 *M* NaCl.
6. TE (10 × stock): 250 m*M* Tris-HCl, pH 7.6 (at room temperature), 50 m*M* EDTA, 0.01% sodium azide. This is diluted 10-fold in ice-cold water and 10 m*M* 2-ME is added just before use.
7. Lysis buffer: 25 m*M* Tris-HCl, pH 7.6 (at room temperature), 5 m*M* EDTA, 5% (v/v) glycerol, 50 mg/L (0.3 m*M*) PMSF (added from an ethanol stock solution immediately before use), 10 m*M* 2-ME.
8. Chromatography resins: Whatman DE 52 DEAE cellulose, or DEAE-Sepharose (Pharmacia, Piscataway, NJ); Ni-NTA-agarose (Qiagen, Chatsworth, CA); Q-Sepharose 16/10 FPLC column (Pharmacia) or Q25 FPLC column (BioRad, Hercules, CA); gel filtration column such as Ultragel AcA 44, Sephacryl S-300, or similar.
9. Materials for Tris-Tricine SDS polyacrylamide gel electrophoresis ***(7)***.

3. Methods

3.1. Bacterial Expression

We describe here expression of m-calpain on a scale of 8 L of bacterial culture, which normally provides 15–20 mg of very highly purified m-calpain after three or four column steps in approx 1 wk. The scale and extent of purification can be varied to suit the experimental needs and laboratory facilities. It is important to complete the purification without delay. If a pause is necessary, the enzyme at any stage should be stored with 50% glycerol at –20°C. The glycerol will have to be removed by dialysis before a subsequent step of purification.

1. Streak a fresh LB/agar plate containing kanamycin (50 µg/mL) and ampicillin (100 µg/mL) from the frozen permanent of *E. coli* strain BL21(DE3) carrying plasmids for large and small calpain subunits, and leave the plate at 37°C overnight.
2. Starting, for example, on day 1 at 8 A.M., pick two single colonies and grow each in 10 mL of LB/kan/amp at 37°C for ~ 8 h with vigorous shaking.
3. During this day, autoclave 2 × 200 mL of LB in 1-L conical flasks, and 2 x 4 L of LB in 6-L conical flasks. The latter should be left overnight at 37°C.
4. Add each 10 mL culture to 200 mL of LB together with kanamycin (50 µg/mL) and ampicillin (100 µg/mL) and grow at 30°C overnight with vigorous shaking.
5. Next morning (day 2), add each 200-mL culture to 4 L of prewarmed LB, and place the 6-L flasks in a water bath at 30°C.

6. Add to each 4-L culture: 400 mg of ampicillin powder; 200 mg of kanamycin powder; 0.4 mL of antifoam.
7. Provide very vigorous aeration by means of a sparger attached to the air supply (*see* **Note 1**).
8. Monitor growth of the *E. coli* at 30°C by observation of the $A_{600\ nm}$. This value should reach ~0.8–1.0 within 3–4 h of growth (*see* **Notes 2** and **3**).
9. When $A_{600\ nm}$ reaches ~1.0, add 400 mg of IPTG (0.15 m*M* final concentration), and continue growth at 30°C with vigorous aeration for 2.5–3 h (*see* **Note 4**).
10. Recover the *E. coli* by centrifugation in 1-L bottles in swing-out buckets of a Beckman 4.2 rotor at 2500*g* for 40 min at 4°C; after centrifuging the first set of 4 L, decant the supernatant, and add the second set of 4 L of culture to the same centrifuge bottles. Centrifuge as before.
11. Resuspend the cells and combine them in ~150 mL of ice-cold STE; recover the cells by centrifugation at 2500*g* for 30 min at 4°C.
12. Resuspend the cells in 120 mL of ice-cold lysis buffer, with freshly added PMSF and 2-ME, and store the suspension in a 150–200 mL glass beaker at –20°C (*see* **Note 5**). This suspension is normally treated on the next day but may be stored at least for a few days at –20°C.

3.2. Purification of Bacterially Expressed m-80k-CHis$_6$/21k Calpain

All procedures are carried out on ice or at 4°C.

1. (Day 3). Thaw the cell suspension cautiously in a beaker of warm water until just a little ice remains.
2. Add fresh PMSF to a final concentration of 50 mg/L (0.3 m*M*), and fresh 2-ME to 10 mM.
3. Place the beaker containing the cell suspension in an ethanol/ice bath.
4. Sonicate the suspension for 4 to 6 times 30 s, with at least 60-s intervals between bursts. Make sure that the suspension does not get warm. Satisfactory lysis can be judged (with some experience) by a change from a white dense suspension (viewed in a Pasteur pipet) to a brown, less opaque, suspension (*see* **Note 6**).
5. Centrifuge the sonicated lysate at 25,000*g* for 30–40 min in a JA20 rotor or equivalent.
6. During this centrifugation, set up 150 mL (settled volume) of DEAE-cellulose stirring with a magnetic stirrer in ~600 mL of TE and fresh 10 m*M* 2-ME.
7. Pour the supernatant from the sonicated lysate centrifugation into the stirred DEAE-celluose suspension. Continue stirring for 30–60 min, then stop the stirrer and permit the DEAE-cellulose to settle for 30–60 min (*see* **Note 7**).
8. Decant 90% of the slightly turbid supernatant solution. Pour the remaining slurry of DEAE- cellulose into a 2.6 × 40 cm column and permit the resin to settle.
9. Wash the column at 1 mL/min with TE for 60 min, and elute the bound protein with a gradient of zero to 0.75 *M* NaCl in TE, in a total volume of 700 mL. The gradient is run overnight at 1 mL/min, and fractions of 8–10 mL are collected (*see* **Note 8**).

10. (Day 4). Assay the eluted fractions for m-calpain activity by means of a conventional casein-based assay. m-Calpain is eluted at approx fractions 35–45 in the conditions described, at a conductivity of 12–18 mmho, or a NaCl concentration of ~0.25 *M* (*see* **Note 9**).

3.3. Subsequent Column Steps

As described in other chapters, there are many possible variations of columns and order of columns. We have found it most reliable, following DEAE-cellulose chromatography, to follow the sequence: (possibly ammonium sulfate precipitation and molecular sieve chromatography), Ni-nitrilotriacetic acid (NTA)-agarose, and finally a high-resolution Q-Sepharose column. For many purposes the molecular sieve column may be omitted, although it does remove a small amount of high molecular mass aggregated material. This procedure avoids or minimizes concentration or dialysis of enzyme solutions between columns. Alternative column types that work fairly reliably for recombinant m-calpain include phenyl-Sepharose and Reactive Red-agarose (Chapter 4).

3.3.1. Ni-NTA-agarose

1. (Day 4). Prepare a column (1 × 10 cm) of Ni-NTA-agarose in 50 m*M* Tris-HCl, pH 7.6, 0.4 *M* NaCl, 4 m*M* imidazole (*see* **Note 10**).
2. Prepare the fraction collection tubes for this column by addition to each tube, immediately before running the column, of 0.2 mL of 50 m*M* Tris-HCl, pH 7.6, 0.1 *M* EDTA, 0.1 *M* 2-ME.
3. Pool the desired fractions from the DEAE-cellulose column—typically providing a pool of 80 mL, which contains ~ 0.25 *M* NaCl and 5 m*M* EDTA.
4. Add to this pool solid NaCl to a final concentration of 0.4 *M* NaCl; 2 mL of 1 *M* Tris-HCl, pH 7.6; and 1 mL of 1 *M* $MgCl_2$ to saturate the EDTA. Add the $MgCl_2$ slowly, and check that the pH remains close to 7.6 (*see* **Notes 11** and **12**).
5. Apply the pooled enzyme solution to the Ni-NTA column at 1 mL/min, followed by a wash of 20–30 mL of 50 m*M* Tris-HCl, pH 7.6, 0.4 *M* NaCl, 4 m*M* imidazole, 5 m*M* 2-ME.
6. Elute the bound calpain with a gradient from 4 m*M* to 250 m*M* imidazole in a total volume of 200 mL of 50 m*M* Tris-HCl, pH 7.6, 0.4 *M* NaCl, 5 m*M* 2-ME, collecting 4 mL fractions (*see* **Note 13**).
7. The His-tagged m-calpain is eluted from the column between 30 and 80 m*M* imidazole, in a volume of ~30 mL. This peak can be detected by absorption at 280 nm or by Bradford protein assay, and an SDS gel is not normally necessary.

3.3.2. Gel filtration

8. (Day 5). Concentrate the pool of m-calpain to ~5 mL either by ammonium sulfate precipitation at 60% saturation, or by ultrafiltration in a Biomax-10K Ultrafree 15 centrifugal filter (Millipore, Bedford, MA), in accordance with the manufacturer's instructions.

9. Apply the sample to a column (2.5 × 90 cm) of AcA 44 equilibrated in 25 m*M* Tris-HCl, pH 7.6, 5 m*M* EDTA, 0.2 *M* NaCl, 2% glycerol, 10 m*M* 2-ME, and elute the column with this buffer at a flow rate of 0.75 mL/min overnight, collecting fractions of ~7 mL. On this column the main peak of m-calpain activity is observed at an elution volume of ~175 mL.
10. (Day 6). Analyze the eluted proteins by activity assay or by means of an SDS gel: a small amount of protein is eluted first, which appears on the gel to be mainly m-calpain and which we assume represents aggregated material, followed by a major peak of active m-calpain.

3.3.3. Q-Sepharose, FPLC column

11. Equilibrate a Q16/10 (Pharmacia, 1.6 × 10 cm quaternary ammonium anion exchange column, or equivalent) in 25 m*M* Tris-HCl, pH 7.6, 5 m*M* EDTA, 10 m*M* 2-ME.
12. (Day 6 or 7) Apply the pool of enzyme from gel filtration, which is in 0.2 *M* NaCl, (or from the Ni-NTA column if gel filtration was omitted), to the Q 16/10 column at 1–2 mL/min, followed by a wash of 25 m*M* Tris-HCl, pH 7.6, 5 m*M* EDTA, 10 m*M* 2-ME, 0.2 *M* NaCl.
13. Elute the bound m-calpain with a gradient from 0.2 to 0.8 *M* NaCl in the above buffer, at a flow rate of 2 mL/min over 60 minutes. The m-calpain is eluted at ~ 0.48 *M* NaCl with a purity of >95% (*see* **Note 14**).
14. The enzyme activity is stable for a few days at 4°C with a little added 2-ME and sodium azide; for long-term storage, add 0.5–1 vol of glycerol and store aliquots at –20°C or –70°C for months without loss of activity.

4. Notes

1. If an air system is not available, it will be necessary to shake the cultures vigorously in volumes of 1 L, since thorough oxygenation is important.
2. The early literature suggested that an $A_{600\,nm}$ of 0.6 was correct for adding IPTG. We have not conducted a complete survey, but satisfactory yields are obtained at least to $A_{600\,nm}$ values of 1.2.
3. If the growth temperature is greater than 30°C, an increasing proportion of the product is found in inclusion bodies. Growth at 22–25°C is equally successful, although it may take 12–16 h before the desired cell densities are achieved, and it may be helpful with mutants, which may be defective in folding or in subunit association.
4. A concentration of 0.4 m*M* IPTG is commonly recommended, but this reagent is expensive and 0.15 m*M* seems to be sufficient: we have not systematically studied the effects of reducing this concentration. The time of continued growth after adding IPTG is also not very critical in the case of m-calpain expression.
5. Freezing of the cell suspension in lysis buffer is not essential. If the timing is appropriate, for example, after overnight growth at 20–25°C, the cell suspension may be sonicated and processed immediately.

6. The glass beaker breaks very occasionally during sonication, but we prefer the visibility offered by glass.
7. As described in Chapter 1, crude supernatant extracts clarified by centrifugation can be applied to DEAE-cellulose either by batch absorption, or by pumping directly onto the column. We prefer batch absorption, which removes much unwanted material and seems to give a cleaner elution pattern. The DEAE-cellulose after absorption of the crude extract can be washed with large volumes of ice-cold TE on a medium porosity sintered glass funnel and then packed into a column.
8. Theory suggests that the total volume of the gradient buffers should be 8 to 10 times the column bed volume of the ion exchange matrix, but for m-calpain the results are satisfactory with only 5 vol.
9. m-Calpain has the advantage that it is eluted after the bulk of the absorbed and eluted protein. For maximum purity, the first 2 or 3 fractions containing m-calpain activity may be rejected, since the concentration of contaminating proteins is still high in these fractions. It is of interest that the m-calpain elution is followed by a large peak of material, which we take to be nucleic acid, since it has a high $A_{260\ nm}/A_{280\ nm}$ ratio and is negative in the Bradford assay. The m-calpain is detected by a standard casein-based assay. If an inactive mutant has been expressed, the 80 kDa subunit can normally be detected on a Coomassie-stained gel of the eluted material, but it may be desirable in some cases to confirm its elution pattern by a Western blot. The use of Tris-Tricine gels *(7)* is strongly recommended, since the calpain small subunits run much more favorably on these gels. In subsequent steps, the m-calpain, if inactive, is sufficiently enriched that it can easily be monitored by Coomassie-stained gels without the time-consuming immunoblotting.
10. The early literature on Ni-NTA-agarose columns suggested a starting concentration of 40 m*M* imidazole, but this is much too high for calpain. The column appears to tolerate up to 10 m*M* 2-ME.
11. The presence of EDTA in the applied sample is incompatible with the Ni^{2+} chelated to the column by the NTA group. This problem can be avoided either by precipitating the protein in 60% ammonium sulfate and redissolving it in buffer lacking EDTA; or by adding sufficient Mg^{2+} to bind all the available EDTA. The reaction of excess Mg^{2+} with EDTA at pH 7.6 generates one equivalent of H^+, which must be adequately buffered. If this is not done, and the pH drops below about 6.0, the calpain tends to precipitate. m-Calpain also will not bind to the Ni-NTA column at lower pH.
12. Rather than eluting with a concentration gradient of imidazole, the column can be eluted simply with 0.15 *M* imidazole. This is faster and reduces the relevant eluted volume, but sacrifices any selectivity in this purification step. Either 0.4 or 0.6 *M* NaCl is recommended by the manufacturer for the Ni-NTA column, but satisfactory results are obtained at 0.2 *M* NaCl, which has the advantage that the eluted enzyme can be applied without any further manipulation directly to the Q-Sepharose column.
13. The Ni-NTA column is regenerated by washing with 0.25 *M* imidazole, and is occasionally cleaned more thoroughly by treatment with 8 *M* urea and 20 m*M* EDTA, followed by recharging with $NiSO_4$ solution.

14. The main peak of eluted m-calpain is preceded in most cases by a small shoulder of material which appears to be m-calpain on gels, and may represent partial and minor oxidation products of the enzyme.

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References

1. Elce, J. S., Hegadorn, C., Gauthier, S., Vince, J. W., and Davies, P. L. (1995) Recombinant calpain II: Improved expression systems and production of a C105A active-site mutant for crystallography. *Protein Eng.* **8,** 843–848.
2. Meyer, S. L., Bozyczko-Coyne, D., Mallya, S. K., Spais, C. M., Bihovsky, R., Kawooya, J. K., Lang, D. M., Scott, R. W., and Siman, R. (1996) Biologically active monomeric and heterodimeric recombinant human calpain I produced using the baculovirus expression system. *Biochem. J.* **314,** 511–519.
3. Graham-Siegenthaler, K., Gauthier, S., Davies, P. L., and Elce, J. S. (1994) Active recombinant rat calpain II: Bacterially produced large and small subunits associate both *in vivo* and *in vitro*. *J. Biol. Chem.* **269,** 30,457–30,460.
4. Elce, J. S., Hegadorn, C., and Arthur, J. S. C. (1997) Autolysis, Ca^{2+} requirement, and heterodimer stability in m-calpain. *J. Biol. Chem.* **272,** 11,268–11,275.
5. Elce, J. S., Davies, P. L., Hegadorn, C., Maurice, D. H., and Arthur, J. S. C. (1997) The effects of truncations of the small subunit on m-calpain activity and heterodimer formation. *Biochem. J.* **326,** 31–38.
6. DeLuca, C. I., Davies, P. L., Samis, J. A., and Elce, J. S. (1993) Molecular cloning and bacterial expression of cDNA for rat calpain II 80 kDa subunit. *Biochim. Biophys. Acta* **1216,** 81–93.
7. Schägger, H. and von Jagow, G. (1987) Tricine-SDS polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166,** 368–379.

7

Purification and Characterization of Crustacean Calpain-like Proteinases

Donald L. Mykles

1. Introduction

Calcium-dependent cysteine proteinases (CDPs or calpain-like proteinases) constitute a large family of related proteins in the tissues of invertebrate species. They vary in native mass from 59 kDa for lobster muscle CDP III to 520 kDa for octopus muscle CDP, and vary also in subunit composition ***(1,2)***. Although quite diverse, invertebrate enzymes share essential biochemical properties with mammalian calpains. The invertebrate proteinases require Ca^{2+} for full activity, and have a much greater affinity for Ca^{2+} than for other divalent cations ***(3–6)***. Invertebrate calpain-like enzymes are strongly inhibited by cysteine proteinase inhibitors, such as E-64 and related epoxysuccinyl peptides, peptide aldehydes (e.g., MDL 28170 and calpeptin), iodoacetate, iodoacetamide, Hg^{2+}, pCMB, and *N*-ethylmaleimide (NEM) ***(1,2,7)***. The serine proteinase inhibitors tosyllysyl chloromethyl ketone (TLCK), tosylphenylalanine chloromethyl ketone (TPCK), leupeptin, and antipain are effective only at higher concentrations. Moreover, cDNA sequences and immunological analysis show significant homologies between mammalian calpains and invertebrate enzymes ***(1,2,7)***.

Four calpain-like enzymes have been identified in crustacean muscles **(Table 1)**. All four degrade myofibrillar proteins (actin, myosin, paramyosin, tropomyosin, and troponin), but with differing specificities ***(3,8–10)***. CDP IIb, which constitutes about 30–40% of the total CDP activity ***(11)***, appears to be related to *Drosophila* Dm-calpain, or CalpA ***(12,13)***. CDP IIb and Dm-calpain have similar subunit masses (91.5–95 kDa) and antibodies raised against the two proteins cross-react ***(14)***. However, they apparently differ in native mass

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Table 1.
Properties of Calcium-Dependent Proteinases (CDPs) From Lobster Striated Muscles.

CDP	Native M_r (kDa)	Subunit M_r (kDa)	Ca^{2+} Requirement (m*M*)[a]
I	310	ND	1
IIa	125	60	1.5
IIb	195	95	2
III	59	ND	0.6

From **ref. *2***, with permission.
[a]Ca^{2+} concentration required for half-maximal activation
ND, not determined

(280 kDa for *Drosophila* vs 195 kDa for lobster CDP IIb ***[4,11,15,16]***), although the identity of the protein encoded by the Dm-calpain cDNA ***(12,13)*** and the protein purified from adult flies ***(4)*** has not been firmly established. CDP IIb undergoes Ca^{2+}-dependent autolysis, although this autolysis has little effect on its Ca^{2+} sensitivity, nor is autolysis required for substrate hydrolysis ***(16)***. Furthermore, unlike mammalian calpains, phospholipid does not lower the concentration of Ca^{2+} required for autolysis or substrate hydrolysis ***(16)***. CDP IIa appears to be the product of a gene distinct from that encoding CDP IIb. The antibody raised against CDP IIb does not react with CDP IIa ***(14)***. However, an antibody raised against a highly conserved peptide sequence encompassing the active site of mammalian calpains ***(17)*** reacts strongly with CDP IIa but not with CDP IIb ***(14)***. Other antibodies raised against mammalian calpains (μ-, m-, and p94-calpain) do not recognize lobster CDP IIa or IIb ***(14)***.

Although biochemically related to mammalian calpains, standard protocols used for the purification of mammalian enzymes are generally not effective for the purification of calpain-like enzymes from invertebrate and plant tissues. It is our experience that affinity columns containing Reactive Red or Blue matrices or hydrophobic columns containing butyl-, octyl,- or phenyl-agarose produce poor yields (*Mykles, unpublished data*) ***(6)***. The method described below is optimized for the purification of the major calpainlike proteinase, CDP IIb, from lobster striated muscles ***(16)***. It has also been used for the partial purification of CDP IIa ***(14)***. The entire procedure can be completed in 4 d: homogenization and ammonium sulfate fractionation on day 1, organomercurial-agarose (OMA) and Q-Sepharose chromatographies on day 2, FPLC gel filtration chromatography on day 3, and FPLC MonoQ chromatography on day 4. The organomercurial-agarose and Q-Sepharose chromatographies can be done on separate days, thus extending the purification to 5 d.

2. Materials

All buffers are stored at 4°C.

1. Buffer A (2 L): 20 m*M* Tris-acetate, pH 7.5, 20 m*M* KCl, 1 m*M* EDTA, 1 m*M* DTT (adjust pH with acetic acid before adding DTT) (*see* **Note 1**).
2. Buffer A without DTT (4 L): 20 m*M* Tris-acetate, pH 7.5, 20 m*M* KCl, 1 m*M* EDTA.
3. Buffer A without DTT or KCl (4 L): 20 m*M* Tris-acetate, pH 7.5, 1 m*M* EDTA. Place in two 2-L wide-mouth flasks or beakers for dialysis.
4. Buffer A with 1 *M* NaCl (500 mL): 20 m*M* Tris-acetate, pH 7.5, 20 m*M* KCl, 1 *M* NaCl, 1 m*M* EDTA, 1 m*M* DTT (adjust pH with acetic acid before adding DTT).
5. Quick organomercurial (OMA) pH equilibrating buffer (600 mL): 200 m*M* Tris-acetate, pH 7.5, 20 m*M* KCl, 1 m*M* EDTA.
6. Sodium acetate buffer for OMA regeneration: 50 m*M* sodium acetate, pH 5.
7. Sodium acetate/$HgCl_2$ buffer for OMA regeneration: 50 m*M* sodium acetate, pH 5, 4 m*M* $HgCl_2$.
8. Buffer B (2 L): 20 m*M* PIPES-NaOH, pH 6.8, 100 m*M* NaCl, 1 m*M* EDTA, 20% (v/v) glycerol, 1 m*M* DTT. Filter with a 0.22 µm membrane filter and degas just before use with the FPLC.
9. Buffer C (500 mL): 20 m*M* PIPES-NaOH, pH 6.8, 500 m*M* NaCl, 1 m*M* EDTA, 20 % (v/v) glycerol, 1 m*M* DTT. Filter with a 0.22 µm membrane filter and degas just before use with the FPLC.

3. Methods

3.1. Synthesis of the Organomercurial-Agarose Gel Matrix

Affi-Gel 501 organomercurial-agarose gel is no longer available from Bio-Rad. This procedure, provided by Bio-Rad, is the same as that used in the manufacture of Affi-Gel 501. Please note that many of the chemicals used in this procedure are very toxic: follow the instructions on Material Safety Data Sheets available from suppliers.

1. Transfer 100 mL of settled Affi-Gel 10 into a Buechner funnel or other vacuum filter device.
2. Wash the gel with 300 mL of anhydrous isopropyl alcohol.
3. Dissolve 1.5 g of *p*-aminophenylmercuric acetate in 300 mL of dimethylformamide.
4. Add the *p*-aminophenylmercuric acetate solution to the 100 mL of Affi-Gel 10. Stir for 4 h at room temperature.
5. Add 1 mL of ethanolamine to the gel slurry and stir for 1 h.
6. Transfer the gel slurry to a Buechner funnel or other vacuum filter device.
7. Wash the gel with 250 mL of dimethylformamide.
8. Wash the gel with 700 mL of anhydrous isopropyl alcohol.
9. Resuspend the gel in 300 mL of anhydrous isopropyl alcohol and store at 4°C.
10. The binding capacity of the gel can be tested using bovine hemoglobin (5 mg/mL) in 0.1 *M* potassium phosphate buffer (pH 8) (40 mL for a 2 mL column). A detailed procedure is available from Bio-Rad.

3.2. Purification of Lobster CDP IIb

3.2.1. Day 1 (Completion Time ~4 h)

Live adult lobsters (0.6–0.8 kg) are available from many supermarkets. An animal should be purchased on the day that a preparation is started. Only live material should be used. All procedures are carried out on ice or at 4°C unless otherwise stated.

3.2.1.1. Dissection (*See* **Note 2.**)

1. Using a pair of bone scissors, quickly remove the two large claws and the abdomen (tail) and place on ice. Wrap the body (cephalothorax) with aluminum foil and place it in a –80°C freezer to kill the animal. Carry out the dissection on a tray of ice covered with a sheet of aluminum foil. Using bone scissors, divide the abdomen into two halves by cutting lengthwise along the midlines of the dorsal and ventral surfaces of the exoskeleton, then separate the muscle by cutting with a scalpel or scissors. Remove the two deep abdominal muscles (deep extensor and deep flexor) from each half by severing their attachments to each segment of the exoskeleton. To remove the closer muscle from the claws, first cut along the length of the dorsal and ventral sides with bone scissors, starting at the proximal end. Connect the dorsal and ventral incisions by cutting along the outer, distal margin of the "hand," or propodus. Remove the outer half of the muscle by severing the attachments to the central blade-like tendon, or apodeme, with scissors. Separate the muscle from the exoskeleton by scraping between the epidermis and exoskeleton with a pair of curved, blunt-ended forceps. Repeat this procedure with the inner half.
2. Rinse tissue in a series of seven 400-mL beakers containing ~300 ml of ice-cold buffer A without DTT.
3. Blot tissue on filter paper to remove excess buffer and place in a tared, 600-mL beaker containing 400 mL of buffer A.
4. Weigh the beaker containing the tissue. Typical yields are 80–120 g from one lobster.

3.2.1.2. Homogenization

1. Transfer the contents (tissue and buffer) of the 600-mL beaker to a chilled Waring blender.
2. Add more buffer A to bring the total volume of buffer to 6.7 times the weight of the muscle (e.g., if the muscle weighs 100 g, then an additional 270 mL of buffer should be added).
3. Homogenize the muscle in the Waring blender at high speed for 1 min. With 30-s extraction intervals on an ice-water bath, repeat the procedure three times.
4. Centrifuge the homogenate in 250-mL screw-cap bottles, at 16,000*g* for 20 min in a GSA (Sorvall) rotor.
5. Pool the supernatant fractions and store the pool on ice.
6. Combine the pellets and rehomogenize them in 200 mL of buffer A with a Waring blender (1 min at high speed).

7. Centrifuge as in **step 4** (*see* **Note 3**).
8. Combine the supernatants from **steps 5** and **7** and measure the volume with a chilled, 1-L graduated cylinder (*see* **Note 4**).

3.2.1.3. Ammonium sulfate fractionation (*See* **Note 5**.)

1. Bring the combined supernatants to 65% saturated ammonium sulfate by adding slowly 0.53 g of granulated ammonium sulfate per milliliter of the original supernatant volume, stirring on a magnetic stirrer until it is completely dissolved (~30 min).
2. Divide between clear 250-mL screw-cap centrifuge bottles and place on ice for 20 min.
3. Balance the bottles and centrifuge at 16,000*g* for 15 min.
4. Discard the supernatant fractions and suspend each pellet in 2.5 mL of buffer A without DTT.
5. Combine the pellet suspensions with 5 ml of buffer A without DTT, which is used to rinse the centrifuge bottles. The pooled suspension is opaque due to residual ammonium sulfate, which prevents the protein from going completely into solution.
6. Dialyze the suspension overnight at 4°C against two 2-L changes of buffer A without DTT and KCl.
7. If the dialyzed solution is still cloudy, add a few more milliliters of buffer A without DTT and KCl, but keep the total volume to less than 30 mL.
8. To get ready for the next day, equilibrate an OMA column (5 × 5 cm, matrix volume 100 mL) with ~500 mL of buffer A without DTT. The pH of the buffer eluting from the column should be about 7.
9. Equilibrate a Q-Sepharose column with ~200 mL of buffer A.

3.2.2. Day 2 (Completion Time ~14 h; can be divided Into two shorter days)

3.2.2.1. Organomercurial-Agarose (OMA) Column Chromatography (*See* **Note 6**.)

1. Clarify the dialyzed solution by centrifugation at 16,000*g* for 10 min, and discard pellet.
2. Load the solution onto the OMA column. Collect the void fraction in a 150-mL flask or beaker and store on ice for later assay.
3. Let the level of the sample drop to the top of the gel matrix and rinse the column with buffer A without DTT, requiring ~1L or until the absorbance at 280 nm returns to baseline.
4. Let the buffer level drop to the top of the gel matrix and elute bound protein with buffer A. Collect the eluate in a 250-mL flask on ice. Continue collecting until the absorbance returns to baseline. This is a good stopping point for a shorter day (*see* **Note 7**).

3.2.2.2. Q-Sepharose Column Chromatography

1. Load the eluate from the OMA column onto a Q-Sepharose column (2.5 × 18 cm, matrix vol 88 mL) equilibrated with buffer A. Collect the void fraction in a flask or beaker and store on ice for later assay.

2. Wash the column with buffer A until the absorbance at 280 nm returns to baseline.
3. Elute the protein bound to the matrix with a linear NaCl gradient from 0 to 1 *M* NaCl in a total volume of 300 mL of buffer A. This is also a good stopping point for the day.
4. Assay the Q-Sepharose fractions for calpain activity using FITC-casein (see below). Use 44 µL of each fraction, and assay each fraction with an EGTA control. Also assay the OMA and Q-Sepharose void fractions (*see* **Note 8**). The major peak of CDP activity, containing primarily CDPs IIa and IIb, elutes as a broad peak between 0.3 *M* and 0.4 *M* NaCl (**Fig. 1**).
5. Pool the active fractions and concentrate to 4-5 mL using a CentriCell 60-mL device (Polysciences, Warrington, PA; 30,000 NMWL) at 4°C as described by the manufacturer.
6. Dialyze the concentrate against 1 L of buffer B.
7. Prepare for the next day by equilibrating the preparative gel filtration column (Superdex 200, 2.6 × 86 cm, matrix volume 460 mL) overnight with buffer B at a flow rate of 0.5 mL/min at room temperature (*see* **Note 9**).

3.2.3. Day 3 (Completion Time ~6 h)

3.2.3.1. FPLC Preparative Gel Filtration Column Chromatography *(See* **Notes 10** and **11**.)

1. Centrifuge the dialyzed solution at 12,000*g* for 10 min or filter through a 0.5-µm syringe filter to remove any particulates. The protein concentration of the supernatant fraction should not exceed 40 mg/mL. The solution can be diluted with buffer B, but the final volume should not exceed 5 mL (the FPLC must be equipped with a 5-mL sample loop).
2. Inject the dialyzed sample via a 5 mL sample loop onto the column. Run the column at 3 mL/min and collect 1-min fractions. Since the void volume of the column is about 171 mL, it is unnecessary to begin collecting fractions until ~70 min after injection. CDP IIb elutes between 82 and 87 min after injection and CDP IIa elutes between 96 and 99 min after injection. There is considerable overlap between the two enzymes; CDP IIb appears as a large peak with a shoulder containing CDP IIa (**Fig. 2**).
3. Assay the fractions for activity using FITC-casein (see below). Use 44 µL of each fraction. A 1-h incubation interval is sufficient. Since calcium-independent proteinase activity is low, there is no need for an EGTA control. Pool active fractions and store on ice.
4. Store the Superdex 200 column in freshly filtered (0.22 µm) and degassed 10% methanol (FPLC grade) by running at 0.5 mL/min overnight until conductivity is zero.

Day 4 (Completion Time ~6 h)

3.2.4.1. *FPLC* MonoQ column chromatography (Pharmacia HR 5/5 column)

1. Equilibrate the MonoQ column (0.5 × 5 cm, matrix volume 1 mL) first with buffer C, followed by buffer B at room temperature.

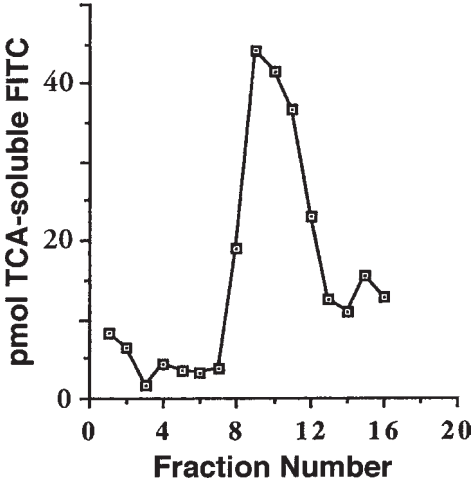

Fig. 1. Q-Sepharose anion exchange chromatography. Active fractions from an organomercurial-agarose column were chromatographed using a 0 to 1 *M* NaCl linear gradient (300 mL total volume; fraction volume approximately 18 mL). A large peak of CDP activity (fractions 8–12) containing mostly CDPs IIa and IIb eluted between 0.3 *M* and 0.4 *M* NaCl; these fractions were pooled and subsequently chromatographed on a Superdex 200 gel filtration column (**Fig. 2**).

2. Inject the sample into the column. The total protein should not exceed 20 mg. If the sample volume exceeds the 5-mL capacity of the sample loop, several 5-mL aliquots can be injected before starting the gradient.
3. Wash the column with buffer B until the absorbance at 280 nm returns to the baseline. Collect the void fraction and store on ice for later assay.
4. Elute the protein with a linear gradient from 0.1 *M* to 0.5 *M* NaCl in a total volume of 20 mL, at a flow rate of 1 mL/min, collecting 0.6 mL fractions. CDP IIb elutes in one or two fractions between 0.4 *M* and 0.41 *M* NaCl **(Fig. 3)**. A typical yield is about 30 µg CDP IIb from 100 g of muscle (**Table 2**).
5. Assay the fractions for activity using FITC-casein (see below). Use 10 µL of each fraction. A 1-h incubation interval is usually sufficient. Since calcium-independent activity is low, there is no need to include an EGTA control.
6. Assess protein compositions of fractions by SDS-PAGE ***(20)***. Combine 10 µL of each active fraction with 5 µL SDS sample buffer without 2-mercaptoethanol (2-mercaptoethanol produces "ghost bands" when gels are stained with silver; its omission has no effect on the mobilities of CDPs IIa and IIb). Proteins are separated on a 10% discontinuous polyacrylamide gel using a BioRad Protean-II minigel apparatus and stained with silver ***(16)***.
7. Pool the active fractions and dialyze overnight at 4°C against buffer B. Store on ice (do not freeze!) (**Note 12**).

A summary of a typical purification is presented in **Table 2** (**Note 13**).

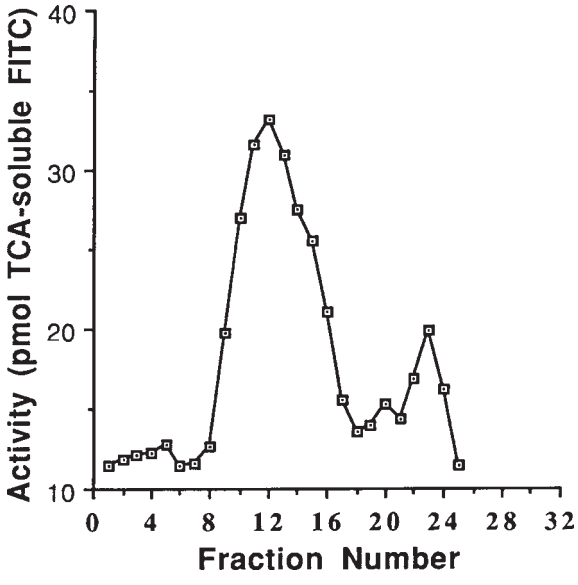

Fig. 2. Superdex 200 gel filtration chromatography. Active fractions (8–12) from a Q-Sepharose column (**Fig. 1**) were chromatographed on a Superdex 200 column (fraction volume 3 mL). CDP IIb (195 kDa) eluted as a large peak (fractions 9–13) with a shoulder (fractions 14–17) containing CDP IIa (125 kDa). Fractions 22–24 contained CDP III (59 kDa), which has not been characterized further. The CDP IIb fractions (9–13) were pooled and chromatographed on a Mono Q column (**Fig. 3**). Fractions 14–17 have been used for the partial purification of CDP IIa using a Mono Q column ***(14)***.

3.3. CDP Assay

The use of FITC-casein provides a highly-sensitive in vitro assay for crustacean calpainlike activities. The synthesis of FITC-casein is described by Twining ***(21)***. The substrate is used for the in-solution assay described below and also for casein zymography, which is described in Chapter 13.

1. In separate beakers, mix the components for the Ca^{2+} cocktail (A) and the components for the EGTA cocktail (B), in volumes sufficient for the number of assays.
   Ca^{2+} cocktail (A) (per assay):
   a. 165 µL of buffer A;
   b. 11 µL of FITC-casein (10 mg/mL in 50 m*M* Tris-HCl, pH 7.2);
   c. 24 µL of 50 m*M* $CaCl_2$;
   EGTA cocktail (B) (per assay):
   d. 165 µL of buffer A;
   e. 11 µL of FITC-casein (10 mg/mL in 50 m*M* Tris-HCl, pH 7.2);
   f. 24 µL of 50 m*M* EGTA.

Table 2
Summary of Purification of CDP IIb From 96 g of Lobster Striated Muscle

Purification step	Total protein (mg)	Specific CDP activity[a]	Purification factor (fold)	Total CDP activity[b]	Yield %
Homogenate	13,510	0.082	1	1,108	100
Supernatant	4,602	0.35	4.3	1,611	145
Am. sulfate	3,789	0.16	2	606	55
OMA column	798	0.25	3	200	18
Q-Sepharose	24.5	4.1	50	100.5	9
Superdex 200	1.6	67.9	828	108.6	10
Mono Q	0.03	2,146	26,170	64.4	6

The apparent increase in total CDP activity in the supernatant fraction was due to the removal of myofibrillar protein substrates present in the homogenate. The large decrease in total activity in the ammonium sulfate fraction was due to the concentration of an inhibitor, which is removed by the OMA column (*see* text).

[a]Micrograms of FITC-casein hydrolyzed per milligram of protein per hour.
[b]Micrograms of FITC-casein hydrolyzed per hour.

2. Dispense 200 µL of each cocktail into separate Eppendorf tubes.
3. Add a 44 µL sample of a column fraction to a tube containing the Ca^{2+} cocktail and a second 44 µL sample to a tube containing the EGTA cocktail. Vortex and incubate 1–3 h at room temperature.
4. Add 10 µL of bovine serum albumin (BSA) (110 mg/mL in deionized water) to each tube, and vortex briefly.
5. Add 100 µL of 21.9% (w/v) TCA to each tube. Vortex and place on ice for at least 20 min, or leave overnight at 4°C.
6. Centrifuge for 3 min in an Eppendorf centrifuge to pellet the precipitated protein.
7 Combine 300 µL of the supernatant fraction and 700 µL of 500 m*M* Tris-HCl, pH 8.5, and vortex briefly.
8. Quantify TCA-soluble FITC with a fluorescence spectrophotometer (485 nm excitation; 525 nm emission). Use a 10-pmol FITC standard (100 µL of 100 n*M* FITC in 6% TCA and 900 µL of 500 m*M* Tris-HCl, pH 8.5).

4. Notes

1. Including EDTA in all buffers prevents Ca^{2+}-dependent autolysis ***(16,22)***.
2. Muscles should be removed in as intact condition as possible to minimize loss of cellular proteins during the washing procedure. Washing muscles before homogenization reduces the amount of hemocyanin introduced into the homogenate. Hemocyanin is an oxygen transport protein free in solution in the hemolymph (blood) at high concentrations (35–40 mg/mL).
3. The pellets can be stored at –80°C and used to purify myofibrillar proteins ***(3,18,19)***.
4. CDPs are stable in relatively crude protein mixtures (e.g., cell-free extract, 65%

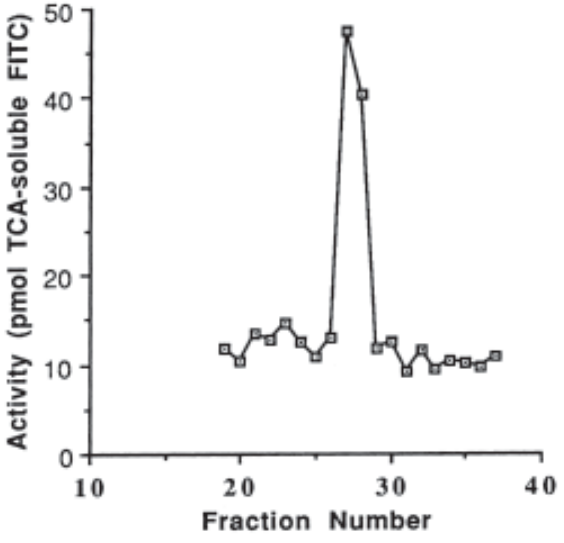

Fig. 3. Mono Q anion exchange chromatography of CDP IIb. Active fractions (9–13) from a Superdex 200 column **(Fig. 2)** were chromatographed using a 0.1 to 0.5 *M* NaCl linear gradient (total volume 20 mL; fraction volume 0.6 mL). CDP IIb eluted as a single peak (fractions 27–28) between 0.40 and 0.41 *M* NaCl.

ammonium sulfate fraction, and Q-Sepharose fraction), but lose activity as contaminating proteins are removed during later stages of the purification.

5. Use only highly purified ammonium sulfate (FPLC- or molecular biology-grade). Reagent-grade ammonium sulfate contains impurities that may adversely affect subsequent steps in the procedure.
6. Chromatography on the OMA column is straightforward. The hydrophobic property of the matrix, however, causes small air bubbles to form and adhere to the matrix when the matrix is suspended in aqueous solutions. This can interfere with optimal packing of the column. The bubbles are removed by degassing the gel suspension before packing the column. The matrix tends to darken with use, but this has no significant effect on the binding capacity or performance of the column.
7. Start regeneration of the OMA column (entire procedure takes about 2 d). Wash the OMA column with the following solutions, in sequence: 1.5 L of sodium acetate buffer (pH 5); 3 L of sodium acetate/$HgCl_2$ buffer (pH 5); 1.5 L of sodium acetate buffer (pH 5); and 600 mL Quick OMA pH equilibrating buffer (pH 7). Store in buffer A without DTT containing 5 m*M* sodium azide.
8. A 2-or 3-h incubation interval is usually required. The Q-Sepharose requires no special treatment, other than the periodic cleaning protocol provided by the manufacturer.
9. This will use 480 mL over 16 h; make sure enough buffer is supplied to avoid the column running dry.
10. The FPLC Superdex 200 gel filtration matrix was packed by us in a Pharmacia

XK 26 column. A prepacked Mono Q column was purchased from Pharmacia. The FPLC should be equipped with a precolumn filter, which prevents particulates from clogging the columns.

11. Including 20% glycerol in buffers used in the Superdex 200 and Mono Q columns preserves enzyme activity.
12. For long-term storage, the purified enzyme should be dialyzed against a solution identical in composition to buffer B, except that the glycerol concentration is increased to 50% (w/v), and stored on ice or at –15°C.
13. Yields of CDP IIb are about 30 µg (about 6% of the total CDP activity in the original muscle homogenate) from 100 g of muscle. Cell-free extracts contain an inhibitory factor that is removed during the OMA step ***(8,11,23,24)***. The concentration of this inhibitor by ammonium sulfate precipitation reduces the apparent total CDP activity in assays **(Table 2)**. The yields are better than they at first appear, since CDP IIb is one of four CDP activities and constitutes 30–40% of the total CDP activity in cell-free extracts ***(11)***.

References

1. Mykles, D. L. (1998) Intracellular proteinases of invertebrates: Calcium-dependent and proteasome/ubiquitin-dependent systems. *Int. Rev. Cytol.* **184,** 157–289.
2. Beyette, J. R. and Mykles, D. L. (1999) Crustacean calcium-dependent proteinases in *Calpain: Pharmacology and Toxicology of Calcium-Dependent Protease* (Wang, K. K. W. and Yuen, P.-W., eds.) Taylor & Francis, Washington, D C. pp. 407–427.
3. Mykles, D. L. and Skinner, D. M. (1982) Molt cycle-associated changes in calcium-dependent proteinase activity that degrades actin and myosin in crustacean muscle. *Dev. Biol.* **92,** 386–397.
4. Pinter, M., Stierandova, A., and Friedrich, P. (1992) Purification and characterization of a Ca^{2+}-activated thiol protease from *Drosophila melanogaster*. *Biochemistry* **31,** 8201–8206.
5. Hatzizisis, D., Gaitanaki, C., and Beis, I. (1996) Purification and properties of a calpain II-like proteinase from *Octopus vulgaris* arm muscle. *Comp. Biochem. Physiol.* **113B,** 295–303.
6. Safadi, F., Mykles, D. L., and Reddy, A. S. N. (1997) Partial purification and characterization of a Ca^{2+}-dependent proteinase from Arabidopsis roots. *Arch. Biochem. Biophys.* **348,** 143–151.
7. Mykles, D. L. (1999) Proteolytic processes underlying molt-induced claw muscle atrophy in decapod crustaceans. *Am. Zool.* **39,** 541–551.
8. Mykles, D. L. and Skinner, D. M. (1983) Ca^{2+}-dependent proteolytic activity in crab claw muscle: Effects of inhibitors and specificity for myofibrillar proteins. *J. Biol. Chem.* **258,** 10,474–10,480.
9. Mykles, D. L. (1990) Calcium-dependent proteolysis in crustacean claw closer muscle maintained in vitro. *J. Exp. Zool.* **256,** 16–30.
10. Mattson, J. M. and Mykles, D. L. (1993) Differential degradation of myofibril-

lar proteins by four calcium-dependent proteinase activities from lobster muscle. *J. Exp. Zool*. **265,** 97–106.
11. Mykles, D. L. and Skinner, D. M. (1986) Four Ca^{2+}-dependent proteinase activities isolated from crustacean muscle differ in size, net charge, and sensitivity to Ca^{2+} and inhibitors. *J. Biol. Chem*. **261,** 9865–9871.
12. Emori, Y. and Saigo, K. (1994) Calpain localization changes in coordination with actin-related cytoskeletal changes during early embryonic development of *Drosophila*. *J. Biol. Chem*. **269,** 25,137–25,142.
13. Theopold, U., Pintér, M., Daffre, S., Tryselius, Y., Friedrich, P., Nässel, D. R., and Hultmark, D. (1995) *CalpA*, a *Drosophila* calpain homolog specifically expressed in a small set of nerve, midgut, and blood cells. *Mol. Cell. Biol*. **15,** 824–834.
14. Beyette, J. R., Emori, Y., and Mykles, D. L. (1997) Immunological analysis of two calpain-like Ca^{2+}-dependent proteinases from lobster striated muscles: Relationship to mammalian and *Drosophila* calpains. *Arch. Biochem. Biophys*. **337,** 232–238.
15. Pinter, M. and Friedrich, P. (1988) The calcium-dependent proteolytic system calpain-calpastatin in *Drosophila melanogaster*. *Biochem. J*. **253,** 467–473.
16. Beyette, J. R. and Mykles, D. L. (1997) Autolysis and biochemical properties of a lobster muscle calpain-like proteinase. *J. Exp. Zool*. **277,** 106–119.
17. Grynspan, F., Griffin, W. R., Cataldo, A., Katayama, S., and Nixon, R. A. (1997) Active site-directed antibodies identify calpain II as an early-appearing and pervasive component of neurofibrillary pathology in Alzheimer's disease. *Brain Res*. **763,** 145–158.
18. Mykles, D. L. (1985) Heterogeneity of myofibrillar proteins in lobster fast and slow muscles: Variants of troponin, paramyosin, and myosin light chains comprise four distinct protein assemblages. *J. Exp. Zool*. **234,** 23–32.
19. Li, Y. and Mykles, D. L. (1990) Analysis of myosins from lobster muscles: Fast and slow isozymes differ in heavy-chain composition. *J. Exp. Zool*. **255,** 163–170.
20. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.
21. Twining, S. S. (1984) Fluorescein isothiocyanate-labeled casein assay for proteolytic enzymes. *Anal. Biochem*. **143,** 30–34.
22. Beyette, J. R., Ma, J.-S., and Mykles, D. L. (1993) Purification and autolytic degradation of a calpain-like calcium-dependent proteinase from lobster (*Homarus americanus*) striated muscle. *Comp. Biochem. Physiol*. **104B,** 95–99.
23. Mykles, D. L. and Skinner, D. M. (1990) Calcium-dependent proteinases in crustaceans in Intracellular Calcium-Dependent Proteolysis (Mellgren, R. L. and Murachi, T., eds.) CRC Press, Boca Raton, FL, pp. 139–154.
24. Treece, C. A. (1994) Partial purification of an endogenous inhibitor of calcium-dependent proteinase in lobster. M.Sc. Thesis, Colorado State University, pp. 1–47.

8

Drosophila Calpains

Purification of a Calpain-like Enzyme from Fruit Flies, and Expression in *Escherichia coli*

Gàspàr Jékely, Marianna Pintér, and Peter Friedrich

1. Introduction

Calpains were initially thought to be specific for vertebrates, but enzymes with calpain-like activity have also been found in invertebrates ***(1,2)***. Calpain-like sequences have been cloned from *Schistosoma mansoni* ***(3)***, *Caenorhabditis elegans* ***(4)*** and *Drosophila melanogaster* ***(5–7)***. The study of *Drosophila* calpains is particularly revealing, since this insect is accessible to many genetic manipulations, including mutant generation and studies on transgenic lines.

Calpains were first detected in fractionated head extracts of *Drosophila melanogaster* ***(2)***. Two partially purified isoforms were described, which could be inhibited by iodoacetate, but not by PMSF, which is characteristic of thiol proteases. Based on their Ca^{2+}-sensitivities and elution positions, the two enzymes were tentatively taken to be homologues of mammalian μ-and m-calpains. The apparent M_r of these *Drosophila* calpains was found by gel chromatography to be 280 kDa.

A Ca^{2+}-activated protease, called CANP, has been purified to homogeneity from *Drosophila*, and was shown to be a 94 kDa protein, with no associated small subunit ***(8)***. This calpain isoform attained half-maximal activation at 0.6 m*M* Ca^{2+}, and could be inhibited by domain I of human calpastatin at a 1:1 molar ratio. These results demonstrate the conservation of the calpain–calpastatin interaction, and support the characterization of the purified CANP as a form of calpain.

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Two cDNAs coding for *Drosophila* calpain homologues, CalpA ***(5,6)*** and CalpB ***(7)*** have so far been cloned and sequenced. Both sequences encode proteins very similar to the large subunits of conventional vertebrate calpains. CALPA, the product of the *CalpA* gene, is a protein of limited tissue expression with a unique insertion of approximately 80 amino acid residues in its calmodulin-like domain. This insert contains a 16 residue-long motif with a high probability of forming a membrane anchoring segment. The other *Drosophila* calpain, CALPB (91 kDa), has an amino acid sequence very similar to that of CALPA, except that the insert is absent **(Fig. 1)**. The two sequences show 68% amino acid sequence identity to each other in the C-terminal part of domain I (32 amino acids), 84% in domain II, 83% in domain III, and 64% in domain IV. The N-terminal regions comprising 80 amino acids in CALPA and 117 amino acids in CALPB are unrelated and show no homology with any known protein sequences.

In this chapter we describe first the method to obtain pure CANP from adult *Drosophila melanogaster* and second, we describe the expression in *E. coli*, solubilization from inclusion bodies, and affinity purification of recombinant His-tagged CALPA.

2. Materials

1. Buffer A: 10 m*M* HEPES-KOH, pH 7.5, 0.5 m*M* EDTA, 0.5 m*M* EGTA, 0.1 m*M* PMSF, 1 m*M* benzamidine, 0.5 m*M* DTE, 0 to 0.5 *M* NaCl. Prepare a stock solution of 100 m*M* HEPES-KOH, pH 7.5, 5 m*M* EDTA, 5 m*M* EGTA and store in aliquots of 50–100 mL at –20°C. The other reagents should be added immediately before use.
2. Fly homogenization buffer: 20 m*M* HEPES-KOH, pH 7.5, 250 m*M* sucrose, 5 m*M* EDTA, 5 m*M* EGTA, 1 m*M* PMSF, 5 m*M* benzamidine, 0.5 m*M* DTE. Prepare a solution of 20 m*M* HEPES-KOH, pH 7.5, 250 m*M* sucrose, 5 m*M* EDTA, 5 m*M* EGTA, and store at –20°C. Add protease inhibitors and DTE before use.
3. Lysis buffer: 100 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, 1 m*M* EDTA, 20 m*M* 2-ME, 1 m*M* PMSF, 5 m*M* benzamidine, 1% Triton X-100.
4. Inclusion body washing buffer: 100 m*M* Tris-HCl, pH 7.5, 2 *M* urea, 20 m*M* 2-ME. Dilute from a stock 1 *M* Tris-HCl, pH 7.5 solution and add 2-ME and crystalline urea.
5. Inclusion body solubilization buffer: 100 m*M* NaH_2PO_4/Na_2HPO_4, pH 7.5, 8 *M* urea, 20 m*M* 2-ME, 20 m*M* imidazole. Prepare from a 1 *M* NaH_2PO_4/Na_2HPO_4, pH 7.5 stock solution, and add crystalline urea and imidazole before use. Set the pH of the solution after addition of imidazole.
6. Ni-nitrilotriacetic acid (NTA) washing buffer: 100 m*M* NaH_2PO_4/Na_2HPO_4, pH 7.5, 6 *M* urea, 20 m*M* 2-ME, 20 m*M* imidazole.
7. Dialysis buffer: 10 m*M* HEPES, pH 7.5, 150 m*M* NaCl, 1 m*M* EDTA, 0.1 m*M* PMSF, 0.5 m*M* benzamidine, 5 m*M* 2-ME. Dilute from a 100 m*M* HEPES, pH 7.5, 1.5 *M* NaCl, 10 m*M* EDTA stock, which should be stored at –20°C.

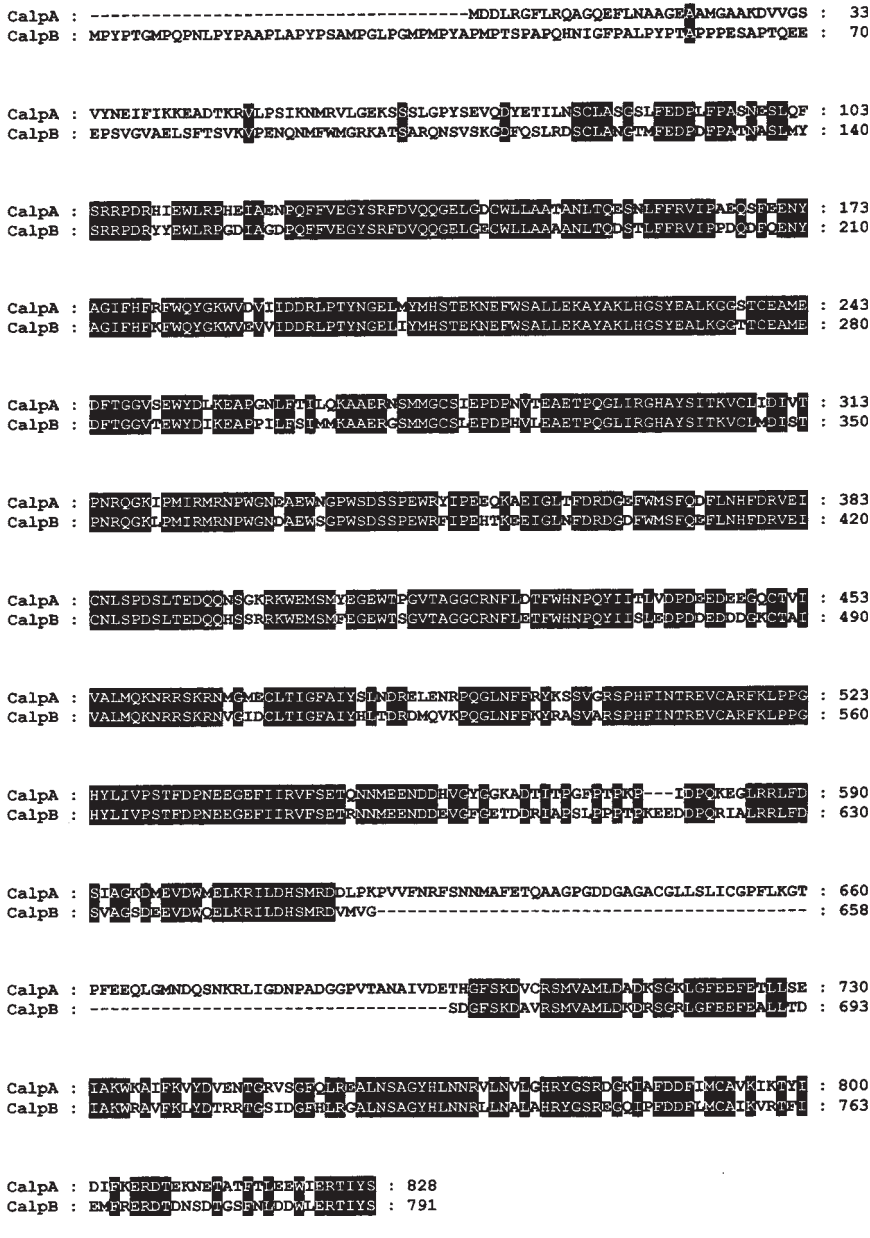

Fig. 1. Alignment of the amino acid sequences of *Drosophila* CALPA and CALPB. The two sequences have dissimilar N-terminal ends, and the inserted region present in CALPA is absent from CALPB. Identical amino acids are boxed.

3. Methods

3.1. Purification of a Ca^{2+}-Activated Thiol Protease (CANP) From Drosophila

See **ref. *8***.

3.1.1. Preparation of a crude extract from flies.

All procedures are carried out at 4°C or on ice, unless otherwise described.

1. Collect 10 g of flies. Homogenize immediately or store them in liquid nitrogen (*see* **Note 1**).
2. Homogenize 10 g of flies in 40 mL of fly homogenization buffer in a glass–glass homogenizer by 13 strokes at 0°C.
3. Centrifuge at 105,000*g* for 60 min.
4. Recover the supernatant and add an equal volume of 100% saturated $(NH_4)_2SO_4$ (in buffer A) slowly and with stirring.
5. Allow the mixture to stand on ice for at least 0.5 h (*see* **Note 2**).
6. Recover the precipitated protein by centrifugation at 20,000*g* for 20 min.
7. Resuspend pellet in 5 mL of buffer A containing 0.5 *M* NaCl.
8. Remove insoluble material by centrifugation at 20,000*g* for 30 min.

3.1.2. Phenyl-Sepharose CL-4B Chromatography

1. Apply the supernatant to a phenyl-Sepharose CL-4B column (0.65 × 10 cm), equilibrated with buffer A containing 0.5 *M* NaCl (*see* **Note 3**).
2. Wash the column with buffer A containing 0.5 *M* NaCl until no further protein elution can be detected with the Bradford protein assay.
3. Elute Ca^{2+}-activated protease-containing fraction with buffer A.
4. Pool fractions containing Ca^{2+}-activated protease activity (*see* **Note 4**).
5. Adjust the conductivity of the pool from the previous step with 5 *M* NaCl to the same value as that of buffer A with 0.5 *M* NaCl (add one tenth volume of 5 *M* NaCl and check the conductivity).

3.1.3. Reactive Red-120 Agarose Chromatography

1. Equilibrate a Reactive Red 120-agarose column (0.65 × 5 cm) with buffer A containing 0.5 *M* NaCl in a cold room (*see* **Note 5**).
2. Apply the pooled calpain fractions from phenyl-Sepharose to the Reactive Red-agarose column.
3. Wash the resin with buffer A containing 0.5 *M* NaCl until no further protein is detected.
4. Elute Ca^{2+}-activated protease activity with buffer A (*see* **Notes 4** and **6**).

3.1.4. Mono Q HR 5/5 Chromatography

1. Apply the eluted calpain from the Reactive Red-agarose column directly onto a Mono Q HR 5/5 column, previously equilibrated with ice-cold buffer A (*see* **Note 7**).
2. Wash with 5 mL buffer A (1 mL/min).

3. Elute calpain with a 0–0.5 *M* NaCl gradient in a total vol of 30 mL of buffer A.
4. Starting from 10 g of flies this procedure yields about 10 µg of highly purified protein in 2–3 mL, a very dilute enzyme solution. This preparation should be used immediately because the enzyme activity decays very quickly (*see* **Note 8**).

3.2. Expression of Recombinant CalpA in E. coli

Drosophila CALPA has been expressed in bacteria, using the CALPA-pET22b expression vector **(Fig. 2)**. The recombinant protein is fused with six histidine residues at the C terminus. This His-6 tag allows metal–chelate affinity purification of the enzyme with a Ni-NTA column. Most of the recombinant protein is found in inclusion bodies, and must be solubilized in a denaturing solution prior to chromatography. The affinity purification is followed by a renaturation procedure, which yields active CALPA.

3.2.1. Expression of CALPA in E. coli.

1. Transform *E. coli* strain BL21(DE3) (not containing pLysS) with the CALPA-pET22b vector, using conventional techniques.
2. Pick one transformed colony from the plate into 5 mL NZYM media containing ampicillin (100 µg/mL).
3. Incubate overnight at 37°C, shaking at 250 rpm.
4. Add the 5 mL overnight culture to 500 mL of NZYM/ampicillin (100 µg/mL), and grow until the OD_{600} reaches 0.7–1.
5. Induce CALPA expression by addition of IPTG to a final concentration of 0.2 m*M*.
6. Continue incubation for 3 h at 37°C, shaking at 250 rpm.
7. Cool the culture on ice and pellet the *E. coli* by centrifugation at 3000*g* for 20 min.

3.2.2. Inclusion Body Preparation and Extraction

1. Resuspend the *E. coli* in 30 mL of lysis buffer.
2. Sonicate at 20 µ for 5 × 15 s on ice.
3. Centrifuge the lysate at 3000*g* for 20 min.
4. Discard the supernatant, and resuspend the pellet in 50 mL of inclusion body washing buffer.
5. Centrifuge at 3000*g* for 20 min.
6. Solubilize the pellet, containing relatively pure inclusion bodies, in 5 mL of inclusion body solubilization buffer at room temperature and allow the mixture to stand at room temperature for 30 min.
7. Dilute the mixture to 50 mL with Ni-NTA washing buffer.
8. Incubate overnight in a cold room.
9. Remove insoluble material by centrifugation at 100,000*g* for 1 h.

3.2.3. Metal–Chelate Affinity Chromatography

1. Equilibrate 10 mL of Ni-NTA resin with Ni-NTA washing buffer, and apply the supernant to the column at a flow rate of 1 mL/min.

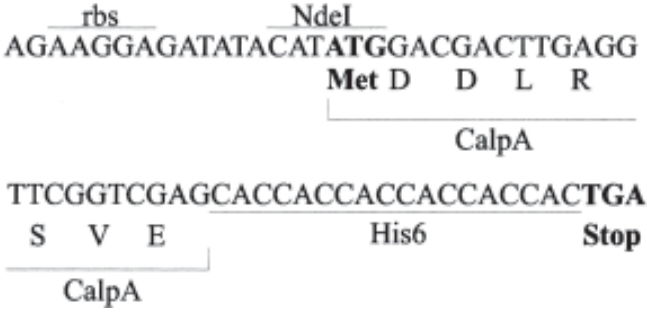

Fig. 2. CALPA-pET22b cloning region. The initiating methionine residue corresponds to the natural start signal of CALPA, while the C-terminal of the recombinant enzyme has been extended with a Val.Glu.His_6 sequence, to allow metal–chelate affinity chromatography

2. Wash the resin with 10 vol of Ni-NTA washing buffer.
3. Elute the His-tagged CALPA protein with a linear gradient of imidazole ranging from 20 to 250 m*M* in a total volume of 20 mL of Ni-NTA washing buffer.
4. Analyse the fractions by means of SDS-PAGE gel electrophoresis, and pool those containing CALPA.

3.2.4. Renaturation of CALPA

1. Dilute the pooled fractions to 50–100 µg protein/mL.
2. Dialyze CALPA against successive changes of dialysis buffer (1 L) containing 4 *M*, 2 *M*, 1 *M,* and 0 urea, in a cold room, for at least 12 h each.
3. This preparation yields about 5 mg of highly pure *Drosophila* CALPA **(***see* **Notes 9** and **10)**.

4. Notes

1. Do not start with less than 10 g of flies. The yield is lower if the flies are stored at –80°C instead of in liquid N_2.
2. The ammonium sulfate precipitate may be left overnight at 4°C, but otherwise the whole procedure should be carried out to the end of **Subheading 3.2., step 2** without interruption (12–14 h).
3. The phenyl-Sepharose CL-4B resin should always be pretreated. First wash the resin with 100 mL of deionized water, then with 300 mL of 2% Triton-X-100. Wash out Triton X-100 with 2–3 L of deionized water, followed by 100 mL volumes of aqueous ethanol in the following concentrations: 10%, 25%, 50%, 75%, 50%, 25%, and 10%. Finally, wash the resin with 300 mL of deionized water. If the resin is reused, it should be treated in the same way.
4. For both phenyl-Sepharose and Reactive Red-agarose columns, by relying on measurement of the conductivity of the eluate, it is possible to pool the Ca^{2+}-activated protease-containing fractions immediately. Waiting for the result of the

protease assay will result in low yield from phenyl-Sepharose; from the Reactive Red 120-agarose column, it results in the loss of all activity.

5. Reactive Red 120-agarose resin should always be pretreated as described ***(9)***. Wash the resin with 20 vol each of the following: deionized water, 4 *M* urea, deionized water, 4 *M* NaCl and deionized water.
6. After elution from the Reactive Red 120-agarose column, only one 94 kDa band is detected with ultrasensitive Coomassie brilliant blue staining on SDS-PAGE.
7. Instead of the MonoQ HR 5/5 column, one can use a small Q-Sepharose fast flow column (0.65 × 3 cm) equilibrated with buffer A. Wash the column with 40 mL of buffer A, then with 40 mL of buffer A containing 0.3 *M* NaCl. CALPA is eluted with buffer A containing 0.5 *M* NaCl.
8. It is a good routine to use 30- and 60-min reaction times at 30°C if the protease assay described in **ref**. ***8*** is followed.
9. The specific activity of recombinant CALPA was about an order of magnitude lower than that of mammalian calpains. It is not yet clear whether this is an intrinsic property of this calpain or whether renaturation of material from inclusion bodies is incomplete. Many efforts have been made to increase the specific activity of the enzyme, but without success. These efforts included changes in the concentration of IPTG, solubilization of inclusion bodies in guanidine HCl-containing buffer, renaturing dialysis in the presence of polyethylene glycol, or at room temperature, and purification of small quantities from the cytoplasm of *E. coli*. The addition of phospholipids resulted in a 4 to 5-fold increase in the enzyme's activity. Taken together, we suspect that the specific activity measured in the presence of phospholipids represents the highest attainable level of CALPA.
10. The other *Drosophila* calpain, CalpB, has also been expressed in bacteria. The recombinant enzyme forms inclusion bodies when expressed at 37°C, but can also be purified from the soluble fraction when expressed at 20°C, in the presence of 0.05 m*M* IPTG. The specific activity of the resulting enzyme is much higher, than that of recombinant CALPA ***(7)***.

Acknowledgments

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References

1. Mykles, D. L. and Skinner, D. M. (1986) Four Ca^{2+}-dependent proteinase activities isolated from crustacean muscle differ in size, net charge, and sensitivity to Ca^{2+} and inhibitors. *J. Biol. Chem.* **261,** 9865–9871.
2. Pintér, M. and Friedrich, P. (1988) The calcium-dependent proteolytic system calpain–calpastatin in *Drosophila melanogaster*. *Biochem. J.* **253,** 467–473.
3. Karcz, S. R., Podesta, R. B., Siddiqui, A. A., Dekaban, G. A., Strejan, G. H., and Clarke, M. W. (1991) Molecular cloning and sequence analysis of a calcium-activated neutral protease (calpain) from *Schistosoma mansoni*. *Mol. Biochem. Parasitol*. **49,** 333–336.

4. Barnes, T. M. and Hodgkin, J. (1996) The tra-3 sex determination gene of *Caenorhabditis elegans* encodes a member of the calpain regulatory protease family. *EMBO J.* **15,** 4477–4484.
5. Emori, Y. and Saigo, K. (1994) Calpain localization changes in coordination with actin-related cytoskeletal changes during early embryonic development of *Drosophila*. *J. Biol. Chem*. **269,** 25137–25142.
6. Theopold, U., Pintér, M., Daffre, S., Tryselius, Y., Friedrich, P., Nassel, D. R., and Hultmark, D. (1995) CalpA, a *Drosophila* calpain homolog specifically expressed in a small set of nerve, midgut, and blood cells. *Mol. Cell. Biol*. **15,** 824–834.
7. Jékely, G. and Friedrich, P. (1998) Characterization of two recombinant *Drosophila* calpains: CALPA and a novel homologue, CALPB. *J. Biol. Chem.* **274,** 23,893–23,900.
8. Pintér, M., Stierandova, A., and Friedrich, P. (1992) Purification and characterization of a Ca^{2+}- activated thiol protease from *Drosophila melanogaster*. *Biochemistry* **31,** 8201–8206.
9. Clark, A. F., DeMartino, G. N., and Croall, D. E. (1986) Fractionation and quantification of calcium-dependent proteinase activity from small tissue samples. *Biochem. J*. **235,** 279–282.

9

Molecular Analysis of p94 and Its Application to Diagnosis of Limb Girdle Muscular Dystrophy Type 2A

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1. Introduction

p94 (also called calpain 3, nCL-1, or CAPN3) is a calpain large subunit homologue, which is predominantly expressed in skeletal muscle ***(1–4)***. The mRNA level of p94 in skeletal muscle is at least 10 times higher than that for the conventional calpain subunits ***(1)***. However, the p94 protein is not easily detectable, since p94 undergoes very rapid and extensive autolysis immediately after translation, and its half-life in vitro is less than 10 minutes ***(5)***. Thus, analysis of p94 at the protein level is very difficult.

In order to avoid this difficulty, we have been using an expression system to analyze the unique autolytic activity of p94. p94 undergoes very rapid autolysis when expressed in COS cells ***(5)***. The full-length 94-kDa protein is hardly detectable, but a 55-kDa degradation product, which corresponds to the C-terminal two-thirds of p94, can be detected (**Fig. 1,** lane 2). This breakdown is caused by autolysis, not by proteolysis by other proteases, because active site-mutated inactive p94 forms such as p94:C129S show stable expression in COS cells ***(5)***.

Expression of p94 in COS cells can therefore be used as the easiest in vivo assay for p94 autolytic activity ***(6)***. Moreover, this method can also be used to analyze the proteolytic activity of p94 on its possible substrates, such as fodrin (α-spectrin) and calpastatin. These assays are important particularly when we consider the various p94 mutants found in limb-girdle muscular dystrophy type 2A (LGMD2A). In 1995, defects in the gene for p94 were shown by Richard et al. ***(7)*** to be responsible for one of the muscular dystrophies, LGMD2A. This was the first demonstration that a defect in a cytosolic enzyme is responsible for muscular dystrophy. The other gene products involved in muscular dystrophies

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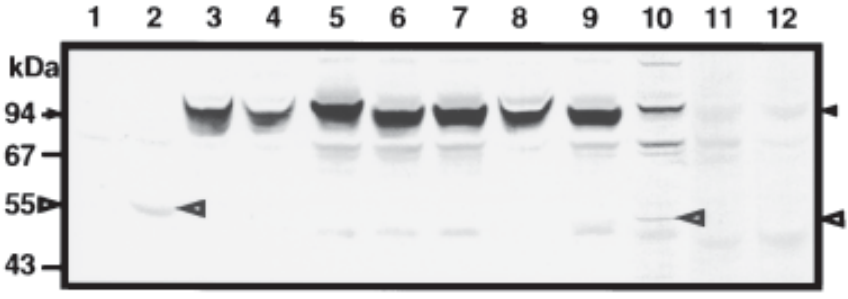

Fig. 1. Autolytic activity of wild-type and mutant p94 when expressed in COS7 cells. Lysates from COS7 cells transfected with wild-type and various mutant human p94 were analyzed by Western blotting with anti-p94 antiserum. *Lanes*: *1*, pSRD; *2*, wild-type p94; *3*, C129S; *4*, L182Q; *5*, G234E; *6*, P319L; *7*, H334Q; *8*, V354G; *9*, R490W; *10*, R572Q; *11*, S744G; *12*, R769Q. Closed and open arrowheads indicate the 94-kDa translation product and a 55-kDa proteolyzed fragment, respectively. L182Q, G234E, P319L, H334Q, V354G, and R490W as well as C129S showed a clear 94-kDa band, indicating that their autolytic activities are completely or almost completely suppressed. R572Q showed a weaker 94-kDa band and a degraded 55-kDa band, suggesting that this mutant has reduced but not completely defective autolytic activity. In contrast, S744G and R769Q retained an autolytic activity similar to that of the wild-type.

so far identified are all structural proteins such as dystrophin, sarcoglycans, and merosin ***(8)***. Therefore, it is very important to determine whether or not the loss of proteolytic activity of p94 is involved in the mechanism of LGMD2A.

In order to analyze proteolytic activity of mutant p94, we used the above-mentioned in vivo autolytic and proteolytic activity assays ***(6)***. Briefly, cDNAs for various p94 mutants are inserted into an expression vector, transfected into COS cells, and p94 and/or its potential substrates, and their degradation products, are detected by their specific antibodies. The results demonstrated that all 10 missense LGMD2A mutants of p94 we examined showed a defect in proteolytic activity against fodrin, strongly suggesting that improper substrate proteolysis causes LGMD2A ***(6)***. Thus, this technique is potentially applicable to diagnosis of LGMD2A **(Note 1)**.

2. Materials

2.1. Buffers

1. Solution D: 6 *M* guanidinium thiocyanate, 37.5 m*M* sodium citrate, pH 7.0, 0.75% (w/v) sodium *N*-dodecanoylsarcosinate, 150 m*M* 2-ME.
2. K-PBS: 30.8 m*M* NaCl, 120.7 m*M* KCl, 8.1 m*M* Na_2HPO_4, 1.46 m*M* KH_2PO_4, 25 m*M* $MgCl_2$, pH 7.3.
3. Buffer A: 100 m*M* Tris-HCl, pH 7.5, 10 m*M* EDTA, 1 m*M* DTT.

4. SDS-PAGE sample buffer: 100 m*M* Tris-HCl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 0.4% (w/v) bromophenol blue, 5% (v/v) 2-ME.

2.2. Preparation and Expression of p94 cDNA

Suggested PCR primers for full-length cDNA amplification could be (*see* **Note 2**):

1. Sense primer (SP): 5'-CAG TTG CTT CCT TTC CTT GAA GGT AGC TG-3' [29 mer, T_m = 65°C]. This primer corresponds to residues -74 to -46 upstream of the initiation codon.
2. Antisense primer (AP): 5'-TCA GGC ATA CAT GGT GAG CTG CAG C-3' [25mer, T_m = 66°C]. This primer corresponds to positions 2466 to 2442, including the stop codon.
3. Expression vector: any expression vector that functions in eukaryotic culture cells can be used. We routinely use the pSRD vector for COS7 cells because its expression rate is very high in these cells ***(11,12)***.
4. Culture cells: any cell line may be used that supports a high expression vector system. We use COS7 cells, as described above, and transfect them by electroporation ***(13)*** (*see* **Note 3**).

2.3. Antisera

1. Anti-p94 antiserum: a very specific and sensitive anti-mammalian-p94 antiserum was generated by using the following peptide as an antigen (of which the C-terminal cysteine residue is used for conjugating the peptide to keyhole limpet hemocyanin) (KLH) ***(5)***:
   NH_2-NTISVDRPVKKKNKPIIFV-C-COOH
   In our hands, both rabbit and goat produced very good antisera.
2. Anti-proteolyzed fodrin antiserum. An antiserum that is specific for the N terminus of the fodrin 150-kDa fragment proteolyzed by calpain was prepared as described in **ref. *14***. Briefly, the antibody was raised against the pentapeptide, NH_2-GMMPR-COOH, corresponding to the N-terminal sequence of the calpain-catalyzed proteolytic fragment of fodrin, and the antibodies were affinity-purified by means of a column conjugated with the same peptide.

3. Methods

3.1. Isolation of Skeletal Muscle RNA

The following is a modified acetic acid/guanidinium thiocyanate/hot phenol/chloroform method, which allows the isolation of sufficient total RNA for reverse transcriptase-polymerase chain reaction (RT-PCR) analysis from as little as 5 mg of skeletal muscle tissue ***(15,16)***. Commercial kits based on this method are available from a number of companies.

1. Transfer 5–500 mg of tissue into a 1.5-mL polypropylene tube already chilled to –80°C.

2. Add 100–200 µL of solution D at room temperature, and immediately homogenize the tissue with a microhomogenizer.
3. Add 400–300 µL (to reach a total of 500 µL) of solution D, 50 µL of 2 *M* sodium acetate, pH 4.0, and 500 µL of water-saturated phenol preheated to 65°C.
4. Mix vigorously for 3–5 min.
5. Add 100–150 µL of chloroform-isoamyl alcohol mixture (49:1) until the solution has just begun to be turbid, and mix well.
6. Incubate on ice for 15 min.
7. Centrifuge at 10,000*g* for 15 min at 4°C.
8. Recover ~400 µL of the aqueous phase into a new 1.5-mL polypropylene tube, add 2 vol of ethanol, and incubate at –80°C for 10 min.
9. Centrifuge at 10,000*g* for 20 min at 4°C.
10. Discard the supernatant, and dissolve the pellet in 150 µL of solution D.
11. Add 300 µL of ethanol to precipitate the RNA.
12. Centrifuge at 10,000*g* for 20 min at 4°C.
13. Discard the supernatant, air-dry the RNA pellet for a few minutes, and dissolve the pellet in 10–100 µL of RNase-free water.
14. Store the RNA at –80°C or lower; 1–10 µg of total RNA can be obtained from 5 mg of skeletal muscle tissue.

3.2. cDNA Cloning of p94

p94 cDNA can be easily obtained by RT-PCR from skeletal muscle RNA. As p94 mRNA is abundant in adult skeletal muscle, it is possible to amplify the cDNA by one-step RT-PCR using the "long-PCR" method. However, nested PCR may be required when only a low amount and/or quality of RNA can be obtained **(Note 4)**.

1. Perform high-fidelity RT-PCR using total RNA obtained in **Subheading 3.1.** and the p94 specific primers (SP and AP) described in **Subheading 2.2.** (*see* **Note 5**).
2. Subclone the amplified cDNA into a high-copy cloning vector or directly into the pSRD vector by TA-cloning or blunt-end ligation.

3.3. Transfection Into COS7 Cells

1. Culture COS7 cells in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum.
2. Transfect the plasmid containing p94 cDNA into COS7 cells by electroporation, as follows (*see* **Note 6**).
3. Harvest cells (6×10^6 cells are required per reaction) by treatment with EDTA and trypsin.
4. Rinse cells twice with 50 mL of PBS.
5. Rinse cells once with 10 mL of K-PBS.
6. Suspend cells in K-PBS to a concentration of 1.2×10^7 cells/mL.
7. Mix 5 µg of expression vector DNA and 0.5 mL of the cell suspension

prepared above (6×10^6 cells) in a 1.5 mL-polypropylene tube, and place the mixture on ice for 10 min.
8. Transfer the cell-DNA mixture into a 0.4-cm electroporation cuvette precooled on ice.
9. Pulse the cuvette at 220 V and 960 μϕ (time constant 20–30 ms).
10. Immediately place it on ice again for 10 min.
11. Place it at room temperature, add 0.5 mL of DMEM without serum (preheated to room temperature), gently mix with the pipet, and incubate at room temperature for 10 min.
12. Add the above cell suspension to 25 mL of DMEM with 10% fetal calf serum, pour it into a 15-cm culture dish (or divide into four 8-cm dishes), and incubate at 37°C in the presence of 5% CO_2.
13. At 36–72 h after transfection, wash cells three times with PBS, scrape them in ~1 mL of PBS, and collect in an ice-cold 1.5 mL-polypropylene tube as quickly as possible after scraping the cells.
14. Collect cells by centrifugation, suspend them in 0.15 mL of ice-cold buffer A, and disrupt by sonication, cooling with ice water.
15. Immediately after disruption, add an equal volume of SDS-PAGE sample buffer to the cell extracts, and heat in boiling water for 5 min.

3.4. Western Blot Analysis of Autolytic Activity

1. Electrophorese the whole-cell extracts, prepared as described in **Subheading 3.3.** on 10% SDS-polyacrylamide gels. Sample volumes (5–10 μL) should be normalized according to the cell number; between 10^3 and 10^5 cells are required for detection of various proteins (*see* **Note 7**).
2. Electrotransfer the proteins onto polyvinylidene difluoride membranes such as Immobilon P (Millipore; Tokyo, Japan) according to the manufacturer's instructions.
3. Detect p94 using anti-p94 antiserum as a primary antibody.

When wild-type p94 is expressed in COS cells, a 55-kDa p94-degradation band can be detected (**Fig. 1**, lane 2, open arrowhead), but the full-length 94-kDa band is very faint or almost invisible (closed arrowhead). By contrast, active site-mutated inactive mutant, p94:C129S, gives a clear 94-kDa band (**Fig. 1**, lane 3). The intensity of the 94-kDa band of p94:C129S is at least 100 times stronger than that of the wild-type p94. Measuring the intensity of the expressed 94-kDa band of the various mutant p94 (**Fig. 1**, lanes 4–12) permits their autolytic activity to be quantified.

3.5. Western Blot Analysis of Fodrinolytic Activity

Electrophorese the whole-cell extracts on 6% SDS-polyacrylamide gels, transfer to a membrane, and incubate with antiproteolyzed fodrin antiserum (*see* **Note 8**).

When wild-type p94 is expressed in COS cells, a clear 150-kDa band can be detected by the antiproteolyzed fodrin antiserum, indicating that significant

fodrinolysis has occurred in the cytosol (**Fig. 2**, lane 2, closed arrowhead). However, the 150 kDa band is barely visible in extracts from cells containing p94:C129S or other LGMD2A-type mutants (**Fig. 2**, lanes 3–12), indicating that they have all lost their fodrinolytic activity ***(6)*** **(***see* **Note 9)**.

3.6. Intermolecular Autolytic Activity Assay

One of the best substrates for p94 is p94 itself, since p94 intermolecularly proteolyzes other p94 molecules ***(18)***. Thus, p94:C129S can be used to assay the "intermolecular autolytic" activity of p94.

1. Transfect COS7 cells with 5 μg both of the p94:C129S expression vector and of an expression vector for wild-type or mutant p94, as described in **Subheading 3.3.** Cotransfection of p94:C129S with the vector without any insert should be performed as a negative control experiment.
2. Harvest cells and analyze for p94, by immunoblotting.

When p94:C129S is expressed alone in COS7 cells under our conditions, it shows a very strong 94-kDa band. Although a low level of randomly degraded fragments of p94:C129S may be detected, these are usually negligible compared with the intensity of the 94 kDa band **(Fig. 3**, lane 2**)**. When coexpressed with wild-type p94 or mutants that still have intermolecular autolytic activity, a 55 kDa degradation band can be detected (**Fig. 3**, lanes 3–7, open arrowheads).

4. Notes

1. The merit of the assay system described here is that it is a functional assay at the protein level regardless of the fact that one can only manipulate p94 at the DNA level. This aspect is very important from the viewpoint of diagnosis of LGMD2A. There are several polymorphisms in the p94 gene sequence, which sometimes include an amino acid residue substitution *(unpublished results)*. Moreover, pig, mouse, and rat p94 are 66, 53, and 49 amino acid residues different from that of human p94. Thus, sequence information is not enough to diagnose LGMD2A, since sequencing cannot distinguish polymorphism and/or unrelated mutations from defective mutations. Our assay, however, is based on the proteolytic activity of p94 so that functionally deficient mutants can be detected. Our recent results strongly suggest that loss of the fodrinolytic activity of p94 is closely related to the LGMD2A condition ***(6)***. If this is the case, an assay of fodrinolytic activity can be applied to diagnosis of LGMD2A.
   Since LGMD2A shows recessive inheritance, there are some further considerations with regard to its diagnosis. First, our assay can establish that a patient is not LGMD2A if he/she has the wild-type activity of p94. Second, if one of the clones derived from a patient shows deficient fodrinolytic activity, it does not necessarily indicate that the patient is LGMD2A, that is, homozygous for the mutated p94 gene(s). To clarify this point, PCR-amplification of the short cDNA fragment

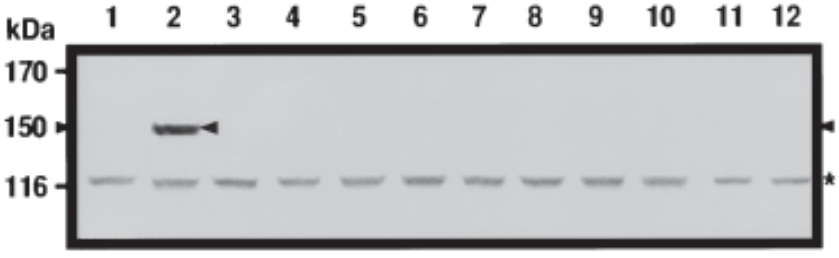

Fig. 2. Fodrinolytic activity of p94 mutants. Lysates from COS7 cells transfected with wild-type and mutant p94 were analysed by Western blotting with an antibody specific to the proteolyzed 150-kDa fodrin fragment. *Lanes*: *1*, pSRD; *2*, wild-type p94; *3*, C129S; *4*, L182Q; *5*, G234E; *6*, P319L; *7*, H334Q; *8*, V354G; *9*, R490W; *10*, R572Q; *11*, S744G; *12*, R769Q. The closed arrowhead indicates the calpain-proteolyzed 150-kDa fragment. Bands indicated by a star are nonspecific signals caused by the second antibody. All mutants showed a complete or almost complete defect in fodrinolytic activity.

containing the detected mutation and direct sequencing of this fragment are required. If the sequencing result shows only the mutated sequence, this patient must be LGMD2A, that is, homozygous for the mutation. If the sequence is a mixture of mutant and wild-type sequence, both types of cDNA have to be cloned, and the fodrinolytic activities have to be examined for both types. Although the procedure is somewhat complicated, there is no other direct diagnosis for LGMD2A at present.

2. These primers show a 100% match to human p94 cDNA sequence, and only 1–3 nucleotides mismatch to those of mouse and rat ***(1,7,9,10)***, suggesting that they can be used for most mammals.
3. Insect cells such as SF9 cells and the baculovirus system can be used, but the procedure becomes much more complicated and thus, cannot be recommended for this purpose.
4. The most important factor in the experiments described here is the high fidelity of cDNA amplification and high efficiency of transfection.
5. High fidelity of cDNA synthesis. It is essential to use a high-fidelity PCR system such as LA-PCR, or *Pfu* DNA polymerase, since many naturally occurring missense point mutants of p94 are found in LGMD2A patients ***(7,17)***. We strongly recommend the use of *Pfu* DNA polymerase or equivalent enzymes, together with a moderate concentration of substrate (ca. 0.2 mM each dNTP), although the amplification yield is very low compared with the yield expected with other thermostable DNA polymerases such as the EX-Taq system. As in all PCR work, sequence analysis of several independent subclones of cDNA is theoretically desirable, but it can become expensive, and is not necessarily required at this point.
6. High efficiency of transfection. The higher the efficiency of transfection, the

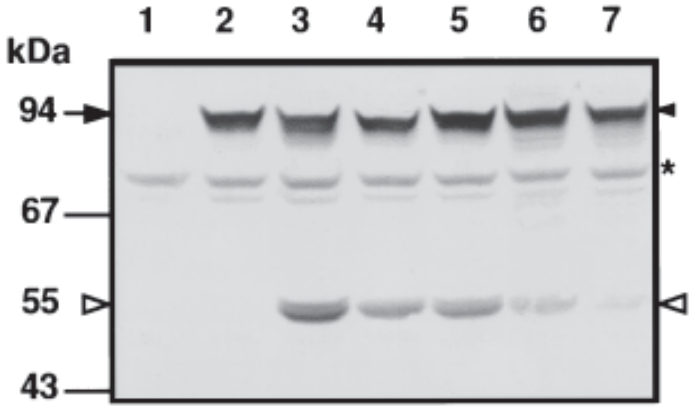

Fig. 3. Intermolecular autolytic activity of p94 mutants. Lysates from COS7 cells transfected with wild-type and mutant p94 were analyzed by Western blotting with anti-p94 antiserum. Lanes: *1*, pSRD; *2*, C129S and pSRD; *3*, C129S and wild-type p94; *4*, C129S and S744G; *5*, C129S and R769Q; *6*, C129S and P319L; *7*, C129S and R490W. Closed and open arrowheads indicate the 94-kDa translation product of C129S and a 55-kDa proteolyzed fragment, respectively. S744G, R769Q, P319L, and R490W generated 55-kDa proteolyzed fragments, indicating that these mutants retain proteolytic activity, although not to the same extent as that of wild-type p94.

clearer the data become, especially for fodrinolytic activity and intermolecular autolytic activity assays. The combination of COS7 cells and electroporation provides very efficient transient transfection. In our experience, 50% to 80% of the total cell population shows a considerable amount of expression of the protein encoded by the transfected expression vector.

7. If the transfection efficiency is high, it is possible to use normal commercially available antifodrin antiserum that recognizes at least the 230 kDa full-length fodrin instead of proteolyzed fragment-specific antiserum. When wild-type p94 is expressed in our system, the 230-kDa fodrin band detected by antifodrin antiserum decreases to 50% to 30% of that of vector-only transfected cells. Fodrinolytic activity can be quantified by densitometry of this 230-kDa fodrin band, although the data are less precise than those obtained using the calpain-proteolyzed fodrin fragment-specific antiserum.
8. Strictly speaking, it cannot necessarily be concluded that p94 directly proteolyzes fodrin. Alternative possibilities are either that μ-or m-calpain themselves are causing the cleavage of fodrin, or that p94 directly or indirectly promotes conventional μ-and/or m-calpain activity, which then cleaves fodrin.
9. The assay of p94 autolysis is much more sensitive than the assay of fodrinolysis, since the latter is judged from a negative result (disappearance of the 150-kDa fodrin cleavage band) while the former is from a positive result (appearance of

the 94 kDa band). Most of the LGMD2A mutants that are deficient in fodrinolysis also lose their p94 autolytic activity, but as a matter of interest, some mutants retain their autolytic activity. Thus, we cannot eliminate the possibility of LGMD2A even if the autolytic activity is not deficient. On the other hand, however, we can conclude that the patient is LGMD2A if autolytic activity is absent.

References

1. Sorimachi, H., Imajoh-Ohmi, S., Emori, Y., Kawasaki, H., Ohno, S., Minami, Y., and Suzuki, K. (1989) Molecular cloning of a novel mammalian calcium-dependent protease distinct from both μ- and m-types. Specific expression of the mRNA in skeletal muscle. *J. Biol. Chem.* **264,** 20,106–20,111.
2. Sorimachi, H., Ohmi, S., Emori, Y., Kawasaki, H., Saido, T. C., Ohno, S., Minami, Y., and Suzuki, K. (1990) A novel member of the calcium-dependent cysteine protease family. *Biol. Chem. Hoppe Seyler* **371,** 171–176.
3. Sorimachi, H. and Suzuki, K. (1992) Sequence comparison among muscle-specific calpain, p94, and calpain subunits. *Biochim. Biophys. Acta* **1160,** 55–62.
4. Ono, Y., Sorimachi, H., and Suzuki, K. (1998) Structure and physiology of calpain, an enigmatic protease. *Biochem. Biophys. Res. Commun.* **245,** 289–294.
5. Sorimachi, H., Toyama-Sorimachi, N., Saido, T. C., Kawasaki, H., Sugita, H., Miyasaka, M., Arahata, K., Ishiura, S., and Suzuki, K. (1993) Muscle-specific calpain, p94, is degraded by autolysis immediately after translation, resulting in disappearance from muscle. *J. Biol. Chem.* **268,** 10,593–10,605.
6. Ono, Y., Shimada, H., Sorimachi, H., Richard, I., Saido, T. C., Beckmann, J. S., Ishiura, S., and Suzuki, K. (1998) Functional defects of a muscle-specific calpain, p94, caused by mutations associated with limb-girdle muscular dystrophy type 2A (LGMD2A). *J. Biol. Chem.* **273,** 17,073–17,078.
7. Richard, I., Broux, O., Allamand, V., Fougerousse, F., Chiannilkulchai, N., Bourg, N., Brenguier, L., Devaud, C., Pasturaud, P., Roudaut, C., Hillaire, D., Passos-Bueno, M.-R., Zatz, M., Tischfield, J. A., Fardeau, M., Jackson, C. E., Cohen, D., and Beckmann, J. S. (1995) Mutations in the proteolytic enzyme calpain 3 cause limb-girdle muscular dystrophy type 2A. *Cell* **81,** 27–40.
8. Campbell, K. P. (1995) Three muscular dystrophies: loss of cytoskeleton-extracellular matrix linkage. *Cell* **80,** 675–679.
9. Sorimachi, H., Forsberg, N. E., Lee, H. J., Joeng, S. Y., Richard, I., Beckmann, J. S., Ishiura, S., and Suzuki, K. (1996) Highly conserved structure in the promoter region of the gene for muscle-specific calpain, p94. *Biol. Chem.* **377,** 859–864.
10. Richard, I. and Beckmann, J. S. (1996) Molecular cloning of mouse canp3, the gene associated with limb-girdle muscular dystrophy 2A in human. *Mamm. Genome* **7,** 377–379.
11. Ohno, S., Akita, Y., Konno, Y., Imajoh, S., and Suzuki, K. (1988) A novel phorbol ester receptor/protein kinase, nPKC, distantly related to the protein kinase C family. *Cell* **53,** 731–741.
12. Takebe, Y., Seiki, M., Fujisawa, J., Hoy, P., Yokota, K., Arai, K., Yoshida, M.,

and Arai, N. (1988) SRa promoter: An efficient and versatile mammalian cDNA expression system composed of the simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat. *Mol. Cell. Biol.* **8,** 466–472.

13. Chu, G., Hayakawa, H., and Berg, P. (1987) Electroporation for the efficient transfection of mammalian cells with DNA. *Nucleic Acids Res.* **15,** 1311–1326.
14. Saido, T. C., Yokota, M., Nagao, S., Yamaura, I., Tani, E., Tsuchiya, T., Suzuki, K., and Kawashima, S. (1993) Spatial resolution of fodrin proteolysis in postischemic brain. *J. Biol. Chem.* **268,** 25,239–25,243.
15. Sakamoto, K., Sorimachi, H., Kinbara, K., Tezuka, M., Amano, S., Yoshizawa, T., Sugita, H., Ishiura, S., and Suzuki, K. (1994) Quantification of calpain-related molecules by specific PCR amplification and its application to human muscular dystrophy. *Biomed. Res.* **15,** 337–346.
16. Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162,** 156–159.
17. Fardeau, M., Eymard, B., Mignard, C., Tome, F. M., Richard, I., and Beckmann, J. S. (1996) Chromosome 15-linked limb-girdle muscular dystrophy: Clinical phenotypes in Réunion island and French metropolitan communities. *Neuromuscul. Disord*. **6,** 447–453.
18. Sorimachi, H., Kinbara, K., Kimura, S., Takahashi, M., Ishiura, S., Sasagawa, N., Sorimachi, N., Shimada, H., Tagawa, K., Maruyama, K., and Suzuki, K. (1995) Muscle-specific calpain, p94, responsible for limb girdle muscular dystrophy type 2A, associates with connectin through IS2, a p94-specific sequence. *J. Biol. Chem.* **270,** 31,158–31,162.

10

Purification of Recombinant Calpastatin Expressed in *Escherichia coli*

Masatoshi Maki and Kiyotaka Hitomi

1. Introduction

Calpastatin is an endogenous inhibitor protein specific for calpain ***(1,2)***. Other calpain-inhibiting reagents such as EDTA, E-64, leupeptin, and calpain inhibitor I (*N*-acetyl-L-leucyl-L-leucyl-L-norleucinal), are frequently used in attempts to assess the role of calpain in degradation of target proteins in cell lysates. However, these reagents are not strictly specific for calpain (for instance, cathepsins B, H, and L are inhibited by E-64, and the proteasome is inhibited by calpain inhibitor I), so that we cannot logically establish that a given degradation is mediated by calpain even if it is inhibited by these reagents. It is clearly important to use inhibitors specific for calpains ***(3)***, and at present calpastatin alone provides the necessary specificity.

A calpastatin molecule consists of four repetitive domains ***(1–4)***, each of about 140 amino acid residues and having 20–30% sequence identity to each other, plus a unique N-terminal domain (domain L) ***(2,4,5)***. Calpain inhibitory activity has been detected in each repetitive domain, but domain 1 possesses the strongest activity ***(6,7)***. Purification of calpastatin from natural sources (animal tissues and cells) is laborious, and the preparations are often contaminated with degradation products, since calpastatin is highly sensitive to other cellular proteases during extraction and purification. Recombinant calpastatins have been successfully purified from *E. coli* ***(8–11)*** and also from insect cells infected with baculovirus ***(11)***. In this chapter we describe protocols for purification of recombinant calpastatins, both as single domains and as full length molecules, following expression in *E. coli*. The method takes advantage of the heat stability of calpastatin, using heat treatment as a very efficient step in the purification.The use

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of immobilized metal affinity chromatography is also described for recombinant calpastatin carrying a His-tag.

2. Materials

2.1. Bacterial Culture

1. *E. coli* strain BL21 (DE3) pLysS or BL21 (DE3) pLysE (Novagen, Madison, WI) **(Note 1)**.
2. Calpastatin expression plasmids: cloned into pET vectors of theT7 RNA polymerase system from Novagen ***(4,10,11)***.
3. Antibiotics: 50 mg/mL ampicillin in water; 12.5 mg/mL chloramphenicol in ethanol; 30 mg/mL kanamycin in water; stored in aliquots at –20°C. Filter sterilization is not necessary.
4. Growth media are autoclaved for 15–20 min at 121°C and cooled. Antibiotics and other supplements are added immediately before use:
   a. L-broth: 10 g/L tryptone or its equivalent, 5 g/L yeast extract, 5 g/L NaCl, 1 ml/L 1*M* NaOH, pH 7.2–7.5;
   b. L-agar: L-broth plus 1.5% (w/v) agar;
   c. ZB medium:10 g/L NZ amine A, 5 g/L NaCl;
   d. ZYG medium: 10 g/L NZ amine A, 5 g/L yeast extract, 5 g/L NaCl. 1 ml/L 1 *M* NaOH, 10 ml/L sterile-filtered 40% (w/v) glucose.
5. 1 *M* stock solution of IPTG: 1 g dissolved in 4.2 mL of water; store aliquots at –20°C.

2.2. Calpastatin Purification

1. Stock solutions of low molecular weight proteinase inhibitors are stored in aliquots at –20°C, PMSF and benzamidine-HCl may be added as solids to lysis buffer just before use:
   a. 200 m*M* PMSF in dry *n*-propanol or ethanol;
   b. 100 m*M* PefablocSC (Boehringer Mannheim) [4-(2-aminoethyl)benzene sulfonyl fluoride-HCl] in water;
   c. 500 m*M* benzamidine-HCl in water.
2. Buffer A: 20 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, 5 m*M* 2-ME.
3. Lysis buffer: buffer A containing 50 m*M* NaCl and supplemented with proteinase inhibitors to final concentrations of 2 m*M* PMSF, 0.1 m*M* PefablocSC, 5 m*M* benzamidine.
4. Buffer B: buffer A, 1 *M* NaCl.
5. Buffer C: buffer A, 0.15 *M* NaCl.
6. Buffers for Ni-NTA affinity purification of His-tagged calpastatin in denaturing solution:
   a. Denaturing lysis buffer: 6 *M* guanidine-HCl, 0.1 *M* NaH_2PO_4, 10 m*M* Tris, adjusted to pH 8.0 with NaOH;
   b. Wash buffer I: 50 m*M* Tris-HCl, pH 8.0, 150 m*M* NaCl;
   c. Wash buffer II: 50 m*M* Tris-HCl, pH 8.0, 800 m*M* NaCl;

d. Wash buffer III: 10 m*M* Tris-HCl, pH 6.8, 150 m*M* NaCl;
e. Elution buffer: 100 m*M* sodium acetate buffer, pH 4.5, 150 m*M* NaCl.

7. Ni-NTA (nitrilotriacetic acid) resin: the 50% suspension in 30% EtOH from QIAGEN (Chatsworth, CA) is equilibrated in denaturing lysis buffer before use.

2.3. Calpastatin Assay

1. 2% (w/v) casein dissolved in 0.5 *M* imidazole-HCl, pH 7.5: dissolve 2 g of casein (Merck, Darmstadt, Germany, nach Hammarsten grade) and 3.4 g of imidazole completely in hot H_2O (~80mL), and cool to room temperature; adjust pH to 7.5 with HCl and make up to 100 mL with H_2O. Store in aliquots of 10–20 mL at –20°C. One aliquot may be stored at 4°C and used for 1 d only.
2. 50 m*M* cysteine, stored at –20°C.
3. 50 m*M* $CaCl_2$, stored at room temperature.
4. 5% (w/v) TCA, stored at room temperature in a brown glass bottle. Handle with care, corrosive.
5. 0.7 *M* Na_2CO_3, 0.1 *M* NaOH: dissolve 7.42 g of Na_2CO_3 and 0.4 g of NaOH in 100 mL water.
6. 2 *M* iodoacetic acid (freshly prepared): dissolve 0.4 g of iodoacetic acid in 1 mL of 2 *M* NaOH.
7. Solutions for the Lowry protein assay:
   a. Solution I: 0.5% (w/v) $CuSO_4$;
   b. Solution II: 1% (w/v) potassium sodium tartrate ($KNaC_4H_4O_6.4H_2O$);
   c. Solution III: 2% (w/v) Na_2CO_3, 0.1 *M* NaOH.
8. Cu^{2+}-alkaline solution: premix solutions I, II, and III in the ratio of 1:1:50 (v/v) immediately before use.
9. 1 *M* Phenol reagent solution (Folin-Ciocalteu's reagent solution): dilute 1.8 *M* (or 2 *M*) commercial reagent solution with H_2O to 1 *M* before use.
10. Calpain: commercially available porcine erythrocyte μ-calpain (CalBiochem, San Diego, CA).

3. Method

3.1. Culture of E. coli

1. Transform *E. coli* strain BL21 (DE3) pLysS with the recombinant plasmid, (or streak from frozen permanent stocks), and select transformants on L-agar plates containing the relevant antibiotic (50 μg/mL of ampicillin for pET-3 derivatives; 30 μg/mL of kanamycin for pET-24 derivatives, 12.5 μg/mL of chloramphenicol if pLysS or pLysE is present).
2. Pick several single colonies with sterile toothpicks, inoculate each single colony repeatedly into 10 mL of ZB medium, and culture at 30°C (to avoid overgrowth) with shaking overnight (starter culture).
3. Transfer one overnight starter culture to 1 L of fresh ZYG medium containing antibiotics, and grow with vigorous shaking at 37°C for 2–3 h until A_{600} reaches 0.4–0.5.

4. Add IPTG to 0.2 m*M* and continue culturing for 2–3 h at 37°C.
5. Take an aliquot of 1 mL of the cell culture to estimate the calpastatin expression level by SDS-PAGE.
6. Harvest cells by centrifugation at 5000*g* for 5 min at 4°C.
7. Suspend the cells with 10–20 mL of 0.15 *M* NaCl and combine them into a single preweighed tube.
8. Re-centrifuge the cells, and estimate their wet weight, which is usually 3–4 g/L of culture.
9. Freeze cells at –20°C or below for at least 30 min.

3.2. Purification by Ion-Exchange and Molecular Sieve Chromatography

All operations are performed on ice or at 4°C, unless otherwise stated.

1. Suspend the frozen cells in 20–40 mL (6–10 vol) of lysis buffer with a glass rod: suspend first with 2–3 mL buffer, and then with the remaining buffer. The suspension becomes viscous due to release of bacterial DNA following enhanced lysis by T7 lysozyme expressed from pLysS. Use a smaller volume of the lysis buffer when the expression level of calpastatin is low.
2. Sonicate the lysed cell suspension on ice under mild conditions until the viscosity is greatly reduced (*see* **Note 2**).
3. Clear the lysate by centrifugation at 12,000*g* for 15 min.
4. Transfer the supernatant to a suitable heat-resistant centrifuge tube.
5. Heat the supernatant in a boiling water bath for 5–10 min until white insoluble material appears.
6. Cool on ice, then centrifuge to remove insoluble materials at 12,000*g* for 15 min.
7. Remove the supernatant solution and recentrifuge it to remove remaining insoluble material.
8. Measure the volume of the supernatant (by weighing) during transfer to a flat-bottom beaker, and cool in an ice-water bath on a magnetic stirrer.
9. While stirring gently, add biochemical grade solid ammonium sulfate slowly to 35% saturation (20.9 g/100 mL of supernatant). After stirring for 30–60 min, remove insoluble material by centrifugation at 12,000*g* for 15 min, and recover the supernatant.
10. Add further ammonium sulfate to 55% saturation (total 35.1 g/100 mL of heat-treated supernatant). The solution should become turbid when the saturation approaches 50% (*see* **Note 3**).
11. Continue stirring for 30–60 min.
12. Centrifuge at 12,000 x g for 15 min.
13. Dissolve the pellet in 2–5 mL of buffer A and dialyze against the same buffer (*see* **Note 4**).
14. Remove insoluble material by centrifugation.
15. Filter the dialysate through a 0.45 µm cellulose acetate membrane (or recentrifuge to remove particles), and apply the filtrate at a flow rate of 1 mL/min to the anion exchange column (HiTrapQ™, 1 mL, Amersham Biotech Pharmacia, Uppsala, Sweden) equilibrated with buffer A containing 0.1 *M* NaCl.

16. Wash the column with at least 10 mL of buffer A containing 0.1 *M* NaCl.
17. Elute calpastatin with a linear gradient of 0.1 *M*–0.25 *M* NaCl in a total volume of 40 mL of buffer A. Calpastatin elutes between 0.15 and 0.2 *M* NaCl **(Fig. 1)**.
18. Collect fractions of 1 mL, and check the purity of calpastatin by SDS-PAGE (*see* **Notes 5** and **6**).
19. Pool the calpastatin-containing fractions, and concentrate if necessary by freeze-drying after dialysis against water.
20. Apply the solution to a gel filtration column equilibrated in buffer A with 0.15 *M* NaCl. Superdex 75 (Pharmacia) is used for the single-domain inhibitor, and Superdex 200 (Pharmacia) for the full-length calpastatin. The column is run at 0.8 mL/min, collecting 1 to 2 mL fractions **(Fig. 2)** (*see* **Note 7**).
21. Screen the column fractions and check the purity of calpastatin by SDS-PAGE **(Fig. 3)**. Store calpastatin solutions at –20°C. If required, dialyze against water extensively and freeze-dry the dialysate.
22. A typical yield of purified protein from 1 L of *E. coli* for domain 1 is 5–20 mg, and for the full-length protein is 1–2 mg.

3.3. Affinity Purification of His-Tagged Calpastatin Under Denaturing Conditions

See **Note 8.**

1. Following expression and harvesting of the cells as in **Subheading 3.1.**, resuspend frozen cells with 20 mL of denaturing lysis buffer. Subsequent operations are carried out at room temperature.
2. Sonicate as described in **Subheading 3.2.2. step 2.**
3. Centrifuge at 12,000*g* for 15 min.
4. Recover the supernatant and clarify it by recentrifugation.
5. To the supernatant solution, add 2.5 mL of Ni-NTA resin (50% suspension in denaturing lysis buffer) and mix at room temperature for 1 h.
6. Centrifuge at 100*g* for 5 min at room temperature, and discard supernatant (*see* **Note 9**).
7. Resuspend resin with 5 mL of the denaturing lysis buffer, and transfer the resin to a small column.
8. Wash with 5 mL of wash buffer I.
9. Wash with 5 mL of wash buffer II.
10. Wash with 5 mL of wash buffer III.
11. Elute with 5 mL of elution buffer (*see* **Note 10**).
12. Add EDTA and 2-ME to final concentrations of 5 m*M* each to the eluted fractions.
13. Adjust pH to 7–7.5 with 1 *M* Tris.
14. Boil the solution for 5 min and centrifuge at 12,000*g* to remove aggregated protein (*see* **Note 11**).
15. Dialyze the supernatant against buffer A.
16. Purify further by gel filtration chromatography.

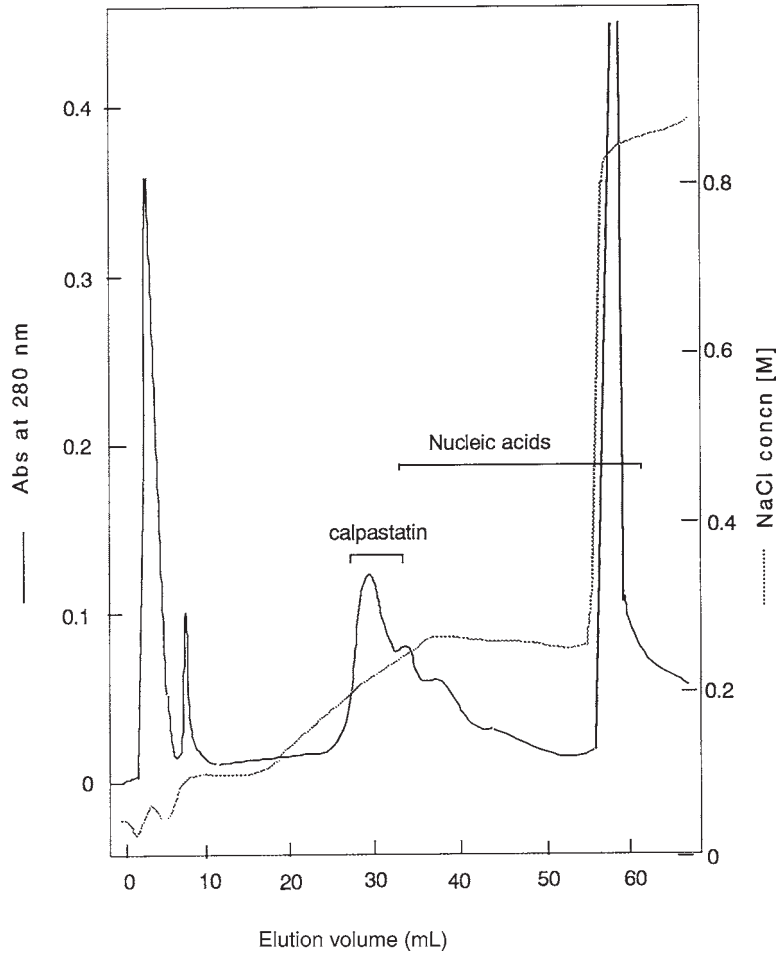

Fig. 1. Anion exchange column chromatography. The dialysate (1 mL aliquot) of the redissolved 35–55 % ammonium sulfate precipitate was applied to a HiTrapQ (1 mL) column, which was washed with buffer A containing 0.1 *M* NaCl, and eluted with a linear gradient of 0.1 to 0.25 *M* NaCl in a total volume of 40 mL of buffer A at a flow rate of 1 mL/min. Calpastatin-containing fractions detected by SDS-PAGE are indicated. The broken line indicates NaCl concentration estimated from conductivity.

3.4. Assay of Calpastatin Activity

The assay of calpastatin activity is based on measurement of the extent of calpain inhibition. Calpain activity is measured by a colorimetric determination of trichloroacetic acid-soluble peptides liberated upon casein digestion,

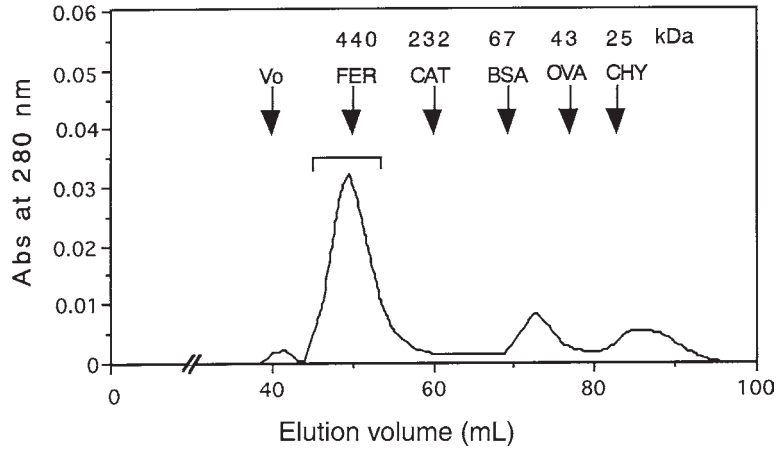

Fig. 2. Gel filtration chromatography of recombinant human calpastatin. A HiTrapQ-purified fraction (3 mL) was applied to a HiLoad 16/60 Superdex 200 preparative-grade column (bed volume, 120 mL) at a flow rate of 0.8 ml/min. Full-length human calpastatin (calculated M_r = 76,480.44) eluted much earlier than contaminants of similar molecular weights. Molecular weight calibration markers are: ferritin (FER, 440 kDa), catalase (CAT, 232 kDa), bovine serum albumin (BSA, 67 kDa), ovalbumin (OVA, 43 kDa) and chymotrypsinogen A (CHY, 25 kDa).

after carboxymethylation of sulfhydryl compounds present in the assay mixture ***(13,14)***.

1. In centrifugeable conical glass tubes, or in 1.5 mL Eppendorf tubes, prepare assay mixtures as follows: 1–100 µL of calpastatin samples, 40 µL of 2% casein, 20 µL of 50 m*M* cysteine, a fixed amount of calpain (normally 20 µL, ~0.5 unit). Adjust the total volume to 180 µL with buffer A (*see* **Note 12**).
2. Preincubate at 30°C for 5 min.
3. Start casein digestion by adding 20 µL of 50 m*M* $CaCl_2$, and continue incubation at 30°C for 30 min.
4. Stop the reaction by adding 200 µL of 5% TCA.
5. Cool on ice for at least 10 min.
6. Centrifuge at 300*g* for 15 min for glass tubes, or at 12,000*g* for 5 min for Eppendorf tubes, at 4°C.
7. Transfer 0.2 mL of the supernatant to a new tube.
8. Neutralize the TCA and make the solution alkaline by addition of 50 µL of 0.7 *M* Na_2CO_3, 0.1 *M* NaOH.
9. Add 50 µL of 2 *M* iodoacetic acid in NaOH in order to carboxymethylate free sulfhydryl groups.
10. Incubate at room temperature for at least 10 min.

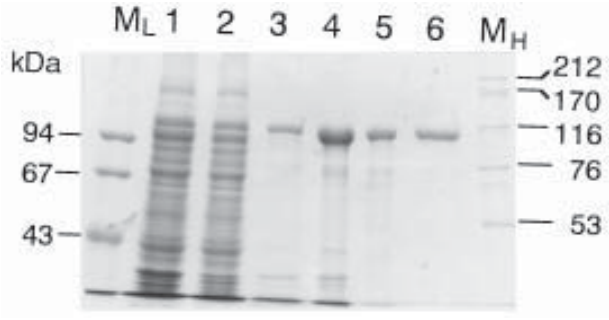

Fig. 3. SDS-PAGE of calpastatin preparations (7.5 % gel). *Lane 1*: total cell lysate of BL21 (DE3) pLysS harboring a full-length human calpastatin expression plasmid (pETCS, *see* **ref. *11***); *lane 2*: lysate supernatant; *lane 3*: heat treatment supernatant; *lane 4*: ammonium sulfate precipitate; *lane 5*: HiTrapQ fraction; *lane 6*: Superdex 200 fraction. M_L and M_H: low and high molecular weight markers, respectively.

11. Add 1 mL of premixed Cu^{2+}-alkaline solution (solutions I + II + III).
12. Allow to stand for about 10 min.
13. Add 100 µL of 1 *M* phenol reagent while vortexing the protein solutions.
14. Allow to stand at room temperature for at least 30 min.
15. Read absorbance at 750 nm **(***see* **Note 13)**.

4. Notes

1. This strain lacks protease genes (*lon*–, *ompT* $^-$), and carries the bacteriophage DE3 lysogen containing the IPTG-inducible T7 RNA polymerase gene. The T7 lysozyme gene is present on the chloramphenicol-resistant plasmid pACYC184 as the plasmids pLysS or pLysE ***(12)***.
2. For instance, sonication for 5–10 min at 20% pulse by Shimadzu USP-300 ultrasonic processor with a microchip at output control 4.5, with the container cooled in an ice-ethanol bath, checking to avoid the sample becoming hot. Check the viscosity periodically with a micropipetter tip.
3. Ammonium sulfate precipitation is important to remove nucleic acids and low molecular weight substances that interfere with anion exchange chromatography. Residual nucleic acids elute around 0.25 *M* NaCl **(Fig. 1)** and partly overlap with the calpastatin peak.
4. Use cellulose dialysis tubing with MWCO 8000 or less (3500) pore size for the recombinant calpastatin of a single inhibitory domain. The single domain 1 protein may be lost when dialysis tubing with larger pore size is used.
5. The fractions should be monitored at 280 nm and 260 nm to observe both protein and residual nucleic acid. Owing to their low content of aromatic residues, calpastatins have low absorption coefficients. For instance, the values of $E^{1\ mg/ml}$ at 280 nm of domain 1 and the full-length human calpastatin are about 0.27 and 0.11, respectively.

6. Calpastatin is a major protein in the heat-treated supernatant, so that SDS-PAGE analysis is sufficient to identify the calpastatin peak in column eluates, and assay of calpastatin activity is not necessary, except to confirm that the isolated protein possesses the desired inhibitory activity.
7. Calpastatins behave abnormally on gel-filtration columns. The single inhibitory domain, which has a theoretical M_r = 14,895, elutes with an apparent molecular weight of 40–50 kDa. The full-length calpastatin, theoretical M_r = 76,480, elutes as a 400–500 kDa protein **(Fig. 2)**.
8. The His-tagged full-length calpastatin can be recovered as a soluble protein in nondenaturing conditions from *E. coli*. Denaturing conditions are however recommended in the case of purification using Ni-NTA resin, since binding of the His-tagged calpastatin to the resin under non-denaturing condition is less efficient, probably because of hindrance by acidic regions of calpastatin.
9. As a precaution, keep the supernatant until successful binding of the His-tagged calpastatin to Ni-NTA-resin and recovery has been established by gel electrophoresis at **Step 12** or **14**.
10. Elution with imidazole buffer at pH 8 instead of acetate buffer at pH 4.5 is also possible.
11. Boiling should be performed only at neutral pH, since boiling at pH 4.5 permits hydrolysis by acid and metal proteases. EDTA is not an effective chelator at pH 4.5, so that the metalloproteases are not inhibited at this pH.
12. Glass tubes are convenient for multiple samples because capping and recapping are not then necessary.
13. Calpain activity is calculated as follows:

$$A_{750}(+\text{calpain}) - A_{750}(-\text{calpain}) = \Delta A_{750}$$

One unit of calpain activity is defined as the amount of calpain which causes 1.0 ΔA_{750} in 30 min in the given conditions. With these units, the specific activity of purified μ-calpain is 1000–1500 U/mg. One unit of calpastatin activity is the amount of inhibitor that inhibits 1 unit of calpain. Note that these unit definitions are not the same as those described previously ***(13)***.

References

1. Parkes, C. (1986) Calpastatins, in *Proteinase Inhibitors* (Barrett, A. J., and Salvesen, G. eds.), Elsevier, Amsterdam, pp. 571–587.
2. Maki, M., Hatanaka, M., Takano, E., and Murachi, T. (1990) Structure-function relationship of calpastatins, in *Intracellular Calcium-Dependent Proteolysis* (Mellgren, R. L. and Murachi, T. eds.), CRC Press, Boca Raton, FL, pp. 37–54.
3. Mellgren, R. L.(1997) Specificities of cell permeant peptidyl inhibitors for the proteinase activities of μ-calpain and the 20S proteasome. *J. Biol.Chem.* **272,** 29,899–29,903.
4. Lee, W. J., Ma, H., Takano, E., Yang, H. Q., Hatanaka, M., and Maki, M. (1992) Molecular diversity in amino-terminal domains of human calpastatin by exon skipping. *J. Biol. Chem.* **267,** 8437–8442.

5. Cong, M., Thompson,V. F., Goll, D. E., and Antin, P. B. (1998) The bovine calpastatin gene promoter and a new N-terminal region of the protein are targets for cAMP-dependent protein kinase activity. *J. Biol. Chem.* **273,** 660–666.
6. Maki, M., Takano, E., Mori, H., Sato, A., Murachi, T., and Hatanaka, M. (1987) All four internally repetitive domains of pig calpastatin possess inhibitory activities against calpains I and II. *FEBS Lett.* **223,** 174–180.
7. Emori, Y., Kawasaki, H., Imajoh, S., Minami, Y., and Suzuki, K. (1988) All four repeating domains of the endogenous inhibitor for calcium-dependent protease independently retain inhibitory activity: Expression of the cDNA fragments in *Echerichia coli*. *J. Biol. Chem.* **263,** 2364–2370.
8. Maki, M., Takano, E., Osawa, T., Ooi, T., Murachi, T., and Hatanaka, M. (1988) Analysis of structure-function relationship of pig calpastatin by expression of mutated cDNAs in *Escherichia coli*. *J. Biol. Chem.* **263,** 10,254–10,261.
9. Uemori, T., Shimojo, T., Asada, K., Asano, T., Kimizuka, F., Kato, I., Maki, M., Hatanaka, M., Murachi, T., Hanzawa, H., and Arata, Y. (1990) Characterization of a functional domain of human calpastatin. *Biochem. Biophys. Res. Commun.* **166,** 1485–1493.
10. Yang, H. Q., Ma, H., Takano, E., Hatanaka, M., and Maki, M. (1994) Analysis of calcium-dependent interaction between amino-terminal conserved region of calpastatin functional domain and calmodulin-like domain of μ-calpain large subunit. *J. Biol. Chem.* **269,** 18,977–18,984.
11. Hitomi, K., Yokoyama, A., and Maki, M. (1997) Expression of biologically active human calpastatin in baculovirus-infected insect cells and in *Escherichia coli*. *Biosci. Biotech. Biochem.* **62,** 136–141.
12. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* **185,** 60–89.
13. Yoshimura, N., Kikuchi, T., Sasaki, T., Kitahara, A., Hatanaka, M., and Murachi, T. (1983) Two distinct Ca^{2+} proteases (calpain I and calpain II) purified concurrently by the same method from rat kidney. *J. Biol. Chem.* **258,** 8883–8889.
14. Ross, E. and Schatz, G. (1973) Assay of protein in the presence of high concentrations of sulfhydryl compounds. *Anal. Chem.* **54,** 304–306.

11

Preparation of Calpastatin Samples for Western Blotting

Masatoshi Maki and Kiyotaka Hitomi

1. Introduction

Determination of calpastatin levels in cells or tissues by Western blotting using antibodies, or by assay of calpain inhibitory activities in vitro, may be required to assess the role and the level of calpastatin, with respect to the levels of other components of the calpain system, in certain cell biological phenomena. Although calpastatin is resistant to heat or denaturants, it is extremely labile to cellular proteases ***(1)***. The wide range and heterogeneity of the apparent molecular masses of calpastatins (17–170 kDa), as revealed by Western blotting of extracts of different tissues and cells, is due both to degradation of the proteins during preparation, and also to alternative splicing and posttranslational modifications ***(2–4)***. In addition to these factors, estimation of molecular weight is unusually difficult because calpastatins migrate abnormally slowly in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), so that their molecular masses are overestimated by 40–60% ***(5)***. Full-length calpastatins (made up of domains L and 1–4) from most tissues of human, rabbit, pig, and others, migrate as 110–120 kDa proteins in SDS-PAGE. However calpastatin from bovine heart migrates as a protein of 140–170 kDa ***(1)***, probably because this calpastatin has an amino-terminal amino acid sequence (XL region) upstream of the previously assigned translation initiation codon ***(6)***. In contrast, erythrocyte calpastatins lack amino-terminal domains and migrate as 70-kDa proteins ***(7,8)***.

An unusual degree of care is therefore required to avoid degradation during sample preparation, in order to establish that differences in size are natural and not artifactual, and for this reason fixation of protein samples by trichloroacetic acid (TCA) treatment before SDS-PAGE is commonly used ***(9–11)***. In this chapter, a

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protocol for preparation of samples from cultured cells for Western blotting is described. Although the method is described for calpastatin, it is equally valuable for any protein that appears to be particularly labile during preparation of cell extracts for Western blotting.

2. Materials

1. Wash buffer: Dulbecco's phosphate-buffered saline (PBS), (8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na_2HPO_4, 0.2 g/L KH_2PO_4), 1 m*M* EDTA, 0.5 m*M* PMSF.
2. 50% (w/v) TCA (strong acid, corrosive), stored in a brown glass bottle at room temperature.
3. Solubilizing solution: 9 *M* urea, 2% Triton X-100, 1% DTT. Dissolve 5.4 g of urea in approximately 5 mL of warm water, and add 2 mL of 10% Triton X-100. Cool on ice. Add 100 mg of DTT and make up the volume to 10 mL. Dispense aliquots of 1 mL and store at –20°C. Thaw to room temperature just before use (*see* **Note 1**).
4. 1 *M* Tris for neutralization: dissolve 1.21 g Tris base in a final volume of 10 mL of water.
5. 5 × SDS gel sample buffer: 0.31 *M* Tris-HCl, pH 6.8, 10% (w/v) SDS, 50% (w/v) glycerol, 25% 2-ME, 0.0125 % bromophenol blue. Dispense aliquots into screw-capped vials and store at –20°C (*see* **Note 2**).

3. Method

Steps 1–8 should be performed on ice or at 4°C.

1. Remove medium from a 60-mm dish containing adherent cells.
2. Wash cells with 3 mL of ice-cold wash buffer.
3. Scrape cells gently with a rubber policeman in 1–2 mL of wash buffer, and transfer cells to a 15-mL plastic tube. Repeat the scraping with 1–2 mL of wash buffer and combine the cell suspensions. In the case of nonadherent cells, harvest cells by centrifugation, wash twice with wash buffer and then go to **step 6**.
4. Centrifuge the cell suspension at low speed (250*g* for 3 min).
5. Suspend cells with 2–3 mL of wash buffer, and collect cells by centrifugation.
6. Suspend cells with 100 µL of wash buffer containing 20 µg/mL leupeptin and 1 µ*M* pepstatin, and transfer the suspension to a 1.5-mL microfuge tube.
7. Add 25 µL of 50% TCA, mix well and allow to stand on ice for 10–30 min (*see* **Note 3**).
8. Centrifuge at 12,000*g* for 5 min.
9. Remove supernatant, and recentrifuge the pellet briefly to permit complete removal of supernatant.
10. Add 80 µL of 9*M* urea solubilizing solution to the pellet, and mix well.
11. Add 20 µL of 5 × SDS gel sample buffer.
12. Sonicate the mixture in order to dissolve the pellets completely.
13. Neutralize the solution with 3–5 µL of 1 *M* Tris base (*see* **Note 4**).
14. Sonicate briefly if the sample is too viscous (*see* **Note 5**).
15. Load samples on to a 7.5 % polyacrylamide slab gel without boiling (*see* **Note 6**).

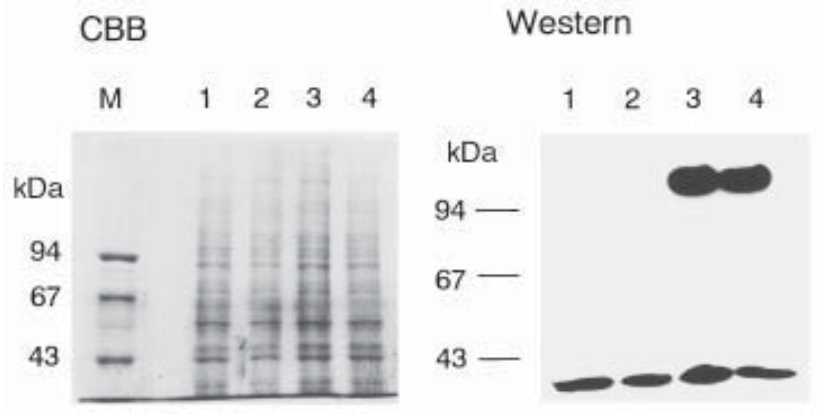

Fig. 1. Western blotting of human calpastatin. Human calpastatin cDNA expression plasmid (pCXN2HACS ***[13]***) was introduced into mouse fibroblasts (NIH3T3 cells) and stably expressing clones were selected. Protein samples were prepared after TCA fixation of the cells as described in the text. ***Left:*** Coomassie brilliant blue R-250 (CBB) staining. ***Right:*** Western blotting using anti-human calpastatin monoclonal antibody, with detection by the chemiluminescent method. The bands at the electrophoresis front represent nonspecific immunoreactants. *Lanes 1, 2*: vector (pCXN2); *Lanes 3, 4*: pCXN2HACS.

4. Notes

1. Urea precipitates when the solution is kept on ice for a long time.
2. Use gloves, especially when handling SDS gel sample buffer, to avoid contamination of the samples with keratins from fingers. Since the solubilizing solution contains DTT, this 5× SDS gel sample buffer does not strictly require 2-ME, but in practice it is convenient to include 2-ME and share the gel sample buffer with that for other unfixed protein samples.
3. The rate and extent of degradation of calpastatin during sample preparation depends to some extent on cell type, and TCA fixation may not be necessary in all cases. Scraping adherent cells after fixation in 10% TCA for 15–30 min is an alternative method **(***see* **Note 7)**. If it is required to prepare calpastatin samples from cells without TCA fixation, immediate heat treatment of cytosol with SDS in the presence of protease inhibitors such as PMSF, *p*-aminobenzamidine, and EDTA is recommended to inactivate cellular proteases. For unknown reasons, however, erythrocyte calpastatin was reported to migrate as a 90–110 kDa protein when cytosol was not heat-treated but as a 70 kDa protein when heat-treated ***(12)***.
4. Neutralization should be carried out cautiously, and can be easily monitored by the change of color of bromophenol blue from yellow to blue. Do not add excess Tris.
5. Neutralized samples become highly viscous due to the presence of high molecular-weight DNA if sonication is not sufficient at **step 12**. Shearing of the DNA by a water bath-type sonicator for 1–2 min reduces the viscosity sufficiently to permit loading of samples onto the gel.

6. Do not boil the final samples before loading them onto the gel. Decomposition of urea by heating may lead to covalent modifications of proteins.
7. If it proves difficult to avoid calpastatin degradation during sample preparation, the cells can be fixed in the dishes with 10 % TCA, scraped into centrifuge tubes and recovered by centrifugation, and the pellets dissolved in 9 *M* urea as in **step 10**.

References

1. Parkes, C. (1986) Calpastatins, in *Proteinase Inhibitors* (Barrett, A. J., and Salvesen, G. eds.), Elsevier, Amsterdam, pp. 571–587.
2. Maki, M., Ma, H., Takano, E., Adachi, Y., Lee, W. J., Hatanaka, M., and Murachi, T. (1991) Calpastatins: Biochemical and molecular biological studies. *Biomed. Biochim. Acta* **50,** 509– 516.
3. Takano, E., Nosaka, T., Lee, W. J., Nakamura, K., Takahashi, T., Funaki, M., Okada, H., Hatanaka, M., and Maki, M. (1993) Molecular diversity of calpastatin in human erythroid cells. *Arch. Biochem. Biophys.* **303,** 349–354.
4. Lee, W. J., Ma, H., Takano, E., Yang, H. Q., Hatanaka, M., and Maki, M. (1992) Molecular diversity in amino-terminal domains of human calpastatin by exon skipping. *J. Biol. Chem.* **267,** 8437–8442.
5. Takano, E., Maki, M., Mori, H., Hatanaka, M., Marti,T., Titani,K., Kannagi, R., Ooi,T. and Murachi,T. (1988) Pig heart calpastatin: Identification of repetitive domain structures and anomalous behavior in polyacrylamide gel electrophoresis. *Biochemistry* **27,** 1964–1972.
6. Cong, M., Thompson,V. F., Goll, D. E., and Antin, P. B. (1998) The bovine calpastatin gene promoter and a new N-terminal region of the protein are targets for cAMP-dependent protein kinase activity. *J. Biol. Chem.* **273,** 660–666.
7. Takano, E., Kitahara, A., Sasaki, T., Kannagi, R., and Murachi, T. (1986) Two different molecular species of pig calpastatin: Structural and functional relationship between 107 kDa and 68 kDa molecules. *Biochem. J.*, **235,** 97–102.
8. Imajoh, S., Kawasaki, H., Emori, Y., and Suzuki, K. (1987) Calcium-activated neutral protease inhibitor from rabbit erythrocyes lacks the N-terminal region of the liver inhibitor but retains three inhibitory units. *Biochem. Biophys. Res. Commun*. **146,** 630–837.
9. Kikuchi, H. and Imajoh-Ohmi, S. (1995) Antibodies specific for proteolyzed forms of protein kinase Cα. *Biochim. Biophys. Acta* **1269,** 253–259.
10. Yu, D., Imajoh-Ohmi, S., Akagawa, K., and Kanegasaki, S. (1996) Suppression of superoxide- generating ability during differentiation of monocytes to dendritic cells. *J. Biochem.* **119,** 23–28.
11. Wang, K. K., Posner, A., and Hajimohammadreza, I. (1996) Total protein extraction from cultured cells for use in electrophoresis and Western blotting. *Biotechniques* **20,** 662–668.
12. Schwarz-Benmeir, N., Glaser, T., Barnoy, S., and Kosower, N. S. (1994) Calpastatin in erythrocytes of young and old individuals. *Biochem. J*. **304,** 365–370.
13. Hitomi, K., Yokoyama, A., and Maki, M. (1997) Expression of biologically active human calpastatin in baculovirus-infected insect cells and in *Escherichia coli*. *Biosci. Biotech. Biochem.* **62,** 136–141.

12

Isolation and Characterization of Calpain Activator Protein from Bovine Brain

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1. Introduction

Although in recent years the molecular structure and function of calpains have been extensively investigated, some aspects of the roles and properties of these proteinases remain obscure, particularly their precise function in the cell, and the mechanisms inducing and promoting their activation.

With regard to the question of activation, and before defining a role for these enzymes, it is necessary to explain the apparent inconsistency between the amount of Ca^{2+} required for activation of the purified enzymes in vitro, and the actual intracellular Ca^{2+} concentration. Some reports have suggested that in particular cell compartments, such as the inner surface of plasma membranes ***(1)***, and, at certain times, the Ca^{2+} concentration can reach values very much higher than the average basal cytosolic level. Even if this correct, while such concentrations might possibly be sufficient to activate μ-calpain, they remain apparently too low to activate m-calpain.

Many physiological compounds have been reported to be able to reduce the Ca^{2+} requirement of calpains, including interactions with the nucleus ***(2)***, with phospholipids ***(3),*** or with other metabolites ***(4)***. In studies aimed at characterizing the involvement of calpain in neutrophil activation ***(5)***, we have, however, identified a protein factor promoting expression of calpain activity at Ca^{2+} concentrations very close to physiological. This protein factor, called calpain activator (CA), has been found also in rat skeletal muscle ***(6)***, and, more recently, in bovine brain ***(7)***. The brain calpain activator, isolated and purified to homogeneity, shows high specificity for μ-calpains from different sources.

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Molecular characterization of CA has demonstrated that it is very closely related to the goat liver protein UK114 *(8)*, the mouse heat-responsive protein HR12, and heat shock proteins of the YJGF and YABJ families (sequences taken from SwissProt sequence data bank; *see also* **ref.** *7)*.

At a functional level, CA induces a large decrease in [Ca^{2+}] required by μ-calpains of different sources to reach 50% of V_{max} **(Fig. 1)**. No effect has been observed on m-calpains *(7)*. CA binds preferentially to human erythrocyte μ-calpain 80-kDa catalytic subunit in a molar ratio of 1:1, whereas no appreciable binding to the 30 kD subunit was observed *(9)*. This protein factor seems to be involved in acceleration of the conformational changes in the proteinase that trigger the overall activation process and precede the autoproteolytic steps *(9)*. Due to the fact that CA is probably bound to or associated with intracellular structures, it may be considered to be the site at which calpain could undergo activation and expression of proteolytic activity.

On the basis of its molecular properties and mechanism of action, CA should be considered, after calpain itself and calpastatin, as the third component of the calcium dependent proteolytic system. We present here methods for the purification and assay of CA.

2. Materials

1. Acid-denatured globin, 20 mg/ml, extensively dialyzed against distilled water *(5)*.
2. Purified human erythrocyte μ-calpain **(Note 1)**.
3. Fluorescamine (Sigma, St. Louis, MO), 30 mg/100 mL of pure acetone.
4. TCA: 50% (w/v) in water, stored at room temperature.
5. Borate buffer: 50 m*M* sodium borate, pH 7.5.

3. Methods

3.1. Assay of Calpain Activator Activity

Calpain activator activity is assayed by adding appropriate amounts of the factor to a calpain assay mixture containing 2 μ*M* Ca^{2+}.

1. In a final volume of 0.5 mL, add:
   a. 50 m*M* sodium borate, pH 7.5;
   b. 1 mg of acid-denatured globin;
   c. 2 units of purified μ-calpain **(Note 1)**;
   d. test sample of calpain activator;
   e. 2 μ*M* Ca^{2+} (added last).
2. Divide this mixture into two equal portions.
3. To one portion, add 40 μL of 50% TCA, and place on ice.
4. Incubate the other portion at 25°C for 10 min, and stop reaction by addition of 40 μL of 50% TCA.
5. Centrifuge the samples at 10,000*g* for 5 min.

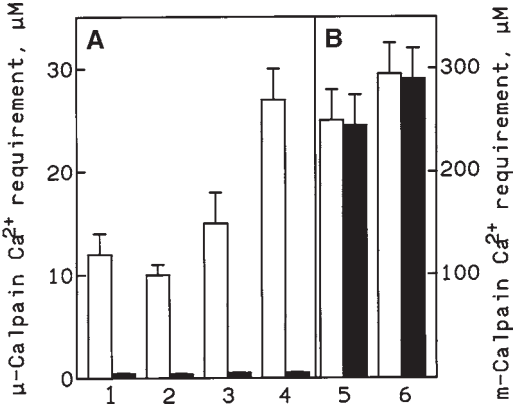

Fig. 1. Effect of CA on the catalytic properties of μ-calpains and m-calpains from various sources. The values indicate the [Ca^{2+}] required to obtain 1/2 V_{max} in the absence (unfilled bars) or in the presence (filled bars) of CA (**A**) Effect of CA on μ-calpains isolated from *1*: rat brain, *2*: rat skeletal muscle, *3*: bovine erythrocytes, *4*: human erythrocytes. (**B**) Effect of CA on m-calpains isolated from *5*: rat brain, *6*: rat skeletal muscle. For experimental details *see* **ref. *7***.

6. Estimate the concentration of free α-NH_2 groups with fluorescamine at pH 8.5 ***(10)***. Dilute 0.2 mL of the clear TCA supernatant in 2 mL of 0.5 *M* sodium borate, pH 8.5, and add 0.2 mL of fluorescamine solution with continuous vigorous stirring.
7. Measure fluorescence at 475 nm, with excitation at 384 nm.
8. One unit of calpain activator activity is defined as the amount causing the appearance of 1 unit of calpain activity ***(11)*** in the presence of 2 μ*M* Ca^{2+} ***(7)***.

3.2. Purification Procedure

A flow chart of the purification procedure is given in **Fig. 2**. All procedures are carried out on ice or at 4°C unless otherwise stated.

3.2.1. Extraction

1. Wash freshly collected bovine brain (80 g) immediately in ice-cold 0.25 *M* sucrose solution to remove blood contamination, and place the tissue on ice. Other tissues may be extracted similarly.
2. Homogenize 10 g portions of the washed brain with a Potter-Elvehjem homogenizer in 40 mL of 50 m*M* sodium acetate, pH 5.8, 0.25 *M* sucrose, 1 m*M* EDTA, 1 m*M* 2-ME (*see* **Note 2**).
3. Sonicate the suspension (6 bursts,10 s each).
4. Centrifuge the lysate at 25,000*g* for 10 min and reject the pellet of particulate material. Collect all supernatants.
5. Adjust the clear supernatant obtained by centrifugation to pH 5.8 by addition of a few drops of 1 *M* HCl, and transfer it to a glass beaker.

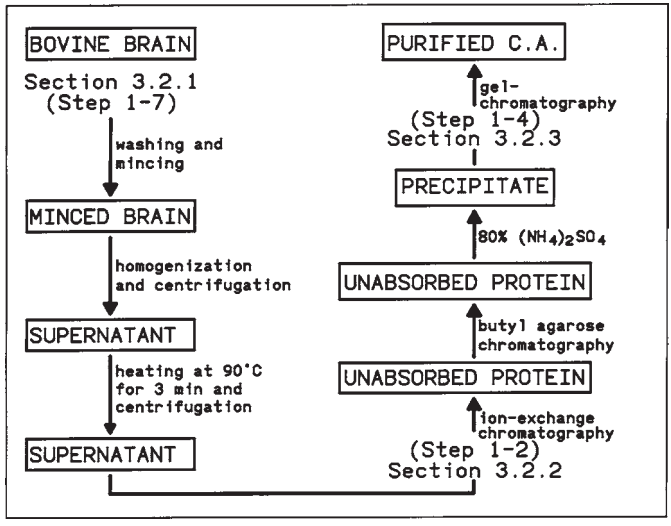

Fig. 2. Flow chart of the purification procedure of calpain activator from bovine brain. The sections in which each of the steps in the procedure is described are indicated.

6. Heat the solution quickly to 90°C by standing the beaker in boiling water, maintain at 90°C for 3 min with continuous vigorous stirring, then cool immediately to 4°C in an ice bath.
7. Remove precipitated protein by centrifugation at 25,000*g* for 10 min (*see* **Note 3**).

3.2.2. Separation of Calpain Activator From Calpastatin and Other Contaminating Proteins

1. Ion exchange chromatography. Bring the pH of the supernatant following heat treatment to 7.5 by adding a few drops of 2 *M* NaOH, and dialyze for 4 h against borate buffer.
2. Filter the dialyzed solution through a 0.8 µm filter to remove insoluble particles, and apply it to a Source 15 Q column (1.5 × 5 cm) (Amersham-Pharmacia Biotech, UK), previously equilibrated in borate buffer.
3. Run the column at 4 mL/min and collect fractions of 10 mL.
4. Measure protein by absorbance at 280 nm and assay the calpain activator protein as described in **Subheading 3.1.**, using 50 µL of each fraction.
5. Pool the fractions containing protein not retained by the column (total volume: ~260 mL), which contain also the calpain activator activity (*see* **Note 4**).
6. Chromatography on butyl-agarose (*see* **Note 5**). Apply the pooled fractions from **step 5** to a butyl-agarose column (1 × 8 cm) equilibrated with borate buffer.
7. Run the column at 0.8 mL/min and collect fractions of 10 mL.
8. Assay calpain activator activity on portions of 50 µL.
9. The calpain activator is not retained by the resin, and is recovered in the flow-through fractions (*see* **Notes 6** and **7**).

3.2.3. Gel Filtration Chromatography

1. Add ammonium sulfate to 80% of saturation (140 g/250 mL), to pools of fractions containing calpain activator following ion exchange or butyl-agarose chromatography (*see* **Note 8**).
2. Stir gently on ice for 1 h, recover the precipitated protein by centrifugation at 25,000*g* for 10 min, and dissolve the precipitate in 2 mL of borate buffer.
3. Apply the solution from the previous step to a Sephadex G-100 column (1.5 × 170 cm) previously equilibrated in borate buffer.
4. Run the column at 12 mL/h and collect fractions of 1.5 mL (*see* **Note 9**).
5. The calpain activator is eluted in a peak before the end of the included volume, corresponding to a molecular mass between 30 and 40 kDa, and at this point reaches its highest specific activity.

4. Notes

1. Human erythrocyte μ-calpain is purified to homogeneity following the procedure described in **ref. *11***. Calpain from human erythrocytes was chosen for its high responsiveness to calpain activator from various tissues. When required, human erythrocyte calpain was replaced with μ-calpain from other sources.
2. The presence of 0.1 m*M* (or greater) EDTA or EGTA is crucial for the extraction step. In the absence of EDTA, the recovery of soluble calpain activator is very low (less than 10% of total). This observation suggests that the calpain activator is normally bound to insoluble cell structures, and that this binding is Ca^{2+}-dependent. The acidic pH of the extraction medium is not strictly essential, but has been chosen to avoid adding large amounts of HCl before heat treatment. 2-ME is only added to the extraction buffer, and is not required in the CA assay mixture.
3. The calpain activator is stable in boiling water for very long periods. The pH is also not very important, since more than 90% recovery of calpain activator activity has been obtained following heat treatment at pH values between 5 and 9. The pH value of 5.8 has been selected because it promotes a large denaturation of contaminating proteins during heating and the highest calpain activator specific activity.
4. This step removes most of the calpastatin, which is retained by the resin.
5. The butyl-agarose column removes any remaining traces of calpastatin.
6. Calpastatin is completely retained by butyl-agarose and can be eluted with 0.2 *M* NaCl in borate buffer. Calpain activator and calpastatin are completely separated by these two last steps.
7. We have recently developed a new procedure to increase the yield and to reduce the time required. The ion exchange column is connected directly to the butyl-agarose column in an FPLC apparatus, since both columns require the same buffer and the calpain activator is not retained by either column, and the combined system is run at 0.8 mL/min. The eluted material contains calpain activator without any contamination by calpastatin.
8. Owing to the "sticky" nature of calpain activator, concentration by ultrafiltration through porous membranes is not recommended, particularly when the protein

concentration is low. Lyophilization causes a large inactivation of calpain activator, probably because of the high salt concentration reached during the process and in the resulting final solution. By contrast, the calpain activator is recovered fully active and in high yield from ammonium sulfate precipitation.

9. FPLC chromatography on a Superose 12 column (1 × 30 cm) at a flow rate of 0.4 mL/min in borate buffer can be used instead of Sephadex G-200. Samples of 0.2 mL are loaded and fractions of 0.4 mL collected. The activator is eluted at a position corresponding approximately to a molecular mass of 30 kDa. SDS-PAGE analysis revealed the presence of a single protein band with a molecular weight of 14.5, suggesting that this protein factor is a homodimer.

Acknowledgments

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References

1. Davies, E. V. and Hallett, M. B. (1998) High micromolar Ca^{2+} beneath the plasma membrane in stimulated neutrophils. *Biochem. Biophys. Res. Commun.* **248,** 679–683.
2. Mellgren, R. L. and Lu, Q. L. (1994) Selective nuclear transport of mu-calpain. *Biochem. Biophys. Res. Commun*. **204,** 544–550.
3. Pontremoli, S., Melloni, E., Sparatore, B., Salamino, F., Michetti, M., Sacco, O., and Horecker, B. L. (1985) Binding to erythrocyte membrane is the physiological mechanism for activation of Ca^{2+}-dependent neutral proteinase. *Biochem. Biophys. Res. Commun*. **128,** 331–338.
4. Croall, D. E. and De Martino, G. N. (1991) Calcium-activated neutral protease (calpain) system: Structure, function, and regulation. *Physiol. Rev.* **71,** 813–847.
5. Pontremoli, S., Melloni, E., Michetti, M., Salamino, F., Sparatore, B., and Horecker, B. L. (1988) An endogenous activator of the Ca^{2+}-dependent proteinase of human neutrophils that increases its affinity for Ca^{2+}. *Proc. Natl. Acad. Sci. USA* **85,** 1740–1743.
6. Pontremoli, S., Viotti, P. L., Michetti, M., Sparatore, B., Salamino, F., and Melloni, E. (1990) Identification of an endogenous activator of calpain in rat skeletal muscle. *Biochem. Biophys. Res. Commun*. **171,** 569–574.
7. Melloni, E., Michetti, M., Salamino, F., and Pontremoli, S. (1998) Molecular and functional properties of a calpain activator protein specific for mu-isoforms. *J. Biol. Chem*. **273,** 12827–12831.
8. Ceciliani, F., Faotto, L., Negri, A., Colombo, I., Berra, B., Bartorelli, A., and Ronchi, S. (1996) The primary structure of UK114 tumour antigen. *FEBS Lett*. **393,** 147–150.
9. Melloni, E., Michetti, M., Salamino, F., Sparatore, B., and Pontremoli, S. (1998) Mechanism of action of a new component of the Ca^{2+}-dependent proteolytic system in rat brain: The calpain activator. *Biochem. Biophys. Res. Commun*. **249,** 583–588.

10. Udenfriend, S., Stein, S., Bohlen, P., Dairman, W., Leingruber, W., and Weigele, M. (1972) Fluorescamine: A reagent for assay of amino acids, peptides, proteins, and primary amines in the picomole range. *Science* **178,** 871–872.
11. Melloni, E., Salamino, F., Sparatore, B., Michetti, M., and Pontremoli, S. (1984) Ca^{2+}- dependent neutral proteinase from human erythrocytes: Activation by Ca^{2+} ions and substrate and regulation by the endogenous inhibitor. *Biochem. Int*. **8,** 477–489.
12. Michetti, M., Salamino, F., Minafra, R., Melloni, E., and Pontremoli, S. (1997) Calcium binding properties of human erythrocyte calpain. *Biochem. J*. **325,** 721–726.

13

Calpain Zymography with Casein or Fluorescein Isothiocyanate Casein

J. Simon C. Arthur and Donald L. Mykles

1. Introduction

Methods for analyzing enzyme activity after electrophoresis in acrylamide gels have been described for a variety of proteins, including several proteases and kinases. The application of this approach to detect calpain activity by casein zymography was first described by Raser et al. in 1995 ***(1)***, and has proved itself by its successful application in several laboratories interested in calpain. Briefly, samples are run under nondenaturing conditions in the presence of EDTA on acrylamide gels in which casein has been copolymerized. After electrophoresis the gels are incubated overnight in buffer containing Ca^{2+} and reducing agent to activate calpain. During this time, casein in the region of an active calpain band is digested to fragments sufficiently small to diffuse out of the gel, and the calpain itself is also extensively degraded. The gel is then stained conventionally in Coomassie brilliant blue, giving rise to a clear band in the dark blue gel. These gels are not very easy to photograph, and a useful modification is to use fluorescein isothiocyanate (FITC)-casein in place of casein in the gels. The FITC-casein system is also more sensitive. In this case, the gels are viewed and photographed with ultraviolet (UV) illumination, and proteinase activity produces dark (nonfluorescent) zones in a bright background. The zymogram method as applied to calpain is still undergoing modifications in many laboratories, as shown for example in Chapter 14, in which some details of the method differ.

Most other intracellular proteases such as cathepsins and proteasomes do not give contaminating bands in this assay as they are either too big to enter the gel, or are unable to generate clear bands on the gel, possibly because they do not cleave the casein into small enough fragments ***(1)***. Some proteases, such as

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trypsin, do give discrete bands, but these can be distinguished from calpain by the fact that they are not Ca^{2+}-dependent. It has been suggested that extensive treatment with serine protease inhibitors such as phenylmethane sulfonyl fluoride (PMSF) or tosyl lysine chloromethyl ketone (TLCK), should minimize their interference.

Of the nonubiquitous calpains, the lens-specific isoform Lp82 gives bands on these casein gels, but calpain 3 or p94 does not ***(2)***.

The casein zymogram has both advantages and disadvantages when compared to standard casein assays for calpain. Unlike most calpain assays carried out in solution, zymograms are unaffected by the presence of calpastatin or other reversibly binding inhibitors of calpain, since these inhibitors in general do not bind in the absence of Ca^{2+}, and separate from calpain during electrophoresis. The zymograms can therefore be used to assess the overall calpain activity in tissue homogenates or cell lysates, in which calpastatin would partially or entirely mask calpain activity in normal assays. Covalent inhibitors such as E-64 remain associated with calpain and no activity is detected in this system ***(1,3)***. Casein zymograms have the very useful ability to resolve μ-and m-calpain activity in a single step from a crude extract, and the method has proved most popular as a tool for detecting μ-and m- calpain activity in cell or tissue extracts, after various prior treatments, without any further manipulation ***(4,5)***. Calpains which have undergone small subunit autolysis before gel loading run normally and are detectable on casein zymograms, with shifts in mobility which we attribute to cleavage of the small subunit from 28 to 20 kDa. By contrast, calpains in which large subunit autolysis has occurred prior to gel loading appear not to be stable in this gel system, and are not detected. Bacterially expressed μ-and m-calpains containing 80 and 21 kDa subunits are readily detected on these gels: they run (as stated earlier) significantly lower on the gel, that is, with increased mobility, than the corresponding 80 + 28 kDa enzymes **(Fig. 1)**. The large subunits of μ-and m-calpain, when expressed alone in bacteria (i.e., in the absence of a small subunit), do not give detectable bands on casein zymograms.

The principal disadvantages of casein zymograms are that they are unsuited for precise quantification (*see* **Note 1**), the process takes much longer than a standard casein assay, and the number of samples that can be assayed simultaneously is limited by the number of gels one is equipped to run.

We present here the practical details required for carrying out the method.

2. Materials

1. Acrylamide solution (acrylamide:bis ratio = 74:1): 29.6% (w/v) acrylamide, 0.4% (w/v) bisacrylamide, in water, filtered, and stored at room temperature in the dark (*see* **Note 2**).
2. Buffer A: 1.5 *M* Tris-HCl, pH 8.8.

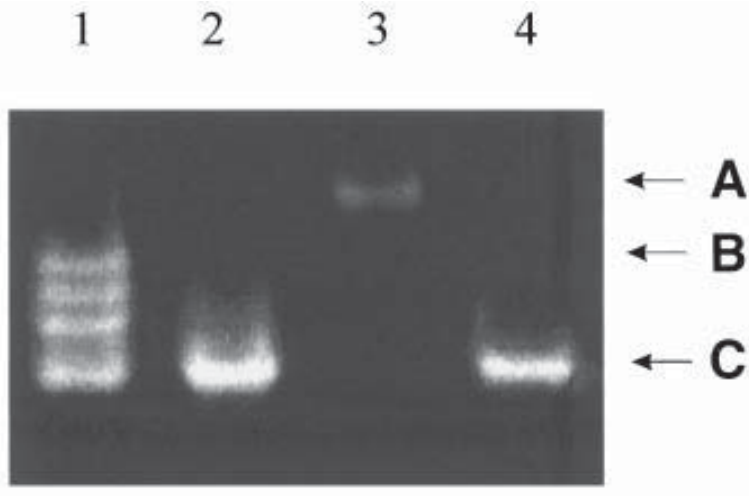

Fig. 1. Casein zymogram of recombinant rat calpains, expressed in *E. coli*. The lanes contained samples of *E. coli* lysate (corresponding to 1 mL of a bacterial culture) containing: 1: rat m-80k/28k calpain, showing several bands representing stepwise degradation of the small subunit which in practice is very difficult to avoid even in the presence of standard proteinase inhibitors; 2: rat m-80k/21k calpain; 3: rat μ-80k/21k calpain; 4: rat m-80k/21k calpain. The arrows indicate: (**A**) the positions of μ-80k/21k calpain; (**B**) m-80k/28k calpain; (**C**) m-80k/21k calpain.

3. Buffer B: 0.5 *M* Tris-HCl, pH 6.8.
4. Casein solution: 10 mg/mL casein in 0.75 *M* Tris-HCl, pH 8.8 (i.e., 50% buffer A) (*see* **Note 3**).
5. FITC-casein solution: 10 mg/mL in 0.75 *M* Tris-HCl, pH 8.8, prepared as described *(7)*.
6. Gel sample buffer: 20% (v/v) glycerol, 0.1 *M* Tris-HCl, pH 6.8, 10 m*M* EDTA, 10 m*M* 2-ME, 0.02% bromophenol blue.
7. Electrophoresis buffer (5 × stock): 125 m*M* Tris base, 625 m*M* glycine, 5 m*M* EDTA, pH 8. This buffer should not require pH adjustment. Dilute to 1 ×, cool to 4°C, and add fresh 10 m*M* 2-ME or 1 m*M* DTT immediately before use.
8. Ca^{2+} incubation buffer: 50 m*M* Tris-HCl, pH 7 at room temperature, 5 m*M* $CaCl_2$, 10 m*M* 2-ME or DTT.
9. Stain solution: 1% (w/v) Coomassie brilliant blue, 10% (v/v) acetic acid, 40% (v/v) methanol, in water.
10. Destain solution: 10% acetic acid, 40% methanol, in water (*see* **Note 4**).
11. Lysis buffer: 50 m*M* HEPES, pH 7.6, 150 m*M* NaCl, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100, 5 m*M* EDTA, 10 m*M* 2-ME, 100 μ*M* PMSF, 10 μg/mL leupeptin (*see* **Note 5**).

3. Methods

3.1. Sample Preparation

1. Homogenize tissue samples in 5–10 vol of lysis buffer at 4°C.
2. Wash cultured cells in PBS and then lyse the cells in one 10-cm dish by scraping in 1 mL of lysis buffer at 4°C. If trypsin is used to remove cells from culture dishes, the cells must be pelleted and washed well in PBS to remove trypsin before lysing the cells.
3. Do not sonicate cell suspensions or lysates—this lysis buffer is sufficient to release calpain.
4. To assay calpain artificially expressed for example in *E. coli*, grow 10 mL of *E. coli* containing the relevant plasmids with appropriate addition of IPTG, recover the cells by centrifugation, resuspend the cells in 0.1 mL of 25 m*M* Tris-HCl, pH 7.5, 5% (v/v) glycerol, 5 m*M* EDTA, 100 µ*M* PMSF, and sonicate with a microtip.
5. Centrifuge tissue homogenates and cell lysates at 12,000*g* for 10 min at 4°C to remove any insoluble material. Recover the supernatant solution.
6. Mix samples with an equal volume of gel sample buffer, or 0.25 vol of a 5 × concentrated form of the gel sample buffer. Do not heat the samples (*see* **Note 6**).
7. For purified calpains, run 1–2 µg per lane. For tissue and cell extracts, run 10–30 µg of soluble protein per lane.

3.2. Gel Preparation and Electrophoresis

1. For the 10% acrylamide resolving gel, mix acrylamide solution, buffer A, casein or FITC-casein solution, and water in the quantities listed in **Table 1**, to make two minigels with finished dimensions of 8 × 5 × 0.075 cm. Acrylamide is toxic and should be handled appropriately. Volumes for FITC-casein are in brackets.
2. Degas solution for 10 min.
3. Add TEMED and ammonium persulfate (**Table 1**), mix gently.
4. Pour the gels to the desired height, and layer water-saturated butanol on top of the gels.
5. Permit the gels to polymerize for at least 1 h at room temperature. They can be wrapped and stored for 1–2 days at 4°C.
6. Mix the components for the stacking gel (**Table 1**), degas briefly, add TEMED and ammonium persulfate, rinse the top of the resolving gel with water, and pour the stacking gel, using a 10-well comb, which provides a maximum loading volume per lane of 25–30 µL.
7. Set up the electrophoresis tank in an ice bath or a cold room, on a magnetic stirrer, and with a peristaltic pump arranged to recirculate the electrophoresis buffer at ~2 mL/min between the anode and cathode compartments. The electrophoresis buffer should be cooled previously to 4°C (*see* **Note 7**).
8. Prerun the gel at 125 V for 15–30 min.
9. Load samples.
10. Run the gel for 2 h at 125 V. The bromophenol blue will run off the gel after about 1 h.
11. For the Ca^{2+}-dependent proteases from lobster, run the gel at 100 V and stop the run when the tracking dye reaches the bottom of the gel *(8)*.

Table 1
Mixtures for Resolving and Stacking Gels

Solution	Resolving gel (mL)	Stacking gel (mL)
Acrylamide (30%)	3.3	0.8
Buffer A, 1.5 *M* Tris-HCl, pH 8.8	1.5	
Buffer B, 0.5 *M* Tris-HCl, pH 6.8		1.25
Casein, 10 mg/mL	2	
Water	3.1	2.95
[FITC-casein, 10 mg/mL]	[0.45]	
[Water]	[4.65]	
	Mix and degas briefly	
TEMED	10 µL	5 µL
10% ammonium persulphate	50 µL	25 µL

12. Stop electrophoresis and remove the gels (*see* **Note 8**).
13. Incubate gels in ~30–50 mL of Ca^{2+} incubation buffer for 20–30 min at room temperature in a shallow tray with lid, with gentle swirling. The presence of fresh reducing agent is important (*see* **Notes 9–11**).
14. If a control is felt to be necessary, an identical gel should be treated in buffer containing 5 m*M* EDTA in place of Ca^{2+} (*see* **Note 12**).
15. Transfer the gels to fresh Ca^{2+} incubation buffer and continue shaking for 20 min.
16. Transfer the gels to fresh Ca^{2+} incubation buffer and continue shaking overnight.
17. Fix casein gels for 20–30 min in destain solution, stain in Coomassie brilliant blue for 30 min, and destain with several washes of destain solution over 30–60 min (*see* **Note 13**) (**Fig. 1**).
18. Photography of sometimes faint clear bands on a Coomassie brilliant blue background is not always easy. Modern image processing machines can scan the gels and print a negative image, which may be easier to see and possibly more suitable for publication.
19. FITC-casein gels are viewed on a UV light box without fixation or staining (**Fig. 2**).

4. Notes

1. A useful estimate of relative amounts of calpain can be obtained by visual comparison of the cleared bands on gels, but precise quantification is difficult, and the response is linear only within a narrow range of predetermined gel loading. Densitometry, scanning, and appropriate software can be used to obtain some degree of objective quantification. No correlation has yet been described between units of µ-and m-calpain activity determined in a standard solution assay, with the size of cleared bands on zymograms. The results of Zhao et al. (Chapter 14) suggest that the response is dependent on pH, so that comparison of µ-and m-calpain activites may be unreliable. This point requires further investigation.

2. The gels are standard discontinuous polyacrylamide gels, very similar to those described by Laemmli ***(6)***, but without SDS. We use a 10% polyacrylamide resolving gel with an acrylamide:bisacrylamide ratio of 74:1, and a 4.8% stacking gel; 12.5% gels and acrylamide/bis ratios of 35:1 have also been used successfully but require longer electrophoresis times and do not result in higher resolution.
3. The 10 mg/ml casein or FITC-casein stock solution should be stirred for at least 45 min to completely dissolve the casein. Addition of low concentrations of 2-ME (0.5 m*M*) help in dissolving the casein, but too much reducing agent interferes with gel polymerization. Once made, the casein solution is stable for 24 h at 4°C; however it is best made fresh or stored in aliquots frozen at –20°C.
4. For economy, we recycle the destain solution by filtration through charcoal to remove the dye.
5. Lysis buffer containing 1% Triton X-100 and 0 or 50 m*M* NaCl is equally effective. A slightly higher NaCl concentration may improve extraction of cytoplasmic protein, but may interfere with electrophoresis if large volumes have to be loaded.
6. Crude extracts can be snap-frozen and stored at –20°C or –70°C, but the calpain activity does not survive repeated freezing and thawing. If repeated analysis of one extract is planned, it should be stored frozen as multiple aliquots, possibly with the addition of 30–40% glycerol or sucrose.
7. The calpains are inactivated by a raised temperature, and care must be taken to keep the gel cold while running. It is also important to circulate the buffer to prevent the formation of pH gradients. We drill small holes in the lid of the electrophoresis tank above the cathode and anode buffer compartments for the pairs of tubes carrying the recirculating buffer.
8. If desired for purposes of comparison, the gels can be blotted after electrophoresis for immunoblotting, instead of incubation in Ca^{2+} activation buffer, but the efficiency of transfer is lower than that for SDS gels. It is useful, however, in separating μ-and m-calpain, which are very often not discriminated by the available anti-calpain antibodies. The small calpain subunit will be found by an appropriate antibody in both the μ-and m-calpain bands. In principle, one could run a casein zymogram as a first dimension, and then place the gel lane across the top of a standard SDS gel to run in a second dimension, before blotting.
9. For μ- and m-calpain from mammalian sources, incubation of the casein gel at room temperature (~20°C) is satisfactory, but the temperature for the overnight incubation should be optimized for the enzymes being studied. For example, calcium-dependent proteases from the tropical land crab (*Gecarcinus lateralis*) are best detected when zymograms are incubated at room temperature (22°C), whereas those from lobster, which live at lower ambient temperatures, are best detected at 4°C.
10. In the following chapter, Zhao et al. describe a casein zymography method with some difference in detail from that described here. Most importantly, it appears that the response of μ-and m-calpain to incubation with Ca^{2+} in these gels is pH-dependent. In our hands, pH 7.6 at room temperature for the Ca^{2+} incubation step appears to be satisfactory for both calpains.

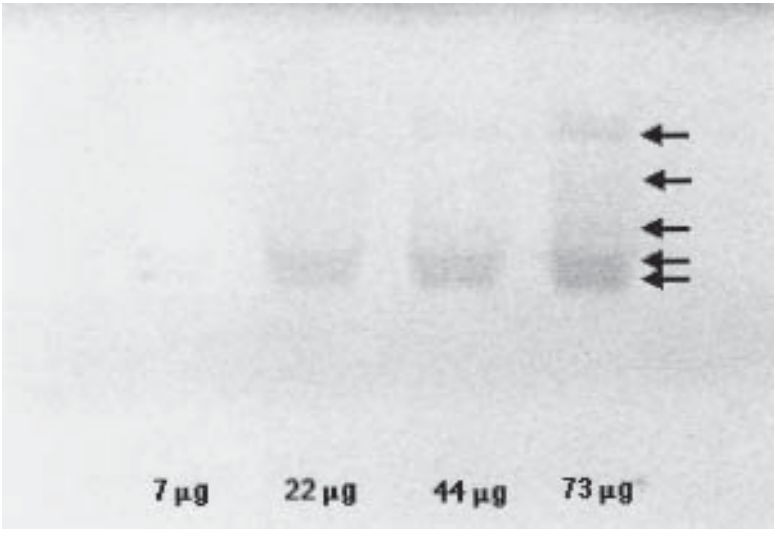

Fig. 2. FITC-casein zymogram of lobster muscle CDP activities. Fractions from organomercurial-agarose chromatography containing all four CDP activities (*see* Chapter 7 in this volume) were electrophoretically separated on a native FITC-casein 10% polyacrylamide gel (*see* Methods) and incubated overnight at 4°C in buffer containing 5 mM $CaCl_2$. A higher-mobility doublet appears at the lowest loading (7 µg protein), indicating greater relative abundance and/or greater specific activity. A lower-mobility band (uppermost band) appears at the 22-µg protein loading. Two intermediate-mobility bands appear at higher loadings (44 µg and 73 µg protein). A control gel incubated in Ca^{2+}-free buffer, in which 5 mM $CaCl_2$ was replaced with 5 mM EGTA, showed no proteolysis at all loadings (Linstrom and Mykles, data not shown). The relationship between the five bands and the four CDPs (I, IIa, IIb, and III) isolated from the same tissue ***(8)*** is not known. Figure provided by Nathan Linstrom and Donald Mykles.

11. It is reported by Dr. Kosower (Chapter 22) that the inclusion of 0.2 units of calpastatin/ml of Ca^{2+} activation buffer stabilizes µ-calpain from myoblasts.
12. In analyzing most crude tissue and cell extracts, a control gel appears to be superfluous, since the only bands normally detected are those corresponding to µ-and/or m-calpain. A control may be more important when studying tissues such as stomach which are enriched in proteases.
13. The gels can be stored in destain solution indefinitely, or in plastic wrap at 4°C.

References

1. Raser, K. J., Posner, A., Wang, K. K. W. (1995) Casein zymography: A method to study mu-calpain, m-calpain, and their inhibitory agents. *Arch. Biochem. Biophys.* **319,** 211–216.

2. Ma, H., Shih, M., Hata, I., Fukiage, C., Azuma, M., and Shearer, T. R. (1998) Protein for Lp82 calpain is expressed and enzymatically active in young rat lens. *Exp. Eye Res.* **67,** 221–229.
3. Fukiage, C., Azuma, M., Nakamura, Y., Tamada, Y., Nakamura, M., and Shearer, T. R. (1997) SJA6017, a newly synthesised peptide aldehyde inhibitor of calpain: Amelioration of cataract in cultured rat lenses. *Biochim. Biophys. Acta* **1361,** 304–312.
4. Zhao, X., Newcomb, J. K., Posmantur, R. M., Wang, K. K., Pike, B. R., and Hayes, R. L. (1998) pH dependency of mu-calpain and m-calpain activity assayed by casein zymography following traumatic brain injury in the rat. Neurosci. Lett. **247,** 53–57.
5. Fukuda, S., Harada, K., Kunimatsu, M., Sakabe, T., and Yoshida, K. (1998) Postischemic reperfusion induces alpha-fodrin proteolysis by m-calpain in the synaptosome and nucleus in rat brain. *J. Neurochem.* **70,** 2526–2532.
6. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.
7. Twining, S. S. (1984) Fluorescein isothiocyanate-labeled casein assay for proteolytic enzymes. *Anal. Biochem.* **143,** 30–34.
8. Mykles, D. L. and Skinner, D. M. (1986) Four Ca^{2+}-dependent proteinase activities isolated from crustacean muscle differ in size, net charge, and sensitivity to Ca^{2+} and inhibitors. *J. Biol. Chem.* **261,** 9865–9871.

14

Casein Zymogram Assessment of μ-Calpain and m-Calpain Activity After Traumatic Brain Injury in the Rat In Vivo

Xiurong Zhao, Jennifer K. Newcomb, Brian R. Pike, and Ronald L. Hayes

1. Introduction

A number of brain insults including traumatic brain injury (TBI) can result in excitotoxic consequences largely attributable to pathological increases in intracellular calcium *(1,2)*. Loss of calcium homeostasis can result in activation of the calcium-dependent proteases, or calpains, that may be one of the principle causes of pathology after acute central nervous system (CNS) injury *(3)*. A zymographic assay for calpains using nondenaturing casein-containing polyacrylamide gels was originally developed for in vitro studies *(4)*. Recently, we have refined this approach to apply to in vivo studies of acute CNS injury *(5,6)*. This technique allows for differential and concurrent measurement of the two major isoforms of calpain, m-calpain and μ-calpain. This technique also provides the opportunity for analyzing protease activity in cytosolic and total membrane fractions (with appropriate sample preparation), an important consideration because calpain translocation may be a determinant of its attack on membrane-bound cytoskeletal protein targets (*7*). The zymographic assay also assesses calpain activity independently of the effects of the endogenous calpain inhibitor, calpastatin. Acute CNS injury such as TBI can also importantly affect brain pH, and both μ-calpain and m-calpain in brain are sensitive to changes in pH *(8,9)*.

This protocol outlines procedures for casein zymographic assays of calpain activity in vivo. Special emphasis has been placed on tissue preparation and incubation procedures to optimize sensitivity and reliability of assessments of activity of both calpain isoforms.

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2. Materials

1. Homogenization buffer: 20 m*M* Tris-HCl, pH 7.6, 5 m*M* EDTA, 5 m*M* EGTA, 1 m*M* dithiothreitol (DTT), 0.5 m*M* PMSF, 10 µg/mL *p*-aminoethyl benzene sulfonyl fluoride (AEBSF) and 5 µg/mL leupeptin.
2. Sample loading buffer: 150 m*M* Tris-HCl, pH 6.8, 20 % glycerol, 2 m*M* 2-ME, 0.004% (w/v) bromphenol blue.
3. Running buffer: 25 m*M* Tris-base, 192 m*M* glycine, 1 m*M* EDTA, 1 m*M* DTT.
4. Incubation buffer: 20 m*M* Tris-HCl, (pH 7.3 for m-calpain, pH 7.5 for µ-calpain), 10 m*M* DTT, 3 m*M* Ca^{2+}.

3. Methods

3.1. Sample Preparation

1. Anesthetize animals with pentobarbital (100 mg/kg, intraperitoneally) and decapitate at the loss of the toe-pinch reflex.
2. Dissect appropriate brain tissue (10–100 mg) into ice-cold homogenization buffer.
3. Homogenize tissue in 1 mL of ice-cold homogenization buffer per 100 mg tissue with 15 strokes in a glass/Teflon homogenizer and then shear using a 1 mL syringe with a 25 gauge needle by 15–20 passes.
4. Centrifuge homogenates at 100,000*g* for 30 min at 4°C to separate cytosolic from total membrane fractions.
5. Perform protein assay.
6. Samples may be used immediately or stored at –70°C **(Note 1)**.
7. Membrane samples may be solubilized with 1% Triton X-100, but denaturing agents such as SDS may not be used.

3.2. Casein Zymogram Electrophoresis Protocol

1. Separating gel: mix the following components together quickly and pour into the gel sandwich (Hoefer [San Francisco, CA], SE 600; use SE 6102 glass plate, 16 × 18 cm) **(Note 2)**.
   a. 32 mL of 2 % casein (w/v, in distilled water);
   b. 32 mL of 38.5 % A/B (acrylamide/bisacrylamide; 37.5/1.0);
   c. 20 mL of 1.5 *M* Tris-HCl, pH 8.8.
   d. 400 µL of 10 % (w/v) ammonium persulfate in distilled water;
   e. 40 µL of TEMED.
2. Stacking gel: (use SE 511–15-1.5 comb; Hoefer):
   a. 10.2 mL of distilled water;
   b. 2.2 mL of 38.5% A/B;
   c. 4.2 mL of 0.5 m*M* Tris-HCl, pH 6.8;
   d. 83 µL of 10% ammonium persulfate;
   e. 17 µL of TEMED.
3. Prerun the gel by running at 125 V for 1 h at 4°C.
4. Combine 1 vol of 2 × gel sample loading buffer with 1 vol of each sample.
5. Do not heat samples.

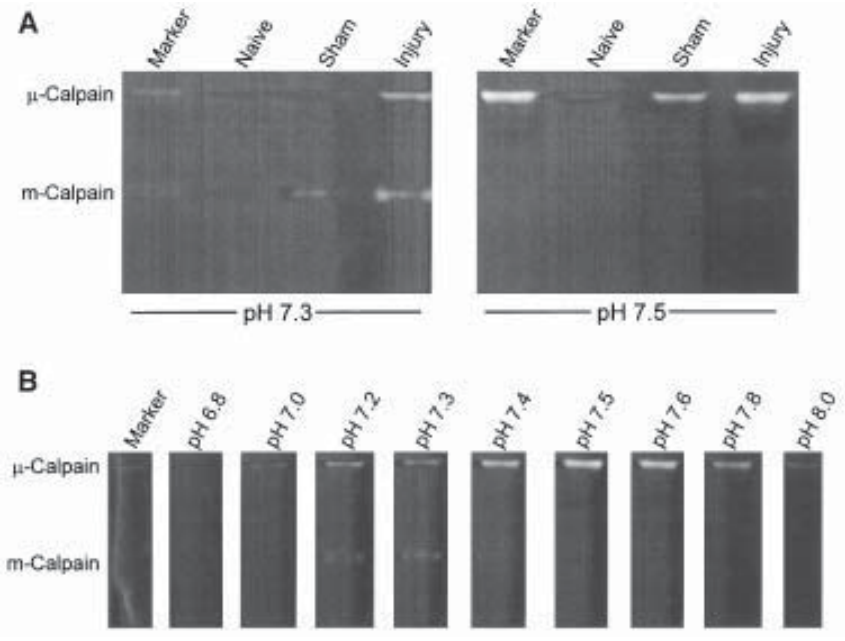

Fig. 1 (**A**). Changes in μ-calpain and m-calpain in cytosolic fractions in cortices following experimental traumatic brain injury. Photograph of representative casein zymograms employing samples from the cortex ipsilateral to the site of injury. Purified μ-calpain and m-calpain were used as markers. Cytosolic fractions were taken from naïve (underwent no surgical procedures), sham-injured and injured cortices 3 h following injury (**B**). Systematic analysis of effects of pH on activation following experimental traumatic brain injury. Photograph of representative casein zymograms employing samples from the cortex ipsilateral to the site of injury. Purified μ-calpain and m-calpain were used as markers. Calpain activity was studied in cytosolic fractions from injured cortices 3 h following traumatic brain injury. Individual casein gels were incubated at pH values ranging from 6.8–8.0. μ-Calpain activity was enhanced between pH 7.2 and 7.8, with 7.5 being optimal. m-Calpain activity was easily detected only between pH 7.2 and 7.4, with 7.3 being optimal.

6. Load approx 100–150 μg of protein into each lane and electrophorese at 125 V for 18–24 h at 4°C (*see* **Note 3**).
7. Rinse the gel in incubation buffer 3 times for 5 min each (*see* **Note 4**). After rinsing, incubate gel in incubation buffer for an additional 24 h at 30 ± 2°C on a shaker (*see* **Note 5**).
8. Stain gel in 0.2% Coomassie blue (w/v, in distilled water) for 2 h and incubate gel in destain solution (5% methanol, 8% acetic acid in distilled water) overnight at room temperature on a shaker.
9. Photograph gel with a Photo-Documentation Camera (Fisher, FB-PDC-34, yellow filter) or any other form of digital imager.

4. Notes

1. If tissue cannot be homogenized immediately, store tissue at –70°C to minimize postmortem calpain activation. Do not freeze sample in liquid nitrogen as this can dry the sample and reduce calpain activity.
2. For in vivo brain samples, large amounts of protein (100–150 µg) may be required in order to observe slight changes in calpain activity. Therefore, use of a large gel electrophoresis apparatus (1.5 mm thickness and 10–15 cm length or longer of separating gel) is recommended for in vivo studies.
3. We have found that electrophoresing the gel at 125 V for 18–24 h results in lower heat accumulation and improved separation of bands.
4. The pH of the incubation buffer should be accurately measured and maintained for each calpain isoform. Calpain isoforms are extremely sensitive to small variations in pH, and failure to control pH could significantly increase variability of measurements. The optimal pH for m-calpain is 7.3 and for µ-calpain is 7.5 ***(5)***.
5. The use of a shaker in **step 4** is necessary as it helps to remove digested casein fragments from the gel.

References

1. Fineman, I., Hovda, D. A., Smith, M., Yoshino, A., and Becker D. P. (1993) Concussive brain injury is associated with a prolonged accumulation of calcium: A ^{45}Ca autoradiographic study. *Brain Res.* **624,** 94–102.
2. Nilsson, P., Hillered, L., Olsson, Y., Sheardown, M. J., and Hansen, A. J. (1993) Regional changes in interstitial K^+ and Ca^{2+} levels following cortical compression contusion trauma in rats. *J. Cereb. Blood Flow Metab.* **13,** 183–192.
3. Kampfl, A., Posmantur, R. M., Zhao, X., Schmutzhard, E., Clifton, G. L., and Hayes, R. L. (1997) Mechanisms of calpain proteolysis following traumatic brain injury: Implications for pathology and therapy: A review and update. *J. Neurotrauma* **14,** 121–134.
4. Raser, K. J., Posner, A., and Wang, K. K. W. (1995) Casein zymography: A method to study µ-calpain, m-calpain, and their inhibitory agents. *Arch. Biochem. Biophys.* **319,** 211–216.
5. Zhao, X., Newcomb, J. K., Posmantur, R. M., Wang, K. K. W., Pike, B. R., and Hayes, R. L. (1998) pH dependence of µ-calpain and m-calpain activity assayed by casein zymography following traumatic brain injury in the rat. *Neurosci. Lett.* **247,** 53–57.
6. Zhao, X., Posmantur, R., Kampfl, A., Liu, S. J., Wang, K. K. W., Newcomb, J. K., Pike, B. R., Clifton, G. L., and Hayes, R. L. (1998) Subcellular localization and duration of µ-calpain and m-calpain activity following traumatic brain injury in the rat: A casein zymography study. *J. Cereb. Blood Flow Metab.* **18,** 161–167.
7. Saido, T. C., Sorimachi, H., and Suzuki, K. (1994) Calpain: new perspectives in molecular diversity and physiological-pathological involvement. *FASEB J.* **8,** 814–822.
8. Chesler, M. and Kaila, K. (1992) Modulation of pH by neuronal activity. *Trends Neurosci.* **15,** 396–402.
9. Nilsson, E., Ostwald, K., and Karlsson, J. O. (1991) Changes in brain calpain activity as a result of *in vitro* ischemia and pH alterations. *Mol. Chem. Neuropathol.* **14,** 99–111.

15

Fluorescence Measurements of Ca^{2+} Binding to Domain VI of Calpain

J. Simon C. Arthur and John S. Elce

1. Introduction

The Ca^{2+} binding properties of calpain are of great interest, both biochemically in the wider context of EF-hand proteins, and physiologically, in the context of calpain regulation. There are two major parameters which one might wish to measure: the actual number of binding sites *(n)* and the binding constants for Ca^{2+}. The latter is normally the macroscopic binding constant for Ca^{2+} of the molecule as a whole (K_d), or the microscopic binding constants for each of the EF-hands, but for cooperative binding these are much more difficult to measure. There is evidence of various kinds to suggest that Ca^{2+} binding causes conformational change in the whole molecule, and this forms the basis for measuring Ca^{2+} binding by means of changes in fluorescence.

The words affinity and sensitivity in the context of Ca^{2+} can be a little confusing, and we use the term K_d to mean the macroscopic dissociation constant for the protein-Ca^{2+} binding equilibrium. This K_d is normally equal to $[Ca^{2+}]_{0.5}$, the Ca^{2+} concentration required for 50 % of the maximum response, measured either by fluorescence, activity, or in some other way. A higher affinity for Ca^{2+} is therefore represented by a lower value of K_d.

The crystal structure of C-terminal domain VI of the small subunit of calpain in the presence and absence of Ca^{2+} has demonstrated that four out of the apparent five EF-hands in this domain are capable of binding Ca^{2+} ***(1,2)***. One of these sites (EF-hand 4) was occupied at >50 m*M* Ca^{2+}, but not occupied at 1 m*M* Ca^{2+} ***(1)***. It seems highly likely that the corresponding four binding sites are available in domain IV of both μ-and m-calpain. If this were correct, it would seem that the maximum value of *n* should be 8, in both μ-and m-calpain,

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but it is quite possible that only some of these 8 sites are occupied under physiological conditions.

It has long been recognized that the apparent binding constants of intact μ- and m-calpain for Ca^{2+}, when measured with purified proteins, are of the order of 10–50 μ*M* and 300 μ*M* Ca^{2+}, respectively, and that these values are much higher than normal intracellular Ca^{2+} concentrations. The possible explanations for this paradox include the effects of phospholipids, calpain activator proteins, or localized areas of high Ca^{2+} concentration within cells, but are not considered further here.

The major difficulty in measurement of Ca^{2+} binding to calpain is that the addition of Ca^{2+} to calpain normally has two undesirable results, namely, aggregation and autolysis, which interfere with most methods of analysis. Ca^{2+} binding to several recombinant fragments of calpain was first measured by means of filter assays with $^{45}[Ca^{2+}]$, yielding values of $n = 2–3$ for various subunits, and $K_d = 80–150$ μ*M* ***(3)***, but this method has not yet been applied to the whole calpain molecule. Equilibrium dialysis was used to measure $^{45}Ca^{2+}$ binding to human μ-calpain, providing values of $n = 8$ and $K_d = 25$ μ*M* for the native enzyme ***(4)***; in the given conditions (1.1 μ*M* carboxymethylated μ-calpain in 50 m*M* sodium borate, pH 7.5, 50 μM Ca^{2+}), the binding sites were independent and of equal affinity, and aggregation apparently did not occur ***(4)***.

Titration of the Ca^{2+} requirement of the enzyme for casein hydrolysis is useful for studying the effects of mutation on calpain activity. Curve fitting of the data to the Hill equation provides a value of K_d, and also gives a value of the Hill constant in the range 3–8, which does not always appear to be related to the assumed or probable number of binding sites ***(5,6)*** *(Dutt et al., unpublished work)*. It is well recognized that the Hill constant does not equal the number of binding sites in an allosteric protein, and caution is required in using the Hill equation ***(7)***.

The major alternative method of studying Ca^{2+} binding that is applicable to calpain, is the observation of changes in fluorescence. These experiments are conducted in relatively dilute protein solution (~ 5–25 μg/mL) where aggregation may be relatively slow. The Ca^{2+}-induced changes in intrinsic tryptophan fluorescence in calpain and its fragments are too small for reliable measurement ***(8)***. It has therefore been found necessary to use the fluorescent dye 2-(*p*-toluidino)naphthalene-6-sulfonate (TNS) ***(9)***. This compound is commonly used as a probe of conformational change, since its fluorescence increases on moving to a more hydrophobic environment, although it should be remembered that the presence of TNS may itself alter K_d values. The binding of TNS may itself help to minimize aggregation of calpain, but it is not possible to use TNS in the presence of other detergents, since this generates a very high background fluorescence. The use of TNS for fluorescence titration for mea-

surement of Ca^{2+} binding to μ-calpain domain IV ***(10)*** and most recently to a related 5-EF hand molecule ***(11)*** has been described.

We describe here the details of a protocol for measuring Ca^{2+} binding to domain VI of the calpain small subunit, by means of fluorescence changes in TNS. Bacterially expressed domain VI exists as a homodimer in solution, but does not aggregate seriously within the time frame of the measurements. This method gave good results for the wild-type protein and for domain VI mutants with a range of EF-hand mutations (**Fig. 1**). We have attempted, by the same means, to measure Ca^{2+} binding to both μ- and m-calpain domain IV fragments, but were not successful, since these domains form a mixture of oligomers in solution and are more prone to aggregation.

2. Materials

All solutions that will be present in the fluorometer cuvette must be filtered through 0.2 μm filters before use (*see* **Note 1**).

1. Buffer A: 100 m*M* Tris-HCl, pH 7.5, 200 m*M* KCl, 10 m*M* 2-ME.
2. Buffer B: 100 m*M* Tris-HCl, pH 7.5, 200 m*M* KCl, 1 m*M* EDTA, 10 m*M* 2-ME (*see* **Note 2**).
3. 1 *M* $CaCl_2$, and a set of appropriate serial dilutions of this solution.
4. 2-(*p*-Toluidino)naphthalene-6-sulfonate (TNS): 5 m*M* in 70% (v/v) ethanol.
5. Bacterially expressed rat calpain small subunit domain VI ***(1)*** (*see* **Note 3**).
6. Fluorometer fitted with a temperature-controlled cuvette holder and a magnetic stirrer under the cuvette.

3. Method

1. Dialyze all protein samples against buffer B overnight at 4°C.
2. Adjust the protein concentration of samples to 1 mg/mL, on the basis of Bradford protein assay, using the same sample of buffer B as used for dialysis (*see* **Note 4**).
3. Centrifuge the protein samples at 12,000*g* for 5 min at 4°C to remove dust or large aggregates. Permit the protein samples to warm to room temperature shortly before use.
4. Set up the fluorometer, stabilized at the desired temperature (normally 20–22°C) with an excitation wavelength of 350 nm and emission wavelength of 444 nm ***(11)***. Slit widths of 5 nm can be used as a starting point; however, they can be increased if the signal is very low.
5. Equilibrate buffer A at the same temperature (20–22°C).
6. Mix in a 1 cm fluorometer cuvette:
   a. 1.95 mL of buffer A;
   b. 20–100 μL of protein solution (final concentration, 5–25 μg/mL, ~ 0.25–1.25 μ*M* of the domain VI monomer) (*see* **Note 5**);
   c. 20 μL of TNS solution (final concentration, 50 μ*M*).
7. Allow 2–5 min for the fluorescence reading to stabilize, then read the fluorescence. Check that the reading is stable and that it is less than one third of the maximum

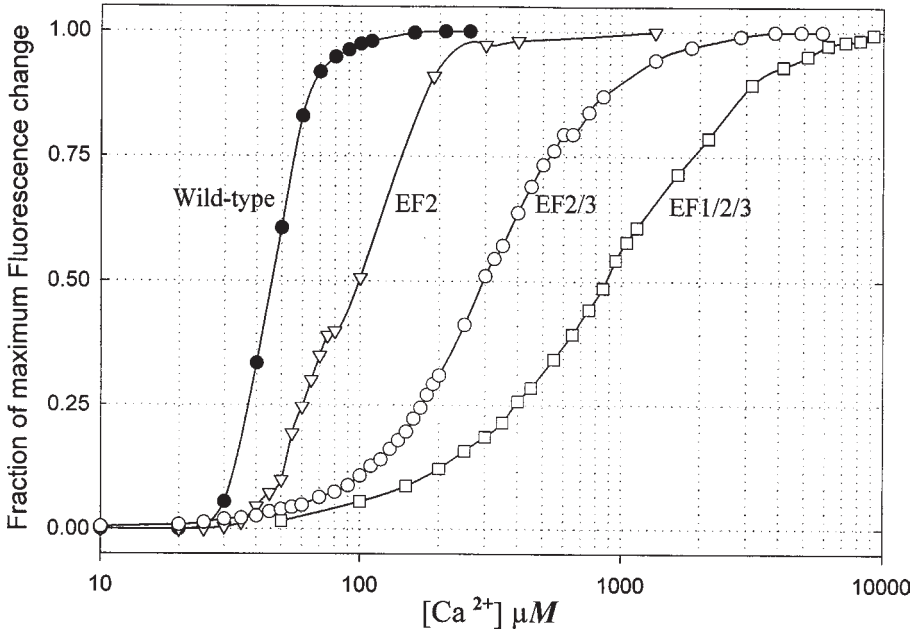

Fig. 1. Change in TNS fluorescence as a function of Ca^{2+} concentration for the domain VI fragment of rat calpain small subunit (●), and for EF-hand mutant proteins which abolish Ca^{2+} binding at EF-hand 2 (▽), EF-hands 2 and 3 (○), and EF-hands 1, 2, and 3 (□).

fluorescence value that can be induced (*see* **Note 6**). Slit widths can be adjusted, or filters introduced to adjust the reading to an acceptable value. On a scale of 1000 units, values of 100–300 are ideal.

8. Set the fluorometer for a time course reading.
9. Add Ca^{2+} stepwise, over the desired concentration range (*see* **Note 7**). After each addition, observe fluorescence for 1 to 2 min, to ensure that the reading is stable. If the reading drifts up or down then it is likely that aggregation of the protein is occurring (*see* **Notes 8** and **9**).
10. Continue addition of Ca^{2+} until the fluorescence ceases to increase.
11. Plot the data as change of fluorescence against the Ca^{2+} concentration (*see* **Note 10**).
12. Add a small excess of EDTA to establish that the fluorescence returns immediately to baseline values.

4. Notes

1. High background noise levels can be due to dust causing light scattering. To minimize this, all solutions should be filtered before use. Small volumes of

protein solution can be either filtered through a spin filter or centrifuged.

2. The value of K_d is highly dependent on pH and ionic strength not only for calpain, but for all EF-hand proteins ***(5)***.
3. Domain VI is very highly expressed in *E. coli*, so that 20–50 mg of pure protein may be readily obtained. Some of the mutant forms are less well expressed ***(1,6)***.
4. For comparison of several proteins, and to avoid errors due to different EDTA concentrations, all protein samples should be dialyzed simultaneously, at very similar protein concentration, and in the same vessel, against a large excess of buffer of accurately known EDTA concentration. The K_d of domain VI in the given buffer conditions is ~48 μ*M* Ca^{2+}, and it is fairly easy to work in this range. For K_d values from 1–10 μ*M*, control and accuracy of the free Ca^{2+} concentration is more difficult, and is normally achieved with Ca^{2+}-EGTA buffers.
5. The protein concentration can be adjusted to allow for differences in the hydrophobicity and tendency to aggregate of different mutants, and for the amount of protein available.
6. The fluorescence of TNS in the absence of protein is negligible, but increases markedly on addition of protein, even in the presence of EDTA. The maximum fluorescence value in calpain measurements will normally be that observed at 0.5–5 m*M* Ca^{2+}, and should be established by preliminary experiment.
7. We add 1 μL of $CaCl_2$ solution at a time, attempting to minimize the change in total volume. Addition of 1 μL of 1 *M* $CaCl_2$ increases the Ca^{2+} concentration in the 2 mL mixture by 0.5 m*M*, and smaller increments are achieved with diluted stock $CaCl_2$ solutions. The aim is to obtain data in the range between 20% and 80% saturation of the protein with Ca^{2+}, as well as at zero and 100% saturation.
8. It is crucial to avoid aggregation, which causes false values. The fluorescence readings should remain constant over time, and should be checked both with and without Ca^{2+}. The most likely cause of a drift up or down in the reading is a slow aggregation of the calpain sample, but change in temperature must be checked and excluded. Aggregation can be confirmed in several ways. A laser light scattering machine can be used to determine the average molecular weight of the protein sample in the presence of Ca^{2+}. Alternatively, the right-angled light scattering of the protein solution can be measured using the fluorometer. To do this, excitation and emission wavelengths are both set to 340 nm and the light scattering measured over time. Aggregation of the sample causes a gradual increase in light scattering.
9. Various means may be adopted to prevent aggregation, including changes in temperature, buffer system (possibly borate ***[4]***), pH, and ionic strength, but all of these parameters also affect the Ca^{2+} affinities of EF-hand proteins **(Note 2)**. Detergents cannot be used with TNS; however, if intrinsic fluorescence is to be used then detergents may prove useful in preventing aggregation. It should be possible to label cysteine residues with fluorescent tags rather than using TNS and this may allow the use of detergents; it has not so far been attempted with calpain. Various calpains have 12–14 cysteine residues, but it is not yet known whether they can be labeled, or whether, once labeled, they undergo changes in environment.

10. The data can be analyzed by non-linear regression (we use Sigma Plot) to the equation:

$$y = M.C^n / (K_d^n + C^n)$$

where $K_d = [Ca^{2+}]_{0.5}$ at which the half-maximal change in fluorescence is observed; n is the Hill constant; $C = [Ca^{2+}]$ concentration; M = (maximum value of fluorescence - baseline value of fluorescence); y = (measured fluorescence value - baseline value of fluorescence). The value of M (as well as those of K_d and n) can be entered as constants that will be evaluated by the regression program while fitting the data.

This equation is based on the Hill equation and the constants do not have any strict kinetic meaning *(7)*. They are, however, useful for comparative purposes. Our titration data fit to the equation extremely well, but it must be pointed out that this does not prove that the Ca^{2+} binding is cooperative. More complex analysis using the Adair equations is theoretically possible, but these equations include microscopic binding constants for each of eight possible Ca^{2+} binding sites in a 21k homodimer. In our hands these binding constants do not all appear to be equal.

References

1. Blanchard, H., Grochulski, P., Li, Y., Arthur, J. S. C., Davies, P. L., Elce, J. S., and Cygler, M. (1997) Structure of a calpain Ca^{2+}-binding domain reveals a novel EF-hand and Ca^{2+}-induced conformational changes. *Nat. Struct. Biol*. **4,** 532–538.
2. Lin, G. D., Chattopadhyay, D., Maki, M., Wang, K. K., Carson, M., Jin, L., Yuen, P. W., Takano, E., Hatanaka, M., DeLucas, L. J., and Narayana, S. V. (1997) Crystal structure of calcium bound domain VI of calpain at 1.90 Å resolution and its role in enzyme assembly, regulation, and inhibitor binding. *Nat. Struct. Biol*. **4,** 539–547.
3. Minami, Y., Emori, Y., Imajoh-Ohmi, S., Kawasaki, H., and Suzuki, K. (1998) Carboxyl-terminal truncation and site-directed mutagenesis of the EF hand structure-domain of the small subunit of rabbit calcium-dependent protease. *J. Biochem*. **104,** 927–933.
4. Michetti, M., Salamino, F., Minafra, R., Melloni, E., and Pontremoli, S. (1997) Calcium-binding properties of human erythrocyte calpain. *Biochem. J*. **325,** 721–726.
5. Elce, J. S., Hegadorn, C., and Arthur, J. S. C. (1997) Autolysis, Ca^{2+} requirement, and heterodimer stability in m-calpain. *J. Biol. Chem*. **272,** 11,268–11,275.
6. Dutt, P., Arthur, J. S. C., Croall, D. E., and Elce, J. S. (1998) m-Calpain subunits remain associated in the presence of calcium. *FEBS Lett*. **436,** 367–371.
7. Weiss, J. (1997) The Hill equation revisited, uses and misuses. *FASEB J*. **11,** 835–841.
8. Hong, H., Johnson, P., and El-Saleh, S. C. (1990) Effects of calcium and calmodulin antagonists on calpain II subunit conformations. *Int. J. Biol. Macromol.* **12,** 269–272.
9. McClure, W. O. and Edelman, G. M. (1966) Fluorescent probes for conformational states of proteins. I. Mechanism of fluorescence of 2-*p*-toluidinyl naphthalene-6-sulfonate. *Biochemistry* **5,** 1908–1918.

10. Yang, H. Q., Ma, H., Takana, E., Hatanaka, M., and Maki, M. (1994) Analysis of calcium-dependent interaction between amino-terminal conserved region of calpastatin functional domain and calmodulin-like domain of μ-calpain large subunit. *J. Biol. Chem*. **269,** 18,977–18,984.
11. Maki, M., Yamaguchi, K., Kitaura, Y., Satoh, H., and Hitomi, K. (1998) Calcium-induced exposure of a hydrophobic surface of mouse LAG-2, which is a member of the penta-EF-hand protein family. *J. Biochem*. **124,** 1170–1177.

16

Kinetic Analysis of Human μ-Calpain Autolysis

Peter Tompa and Peter Friedrich

1. Introduction

The relation of autolysis to calpain activation has a long and controversial history. Early on, it was realized that calcium-induced calpain activation is accompanied by autolytic truncation of the enzyme in vitro and that sensitization to calcium occurs at the same time ***(1)***. The issue of the mechanistic role of autolysis in calpain activation, however, has still not been settled. Several aspects of autolysis have been addressed, such as the temporal order of autolysis and activation ***(2,3)***, the relation of autolysis to sensitization to calcium ***(4,5)***, and possible differences in the mode of autolysis between μ-and m-calpain ***(5,6)***. Much work has also been devoted to the question of the physiological relevance of calpain autolysis. Calpain action was found to be accompanied by autolysis in vivo ***(7)***, but opposing examples are also known ***(8)***. Pathological involvement of autolysis has also been suggested ***(9)***. The prevailing view is that autolysis is possibly a major mechanism of calpain activation, but that other mechanisms of calpain activation may also act under various conditions ***(10)***.

A great deal of the controversy stems from incorrect kinetic treatments in the analysis of autolysis and activation. Percent conversions measured over lengthy incubation times have sometimes been determined and compared in a way that makes the conclusions invalid. One example of this faulty practice is that calcium sensitivity of calpain activation has been recorded as the calcium concentration needed to convert 50% of a substrate under the given conditions. Naturally, a change in the calpain concentration or incubation time would make the same conversion occur at a different calcium concentration, that is, such a value of half-saturation varies with the experimental conditions. To overcome this problem, it is important that rate constants of autolysis and activation be

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determined under assay conditions of true initial velocity. We give a brief description of how these processes should be followed in vitro so that they can be compared in a kinetically correct manner ***(3,11)***.

2. Materials

1. Human erythrocyte μ-calpain is prepared as described ***(3)***. The typical yield is about 5 mg from 400 mL of blood concentrate (corresponding to 800 mL of blood). Pure calpain, at a concentration of about 1 mg/mL, can be stored in calpain buffer at 0°C.
2. Calpain buffer: 10 m*M* HEPES, pH 7.5, 1 m*M* EGTA, 0.5 m*M* DTE, 1 m*M* benzamidine, 0.1 m*M* PMSF.
3. Fluorescent calpain substrate 5-([4,6-dichlorotriazin-2-yl]amino)-fluorescein-labeled-microtubule-associated protein 2 (MAP2-DTAF) ***(11)***.
4. SDS gel sample buffer containing EGTA: 0.03 *M* Tris-HCl, pH 6.8, 4% (w/v) SDS, 30 m*M* EGTA, 10% (v/v) glycerol, 0.2 *M* DTE, 0.006% (w/v) bromphenol blue.
5. 200 m*M* DTE.

3. Method

3.1. Determination of the Rate Constant of the Autolysis of μ-Calpain Large Subunit

1. In order to preactivate the enzyme, add DTE to a final concentration of 2 m*M* to a 1 mg/mL calpain solution (*see* **Note 1**).
2. Incubate the calpain with the DTE for 20 min on ice, and then equilibrate the calpain solution and all other reagents to the desired temperature before making measurements.
3. Prepare tubes containing approximately 0.1 mg/mL μ-calpain in 100 μL of calpain buffer.
4. Take a 20-μL sample at time zero with no calcium.
5. Add calcium to yield the desired free calcium concentration (*see* **Note 2**). To saturate μ-calpain, use calcium at 1 m*M* or higher (cf. ***3***) (*see* also **Fig. 4**).
6. For conditions involving very high initial rates of autolysis, prepare separate mixtures for each data point (*see* **Note 3**).
7. Withdraw 20-μL aliquots from the reaction mixture at various times starting at about 15 s; stop autolysis by addition of SDS gel sample buffer, and boil the samples immediately (*see* **Note 4**).
8. Run samples on an SDS-PAGE of about 6–7% (*see* **Note 5**).
9. Stain the gel with Coomassie brilliant blue **(Fig. 1)** (*see* **Note 6**).
10. Determine the intensity of the 80 kDa band by densitometry. Make sure that intensities are in the range where response of the densitometer is linear. If autolysis is too fast (i.e., a detectable 80-kDa band is not seen even at the shortest time), repeat from **step 5** with reduced incubation times.

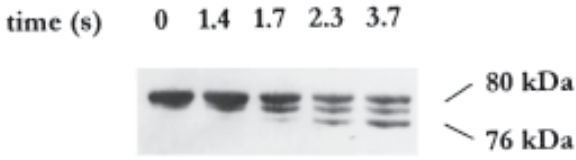

Fig. 1. Autolysis of μ-calpain large subunit. 0.6 μ*M* μ-calpain at 10 μ*M* free Ca^{2+} was incubated at 25°C, and aliquots were withdrawn at 0 s (control), 1.4, 1.7, 2.3, and 3.7 s. The samples were run on 6% SDS-PAGE and stained with Coomassie brilliant blue.

11. Plot logarithm of 80-kDa band intensities against incubation time **(Fig. 2)**, and fit linear functions to the points. Autolysis follows apparent first-order kinetics so that the slope of these straight lines yields the apparent first-order rate constants.

3.2. Determination of the Rate Constant of μ-Calpain Activation

This assay is performed using MAP2-DTAF as a fluorescent substrate of calpain, as described in Chapter 17.

1. Preactivate calpain as above.
2. Set the excitation and emission wavelengths of the fluorimeter at 490 and 520 nm, respectively, and the response time at the minimum of the instrument so that fast changes can be followed.
3. Place 0.02 μ*M* μ-calpain and 0.2 μ*M* MAP2-DTAF in calpain buffer into the cuvette of the instrument.
4. Add calcium to the desired concentration and mix rapidly to start the reaction. Record the progress curve of substrate consumption until a constant rate is achieved **(Fig. 3)** (*see* **Note 7**).
5. Fit a straight line to the linear steady-state part of the progress curves, and extrapolate the line to the *x*-axis (time) to determine the length of the lag-phase. This time ("transit time") is the reciprocal of the first-order rate constant of activation ***(12)***.
6. Comparison of the rate constants for autolysis of the calpain large subunit **(Subheading 3.1.)** and for activation of the enzyme for MAP2-DTAF hydrolysis **(Subheading 3.2.)** shows that these values are essentially equal over a wide range of calcium concentration **(Fig. 4)** ***(3)***.

4. Notes

1. It is essential to use the freshest possible calpain. Purified calpain, although retaining activity, slowly changes in some uncontrollable manner and various kinetic parameters drift over time. The reasons for this deterioration are not all clear, but oxidation is one important factor. Storage of the enzyme in the presence of reducing agent, and preincubation of calpain with DTE before the experiment is essential. Storage in the presence of sodium azide (0.001%) is also recommended.

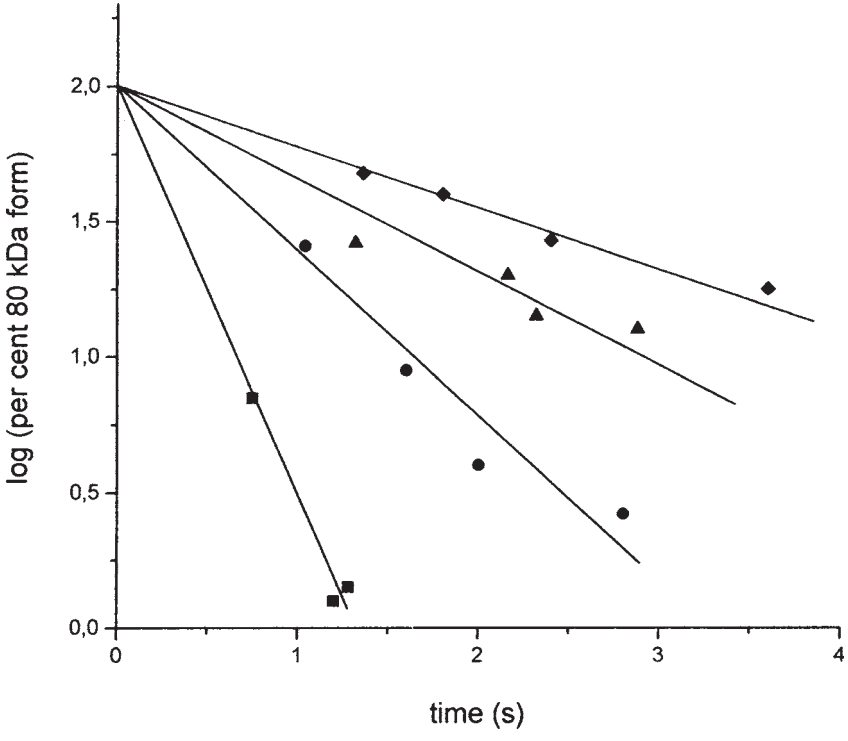

Fig. 2. Time course of μ-calpain autolysis. The disappearance of the 80 kDa form (expressed as a percentage of the sum of the 76, 78, and 80 kDa forms) was observed at 25°C at 10 (◆), 100 (▲), 250 (●), and 1000 (■) μ*M* Ca^{2+}. The slopes of the straight lines yield the apparent first-order rate constants of autolysis. (From **ref. *3*** with permission.)

2. Precision and control of the concentration of free calcium is not trivial in the range where EGTA and calcium concentrations are commensurable, since a 10% deviation in either concentration may cause unacceptably large errors. For example, at 1 m*M* EGTA, pH 7.5 (log K_{app} = 7.415) ***(13)***, addition of 0.95 m*M* total Ca^{2+} results in 0.72 μ*M* free Ca*2+* concentration. However, if total Ca^{2+} is 1.05 m*M*, the free Ca^{2+} concentration is 50.7 μ*M*, almost two orders of magnitude higher. Thus, a seemingly small uncertainty in the concentration of stock solutions may cause enormous systematic errors in the experiments. This may have contributed to some of the inconsistent results in the literature ***(3)***. Inadequate control of pH and neglect of the large pH-effect on EGTA-Ca^{2+} dissociation will also cause significant errors. For example, with 1 m*M* EGTA and 0.95 m*M* Ca^{2+}, the free Ca^{2+} concentration is 0.72 μ*M* at pH 7.5 (log K_{app} = 7.415), but rises to 9.4 μ*M*, more than 10 times higher, at pH 6.9 (log K_{app} = 6.227) ***(13)***. New stocks of calcium and EGTA solutions should be titrated against each other with a calcium-selective electrode.

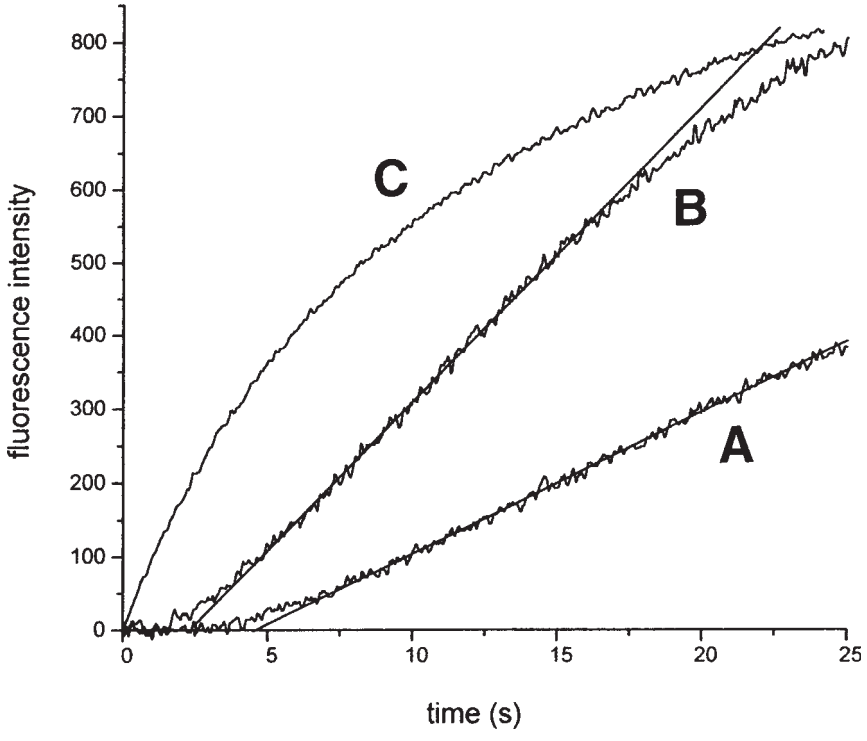

Fig. 3. Progress curve of substrate degradation by μ-calpain measured with MAP2-DTAF. Degradation of 0.2 μ*M* DTAF-MAP2 was initiated by the addition of 0.02 μ*M* μ-calpain at 8 μ*M* **(A)** and 28 μ*M* **(B)** Ca^{2+}, and by addition of preautolyzed μ-calpain at 28 μ*M* Ca^{2+} **(C)**. Transit times of activation were determined by fitting straight lines to the linear phase of progress curves and extrapolating them to zero substrate conversion. (From **ref. *3*** with permission.)

3. For high calcium concentrations, very fast sampling may be needed that cannot be achieved by taking successive aliquots from one reaction mixture, (and ideally requires automated rapid reaction apparatus). In this case separate samples should be prepared for each time point, and each sample should be started and stopped individually. For very short incubation times, fill one pipette with calcium and the other with SDS gel-sample buffer and place them close at hand. Holding the tube with calpain at a vortex, add calcium to start the reaction, take the other pipette and stop the reaction immediately. Call out both moments so that another person with a stop-watch can measure the time elapsed. Do not aim for round numbers (e.g., 2.0, 3.0 s), but record the actual time of incubation. Incubation times in the order of 2–3 s can be reliably achieved.
4. Determination of high autolysis rates requires fast sampling and rapid and complete termination of the reaction. If the SDS gel sample buffer does not

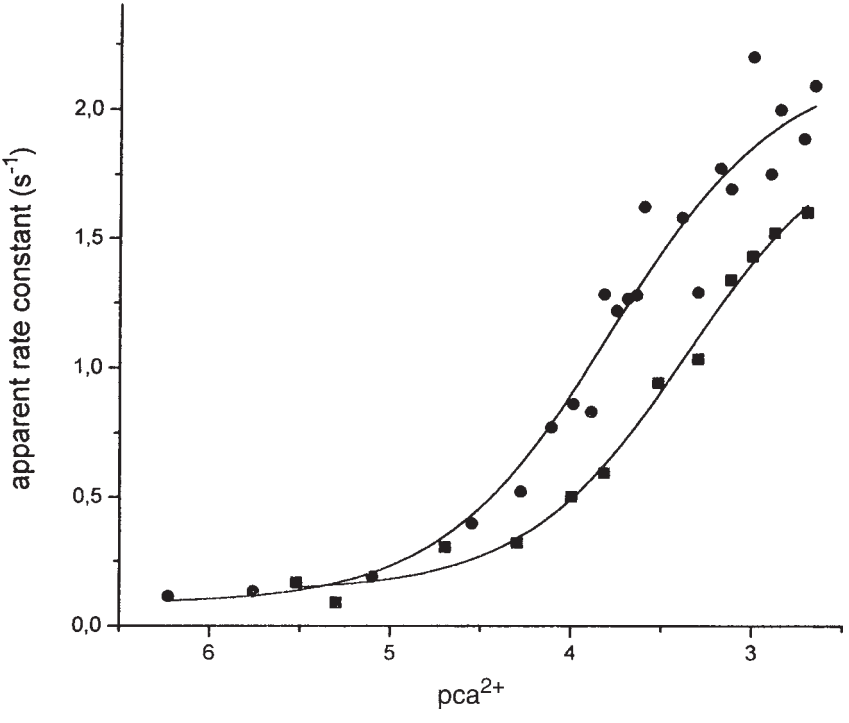

Fig. 4. Autolysis and activation of μ-calpain run in close parallel. The apparent rate constants of μ-calpain autolysis (■, cf. **Fig. 2**) and activation (●, cf. **Fig. 3**) were determined at various calcium concentrations.

contain EGTA, significant further autolysis of calpain can occur during addition of this buffer and while warming to 100°C.

5. If the 80, 78, and 76 kDa bands are not well-resolved on the gels, it is probably necessary to improve the gel running conditions until the bands are more fully resolved. However, it is possible to fit the resulting densitometry pattern to three peaks in order to quantify the 80 kDa peak. It may be noted that, whereas human μ-calpain shows these autolytic changes, rat μ-calpain autolysis is not detectable by gel electrophoresis.
6. Calpain autolysis is mostly intramolecular ***(14)*** and follows first-order kinetics. The rate constant, therefore, does not vary with enzyme concentration, but artifacts may arise at very low enzyme concentrations. Calpain autolysis products appear to be sticky, unstable, and poorly recovered, so that their relative amounts may change before detection. A certain form may completely escape detection if its concentration is much lower than that of the others. This point may be especially critical when a decision on the activation mechanism hinges on the presence or absence of a certain form in physiological samples.These problems

may be minimized either by raising enzyme concentration, or by adding a protective protein (BSA) to the samples and avoiding excessive mixing and exposure of the reaction mixture to new surfaces. At very low enzyme concentrations it may be necesssary to use Western blotting to detect and quantify the autolysis products, but artifacts may occur here also, if the individual autolysis forms differ in their efficiency of transfer, binding to membranes, and immunodetection. Simple Coomassie staining is preferable to blotting for densitometry quantification.

7. To achieve rapid mixing without removing the cuvette from the fluorometer, prepare a plastic mixing rod with a small cavity for an aliquot of calcium solution at the tip and add this to the cuvette by means of two gentle strokes. It also increases temporal resolution if the fluorometer is fixed to record with its lid open. With a little practice, the change in fluorescence intensity can be reliably recorded within a couple of seconds. This is especially critical at high calcium concentrations where the initial lag-phase of the reaction is short.

Acknowledgments

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References

1. Suzuki, K., Tsuji, S., Kubota, S., Kimura, Y., and Imahori, K. (1981) Limited autolysis of Ca^{2+}-activated neutral protease (CANP) changes its sensitivity to Ca^{2+} ions. *J. Biochem. (Tokyo)* **90,** 275–278.
2. Saido, T. C., Nagao, S., Shiramine, M., Tsukaguchi, M., Sorimachi, H., Murofushi, H., Tsuchiya, T., Ito, H., and Suzuki, K. (1992) Autolytic transition of μ-calpain upon activation as resolved by antibodies distinguishing between the pre-and post-autolysis forms. *J. Biochem.* **111,** 81–86.
3. Baki, A., Tompa, P., Alexa, A., Molnár, O., and Friedrich, P. (1996) Autolysis parallels activation of μ-calpain. *Biochem. J.* **318,** 897–901.
4. Cong, J., Goll, D. E., Peterson, A. M., and Kapprell, H.-P. (1989) The role of autolysis in activity of the Ca^{2+}-dependent proteinases (μ-calpain and m-calpain). *J. Biol. Chem.* **264,** 10,096–10,103.
5. Elce, J. S., Hegadorn, C., and Arthur, J. S. C. (1997) Autolysis, Ca^{2+} requirement, and heterodimer stability in m-calpain. *J. Biol. Chem.* **272,** 11268–11275.
6. Tompa, P., Baki, A., Schád, É., and Friedrich, P. (1996) The calpain cascade: μ-calpain activates m-calpain. *J. Biol. Chem.* **271,** 33,161–33,164.
7. Saido, T. C., Suzuki, H., Yamazaki, H., Tanoue, K., and Suzuki, K. (1993) In situ capture of μ-calpain activation in platelets. *J. Biol. Chem.* **268**, 7422–7426.
8. Molinari, M., Anagli, J., and Carafoli, E. (1994) Ca^{2+}-activated neutral protease is active in the erythrocyte membrane in its nonautolyzed 80-kDa form. *J. Biol. Chem.* **269,** 27,992–27,995.
9. Saito, K., Elce, J. S., Hamos, J. E., and Nixon, R. A. (1993) Widespread activation of calcium-activated neutral proteinase (μ-calpain) in the brain in Alzheimer

disease: A potential molecular basis for neuronal degeneration. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 2628–2632.
10. Johnson, G. V. and Guttmann, R. P. (1997) Calpains: Intact and active? *BioEssays* **19,** 1011–1018.
11. Tompa, P., Schád, É., Baki, A., Alexa, A., Batke, J., and Friedrich, P. (1995) An ultrasensitive, continuous fluorometric assay for calpain activity. *Anal. Biochem.* **228,** 287–293.
12. Frieden, C. (1979) Slow transitions and hysteretic behavior in enzymes. *Annu. Rev. Biochem.* **48,** 471–489.
13. Tsien, R. and Pozzan, T. (1989) Measurement of cytosolic free Ca^{2+} with quin2. *Methods Enzymol.* **172,** 230–262.
14. Inomata, M., Kasai, Y., Nakamura, M., and Kawashima, S. (1988) Activation mechanism of calcium-activated neutral protease. *J. Biol. Chem.* **263,** 19,783–19,787.

17

A Sensitive and Continuous Fluorometric Activity Assay Using a Natural Substrate

Microtubule-Associated Protein 2

Peter Tompa, Éva Schád, and Peter Friedrich

1. Introduction

Calpain activity can be assayed by a variety of methods which almost invariably rely on measuring the liberation of trichloroacetic acid (TCA)-soluble peptides from a protein substrate, usually casein. The soluble peptides are then measured either by their absorption at 280 nm, or by their radioactivity if the casein was radioactively labeled. The major difficulty in this type of assay is that the small peptides need to be separated from partially digested or undigested casein, which makes the assays tedious, and precludes continuous monitoring of activity. A continuous assay is possible with fluorescent peptide substrates ***(1)***, but these are relatively poor substrates for calpain so that such an assay is insensitive. A continuous and sensitive assay, suitable therefore also for kinetic studies, has been developed, using MAP2-DTAF as substrate (5-[{4,6-dichlorotriazin-2-yl}amino])-fluorescein[DTAF]-labeled microtubule-associated protein 2 [MAP2] ***(2)***. The assay depends upon the observation that release of small peptides carrying only one fluorescent label, by digestion of a protein labeled with many fluorescent groups, is accompanied by a significant increase in fluorescence of the solution, without any need for separation. Detailed consideration of the conditions and kinetics of the assay is given in ***(3)***, but the assay is most useful in the range of 10–180 ng of calpain, and is therefore 10^2–10^3-fold more sensitive than the commonly used calpain assays.

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2. Materials

1. Calpain buffer: 10 m*M* HEPES, pH 7.5, 1 m*M* EGTA, 0.5 m*M* DTE, 1 m*M* benzamidine, 0.1 m*M* PMSF.
2. Labeling solution: prepare a stock of 1 *M* Na_2CO_3, 30 m*M* benzamidine. Store in aliquots at –20°C.
3. MAP2: approximately 1 mg of MAP2 is prepared from 25 rat brains as described ***(4)***. The purified protein is stored in small aliquots at –80°C at a concentration of 1 mg/mL in 0.1*M* PIPES, pH 6.6, 1 m*M* $MgSO_4$, 1 m*M* EDTA. This is sufficient substrate for approximately 1000 assays. Commercially available MAP2 may also work but should be checked for impurities as their presence may compromise the assay.

3. Method

3.1. Labeling of MAP2 With DTAF

1. Add 3.8 µL of labeling solution to 50 µL of 0.5 mg/mL MAP2 at 4°C. This will bring the pH to about 8.5.
2. Add 0.5 mg DTAF to this MAP2 solution, and mix by vortexing (*see* **Note 1**). Incubate the dye with MAP2 for about 5 min at 4°C in the dark.
3. Prepare a small column (about 1 × 0.5 cm) of Sephadex G25 in a Pasteur pipette or a pipette-tip, equilibrated in calpain buffer and 2 m*M* benzamidine (*see* **Note 2**). The column is too small to run dry under gravity.
4. Layer the labeled MAP2 solution and successive 50 µL portions of calpain buffer on top of the column, collecting the eluate in 50 µL fractions and waiting about 10 s every time for flow to stop.
5. Monitor the elution by measuring the fluorescence intensity of the fractions at excitation and emission wavelengths of 470 and 520 nm, respectively. The labeled MAP2 is recovered around fraction 4. Pool peak fractions and use immediately, or store at –20°C (*see* **Note 3**).
6. If required, the extent of labeling can be calculated from the absorbance of labeled MAP2 at 495 nm, using the absorption coefficient of DTAF, $A_{0.1\%} = 127.5$ ***(2)***.

3.2. Measuring Calpain Activity With MAP2-DTAF

1. Set the excitation and emission wavelengths of the fluorometer at 470 and 520 nm, respectively. For instrumental setup, refer to **Note 4**. For small assay volumes, a 3 × 3 mm quartz fluorescence cuvette allows measurements to be made in 50-µL sample volumes.
2. Place approximately 20 µg/mL MAP2-DTAF and 1 m*M* Ca^{2+} (final concentrations) in calpain buffer in the cuvette.
3. Start the reaction by addition of 2–5 µL of an enzyme sample containing 0.5–5 µg/mL calpain (*see* **Note 5**) and record the change in fluorescence intensity.
4. The initial rate of change of fluorescence intensity **(Fig. 1)** is proportional to the concentration of calpain **(Fig. 2)** (cf. **Note 6**).

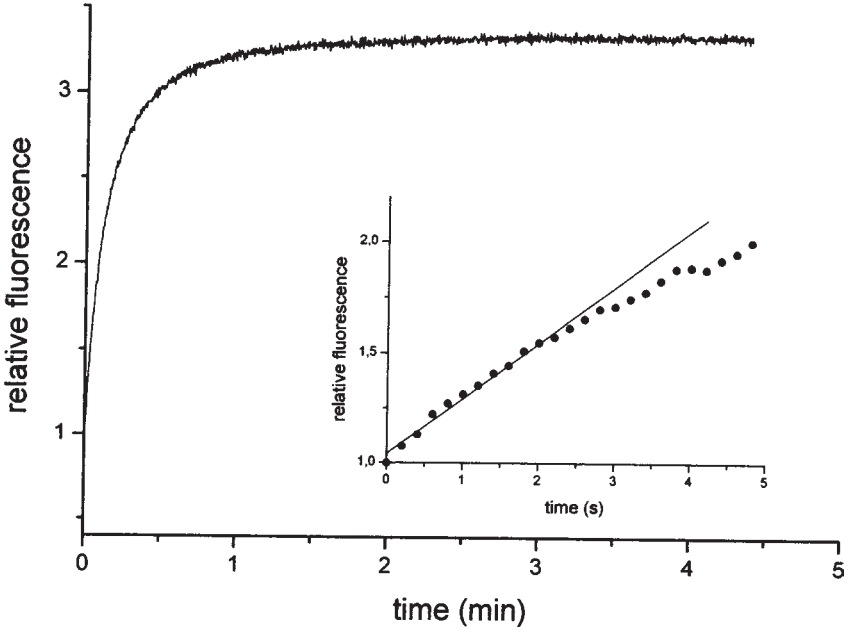

Fig. 1. Determination of the initial rate of substrate consumption by calpain. 18 µg/mL MAP2-DTAF was mixed with 1.8 µg/mL calpain (final concentrations) in 50 µL calpain buffer at 1 m*M* Ca^{2+} and the progress curve of fluorescence intensity was recorded. The straight line fitted to the initial phase of the curve (insert) demonstrates the determination of the slope.

5. Repeat the assay, varying factors such as the nature and pH of the buffer, ionic strength, and concentrations of calpain, Ca^{2+}, and labeled substrate, as required.

4. Notes

1. We have found that the labeling is normally not affected by the quality of MAP2 and DTAF used. However, the assay seems to fail at times in that calpain addition causes only small or negligible fluorescence change. The reason is not always apparent, but quality and freshness of the ingredients may be critical. MAP2 should not be contaminated with other proteins because these also become labeled and add to the background but fail to contribute to the signal that develops upon proteolysis. Fresh DTAF should be used.
2. The simplest way to separate MAP2-DTAF from the unreacted dye might seem to be a gel filtration spin-column. With this method, however, recovery of the protein is incomplete and the resulting dilution compromises the signal-to-noise ratio in the assay. We prefer conventional gel filtration by gravity in a small column, a method that is still sufficiently fast to allow labeling to be carried out just before the assay.

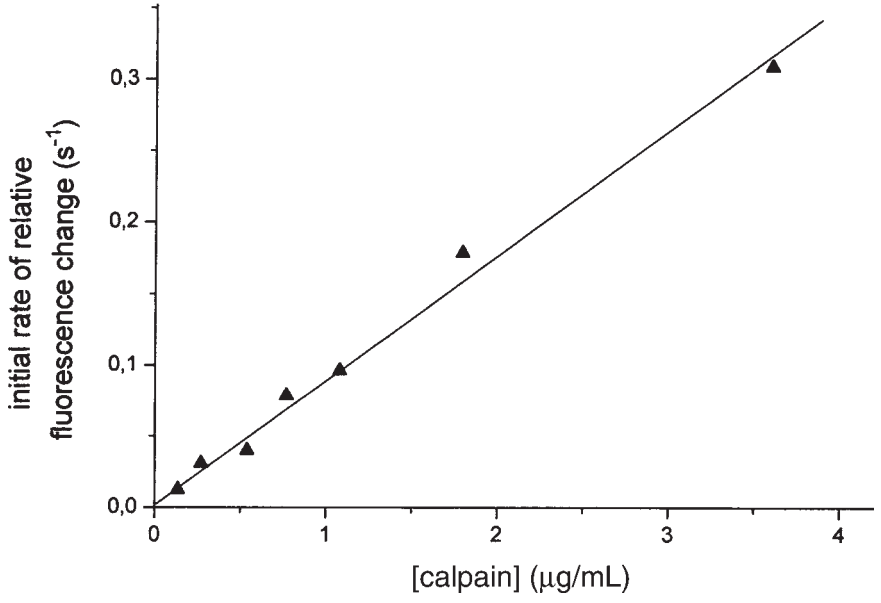

Fig. 2. Initial rate of relative fluorescence intensity change as a function of calpain concentration. 18 µg/mL MAP2-DTAF was mixed with various concentrations (0.136–3.6 µg/mL) of calpain in 50 µL calpain buffer at 1 m*M* Ca^{2+} and the initial rate of fluorescence intensity change was determined as in **Fig. 1.** The initial rate was proportional to calpain concentration in the given range.

3. Although labeled MAP2 can be stored frozen without much diminution in the fluorescence intensity, the assay seems to perform better if the substrate is prepared fresh and kept on ice on the day of its use. The labeling and gel filtration can be carried out within about 1 h.
4. To reduce the noise it is advisable to open the slits of the fluorometer as much as possible to reduce the gain on the photomultiplier. This may be particularly important with fast kinetic measurements where the response time of the instrument is best set to the minimum so that it does not interfere with the reaction itself.
5. Calpain in a small volume is preferably mixed into the reaction mixture with a small mixing rod that has a small cavity at the end for the enzyme. For fast kinetic measurements, rapid mixing is essential, and can be achieved within a couple of seconds with sufficient practice. It is even possible to set the instrument to record with its lid open, which reduces the dead-time of the measurement significantly. In this case the ambient light should be dimmed and the experimenter should not move about while the initial phase of the reaction is in progress. The reaction can also be started with the addition of Ca^{2+} which, however, decreases MAP2-DTAF fluorescence and therefore causes an initial drop in fluorescence intensity.

6. MAP2-DTAF is an unconventional and nonhomogeneous substrate in that it presents multiple cleavage sites to the enzyme, presumably all different with regards to their calpain sensitivity and contribution to the developing fluorescent signal. As a result, the progress curve recorded with this substrate does not have a substantial initial linear section (~2–5 s) that could be used as a valid estimate of initial velocity. Therefore, it is of the utmost importance to record the change in fluorescence intensity as early as possible and to calculate the initial slope of the curve by numerical curve-fitting. In every other aspect, MAP2-DTAF behaves as a regular substrate, that is, its initial consumption rate is linear with enzyme concentration, and it shows saturation kinetics that yield a linear function on a double reciprocal plot.

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References

1. Sasaki, T., Kikuchi, T., Yumoto, N., Yoshimura, N., and Murachi, T. (1984) Comparative specificity and kinetic studies on porcine calpain I and calpain II with naturally occurring peptides and synthetic fluorogenic substrates. *J. Biol. Chem.* **259,** 12,489–12,494.
2. Blakeslee, D. and Baines, M. G. (1976) Immunofluorescence using dichlorotriazinyl-amino-fluorescein (DTAF). I. Preparation and fractionation of labelled IgG. *J. Immunol. Methods* **13,** 305–320.
3. Tompa, P., Schád, É., Baki, A., Alexa, A., Batke, J., and Friedrich, P. (1995) An ultrasensitive, continuous fluorometric assay for calpain activity. *Anal. Biochem.* **228,** 287–293.
4. Alexa, A., Tompa, P., Baki, A., Vereb, G., and Friedrich, P. (1996) Mutual protection of microtubule-associated protein 2 (MAP2) and cyclic AMP-dependent protein kinase II against μ-calpain. *J. Neurosci. Res.* **44,** 438–445.

18

Measurement of Calpain Activity In Vitro and *In Situ* Using a Fluorescent Compound and Tau as Substrates

Rodney P. Guttmann and Gail V. W. Johnson

1. Introduction

Calpains play important roles in numerous physiological and pathological processes ***(1,2)*** by catalyzing the limited proteolysis of a wide variety of protein substrates. The activity of calpain toward a specific substrate is regulated not only by calcium, but also by numerous other factors including calpain activator proteins ***(3)***, redox state ***(4,5)***, and the phosphorylation state of the substrate ***(6–8)***. In order to determine the relative contributions of these and other factors to the activity of calpain, it is necessary to be able to measure calpain activity both in vitro and *in situ*, and these assays are the subject of this chapter.

In many cases, the classical type of calpain assay using casein hydrolysis and trichloroacetic acid (TCA) precipitation may be sufficient, but in **Subheading 3.1.** of this chapter, two other protocols for measuring calpain activity in vitro are described. Calpain activity can be determined either by using a "standard" calpain substrate (such as the fluorescent peptide described here), or by using a defined protein (in this case the tau protein). The reader should be aware that a variety of fluorescent substrates ***(9)*** and well-characterized protein substrates ***(5,6,10–12)*** have been described and can be easily introduced into the protocol described below.

In **Subheading 3.2.** of this chapter methods are described to measure calpain activity in cultured cells *in situ*, using either a membrane-permeable fluorescent peptide as substrate, or endogenous substrates. These measurements will allow evaluation of the effects of different treatments or experimental paradigms both on the total detectable calpain activity in the cells, and also on its activity

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against specific substrates. As in all calpain assays without some form of prior separation step, it must be noted that the presence of endogenous inhibitors, especially calpastatin, may mask some or all of the real total calpain activity.

2. Materials

2.1. Equipment

1. A single-wavelength fluorescence detector capable of 380 nm excitation (10-nm bandwidth) and 460 nm emission (20-nm bandwidth) with stirring capability and temperature control such as a PTI Delta Scan system (Photon Technologies Inc., Monmouth Junction, NJ 08852).
2. A quartz cuvette for fluorescent measurement and microstir bars that fit properly within the cuvette (Fisher, Pittsburgh, PA 15205-9913). A 1 mL cuvette is usually preferred.
3. A computer system and software for data analysis (PTI software, PTI).
4. A fluorescent microplate reader capable of 380 nm excitation and 460 nm emission. The Bio-Tek FL600 is one possible choice (Bio-Tek, Winooski, VT 05404-0998)
5. 24-well microtiter plates to which cells are readily adherent and which are transparent to the specified wavelengths (Corning/Costar, Cambridge, MA 02140).

2.2. Reagents and Solutions

1. 200 m*M* HEPES, pH 7.4. HEPES is used at a final concentration of 50 m*M*.
2. $CaCl_2$ stock solutions (200–500 m*M*) in distilled H_2O. These solutions should be made up as accurately as possible and stored at 4°C (*see* **Note 1**).
3. 100 m*M* DTT in distilled H_2O, stored in aliquots at –20°C.
4. *N*-Succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine-7-amido-4-methylcoumarin (Succ-LLVY-AMC) (Bachem, King of Prussia, PA 19406) is prepared as a stock solution of 10 mg/ml (13.1 m*M*) in dimethylformamide (DMF) and stored in aliquots at –20°C in the dark.
5. Purified μ-calpain (calpain I) (Stock concentration varies with lot number) (Calbiochem, San Diego, CA 92121). Stock solutions are stored at 4°C for several weeks.
6. Tau, to be used at final concentration of 0.2 to 2.0 mg/mL. (Tau can be purified as described ***(14)*** or purchased from Cytoskeleton, Denver, CO 80206).
7. 100 m*M* EGTA in distilled H_2O, pH 7.5.
8. Cell culture medium (RPMI 1640) (Gibco, Rockville, MD 20849-6482), 1% glutamine, 1% Pen-Strep, 5% Fetal Clone II (Hyclone, Logan, UT), 10% horse serum.
9. Calcium ionophore, such as ionomycin (Sigma), which is used at a final concentration between 0.2 and 2.0 μ*M* and is prepared as a 1000 X stock solution in DMSO. Aliquots can be stored at –20°C for up to a few weeks and should not undergo numerous cycles of freeze–thaw.
10. Antibodies: for tau (Zymed, San Francisco, CA 94080, Chemicon, Temecula, CA 92590 or Sigma); for calpain (Chemicon).

11. Laemmli stop buffer, 2 × stock: 0.1 *M* Tris-HCl, pH 6.8, 4% (w/v) SDS, 10 m*M* EDTA, 20% (v/v) glycerol, 0.2 *M* DTT, 0.2% (w/v) bromophenol blue.

3. Methods

3.1. Measurement of Calpain Activity In Vitro With Succ-LLVY-AMC

The effects of different agents on the activity of calpain can be determined using the highly fluorescent peptide substrate *N*-succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine-7-amido-4-methylcoumarin (Succ-LLVY-AMC) ***(4,15–18)***. The assays are described here using μ-calpain, but are equally applicable to m-calpain, using an appropriate Ca^{2+} concentration. The assay is based on the shift in fluorescence that occurs when the fluorescent AMC moiety is cleaved from the peptide. Fluorescence of free AMC is maximal at an excitation wavelength of 380 nm and emission wavelength of 460 nm and these values are different from those observed for AMC when covalently linked to Succ-LLVY. Proteolytic release of the AMC moiety therefore causes an increase in fluorescence at detector settings of 380 nm excitation and 460 nm emission.

1. Turn on the fluorescence detector and computer according to specific instructions given by the manufacturer. For optimal results, the experiments should be carried out in the lowest possible ambient light, to protect the photomultiplier tube from direct light exposure.
2. Allow equipment to stabilize (light source and water bath will need approximately 30 min).
3. Thoroughly clean a 1 cm quartz cuvette (*see* **Note 2**).
4. Add components to the cuvette in this order, to a final volume of 1 mL, stirred:
   a. 0.25 mL of HEPES buffer;
   b. 12 μL of Succ-LLVY-AMC (stored on ice) (*see* **Note 3**);
   c. 0.1–1 U of calpain;
   d. 1–10 μL of DTT;
   e. water as required, allowing for the later addition of calcium;
   f. equilibrate the mixture in the fluorometer, and establish a baseline;
   g. initiate reaction by adding calcium (*see* **Note 4**).
5. The initial rates of calpain activity are calculated from the fluorescence progress curves by standard kinetic analysis (*see* **Notes 5** and **6**).
6. It is possible to stop the reaction at any time by addition of EGTA to a final concentration of 10 m*M*, or 10-fold greater than the concentration of calcium. The sample can then be used to observe the extent of calpain autolysis by immunoblot analysis.

3.2. Measurement of Calpain Activity In Vitro by Observation of Proteolysis of the Microtubule-Associated Protein Tau

In addition to measuring calpain activity using fluorescent peptide substrates, it may be desirable to determine calpain activity using specific

protein substrates ***(4,5)***. This will permit evaluation both of calpain activity toward putative physiological substrates, and also of the effects of posttranslational modifications of the substrate, such as phosphorylation, on susceptibility of the substrate to calpain proteolysis. Although the following assay is described for tau ***(14)***, it can be modified to examine the proteolysis of virtually any putative calpain substrate.

1. In a 1.5-mL Eppendorf tube, place 25 µL of HEPES buffer, 1 µL of DTT, 20–200 µg of tau, 0.01–0.1 U of calpain, and H_2O to a final volume of 100 µL (allowing for calcium addition).
2. Add calcium to initiate the reaction, (usually to a final concentration of 0.1–2 m*M*) followed by brief vortexing. The reaction can be carried out at room temperature or 37°C in a shaking water bath. Control incubations are set up in the absence of Ca^{2+}, with 1 m*M* EDTA.
3. Remove aliquots (usually 10 µL) at various time points (from 30 s up to 30 min) and add them to an equal volume of 2 × Laemmli stop buffer.
4. Heat samples in a boiling water bath for 5 min **(Note 7).**
5. Centrifuge samples at 12,000*g* for 5 min in a tabletop microfuge, to remove any SDS-insoluble material, and transfer the supernatants to clean microfuge tubes.
6. Samples can be stored at –20ºC for several months.
7. Run SDS-PAGE on 0.1–1 µg protein/lane, and perform immunoblot analysis using established procedures. For tau, 8–10% gels are ideal, and for human µ-calpain 7.5% gels give optimal separation of intact calpain and the autolytic forms (70/78/76 kDa) ***(1,2)***.
8. Develop the blots by enhanced chemiluminescence (ECL)(Amersham Life Science, Arlington Heights, IL), and quantitate the results by densitometry with the Bio-Rad imaging densitometer model GS-670 or its equivalent **(Note 8)**.
9. Data can be expressed in terms of the loss of intact tau protein and plotted as percent initial substrate remaining versus time.

3.3 Measurement of Calpain Activity In Situ, Using a Membrane-Permeable Fluorescent Substrate

Subsequent to examining the effects of different modulating factors on calpain activity in vitro, it is necessary to correlate these finding with events that occur *in situ* or in vivo. Additionally, *in situ* and in vivo studies are necessary to attempt to establish authenticity of putative calpain substrates, and to determine how alteration of cell processes modulates their proteolysis by calpain.

Succ-LLVY-AMC is membrane-permeable and therefore can be used to measure *in situ* proteolytic activity of calpain in cultured cells. The protocols given here have been optimized for the human neuroblastoma cell line SH-SY5Y. However, other systems have been used ***(19)*** under similar conditions. In addition, calpain is present in all tissues and virtually all organisms studied ***(20–22)***, thus these conditions should be easily modified for a specific cell line or tissue.

1. Grow cells in cell culture medium in 24-well plates to confluency of 70–80% in a humidified CO_2 incubator at 37°C.
2. Rinse cells three times with serum-free medium without phenol red.
3. Add 500 µL of serum-free medium without phenol red containing 80 µ*M* Succ-LLVY-AMC to each well and return the plate to the incubator for 10–30 min (*see* **Note 9**).
4. Place the 24-well plate into the microplate reader and make a background fluorescence measurement (the cells may be gently shaken before reading). The data are appropriately stored using the available software package.
5. Add ionomycin (from 0.2 to 2.0 µ*M* ionomycin is sufficient for most cell types), shake the plate gently, and take a reading immediately. Return the plate of cells to the CO_2 incubator (*see* **Note 10**).
6. Perform plate readings every 10 to 30 min with the plate returned to the CO_2 incubator as rapidly as possible after each reading in order to maintain optimal cell viability and pH.
7. To evaluate the results, the background levels (the first data taken in **step 4**) should be subtracted from subsequent data for each individual well (to allow for well-to-well variability).
8. The results are expressed in terms of arbitrary units (increase in fluorescence) for each well versus time.

3.4. In Situ Proteolysis of the Endogenous Substrate Tau

In addition to determining the calpain activity level in cultured cells with a fluorescent substrate, proteolysis of endogenous substrates *in situ* can be followed by immunoblot methods. In this example, the tau protein is used ***(5)***, but other substrates can be examined similarly using appropriate gel conditions, such as spectrin, and MAP-2 ***(25,26)***.

1. Grow cells in cell culture medium to 70–80% confluency in 35-, 60-, or 100-mm culture dishes depending upon the expression level of calpain and the calpain substrate to be examined. For SH-SY5Y cells, 60-mm Corning plates are used.
2. Rinse cells three times with serum-free medium.
3. Add an appropriate volume of serum-free medium.
4. Add ionomycin (0.2–2 µ*M*) and incubate for up to 60 min **(Note 11)**.
5. Rinse cells in serum-free medium.
6. Scrape the cells into 200–500 µL of 2X Laemmli stop buffer without dye and without DTT **(Note 12)**, and heat the cell lysates immediately in a boiling water bath for 10 min.
7. Determine the protein concentration of each sample by means of the bicinchoninic (BCA) assay (Pierce, Rockford, IL).
8. Dilute the samples to a uniform protein concentration with 2X Laemmli stop buffer containing 5 m*M* DTT and bromophenol blue as the tracking dye; heat them again in a boiling water bath for 5 min.
9. Run 10–50 µg of total protein per lane on an SDS polyacrylamide gel, and perform immunoblot analysis and quantitation as described above.

4. Notes

1. Standardization of Ca^{2+} concentration is highly recommended and can be done with a Ca^{2+}sensitive ion probe such as Orion Research model EA940 (Fisher, Pittsburgh, PA). Final concentrations of Ca^{2+} in the assays will range from 0.1 μ*M* to 5 m*M*. After dilution, free Ca^{2+} concentrations lower than 100 μ*M* should be determined with a Ca^{2+}-sensitive indicator such as Fura-2 (Teflabs, Austin TX 78747), while concentrations greater than 100 μM can be confirmed with the Ca^{2+}-sensitive ion probe ***(5,13)***.
2. Wash the cuvette thoroughly with water and methanol to remove any traces of protein or calcium.
3. The K_m of calpain for Succ-LLVY-AMC has been reported to be between 100–200 μ*M* ***(4)***. Frozen stocks may be kept for several weeks or months, but once thawed, the aliquots should not be refrozen or reused. The final concentration of Succ-LLVY-AMC used in the in vitro reactions is 170 μ*M* (0.117 mg/mL).
4. Before calcium addition, make sure that the baseline is stable for several minutes, as addition of some compounds may increase or decrease basal fluorescence. This will also demonstrate that there is no Ca^{2+} already present. Perform control experiments to establish that measurements lie within the optimal range for the instrument, and to determine the contribution of specific reagents to any change in fluorescence.
5. Fluorescence change can be observed for several minutes, but the trace will not be linear for very long, at least partly because of calpain autolysis and degradation in the presence of Ca^{2+} ***(4)***.
6. Succ-LLVY-AMC is a preferred substrate for calpain, but can be cleaved by other proteases ***(4)***. For in vitro studies, this fact is advantageous because the assay can be used to determine whether certain modulatory factors affect other protease activities as well ***(4)***. However, it may also be desirable to establish the extent of non-calpain-catalyzed hydrolysis of the substrate ***(17,24,25)***. This can be approached by addition of various protease inhibitors.
7. It is not commonly appreciated that heating in Laemmli stop-buffer can be deleterious for some proteins, and that incubation at 25–37°C for 20 min in the stop-buffer may be sufficient to denature and dissolve all proteins. Comparisons should be made of gels and immunoblots with and without boiled samples.
8. It is important to establish appropriate antibody dilutions and incubation times to obtain a linear correlation between intensity of staining and quantity of antigen.
9. Controls should include wells which receive only the solvent DMF without Succ-LLVY-AMC, and wells with and without ionophore.
10. Keep the dish warm and buffered (i.e., in the incubator) as much as possible. If the cells can be maintained in a medium that does not require CO_2 for buffering (e.g., HEPES), and the plate reader can maintain a temperature of 37ºC, the plate can be left in the reader for the duration of the experiment.
11. To investigate the specific role of calpain in degradation of endogenous substrates, parallel incubations in the presence of selective protease inhibitors ***(5,27)***, and in Ca^{2+}-free media, are recommended.
12. DTT interferes with the BCA protein assay, and with other protein assays.

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References

1. Goll, D. E., Thompson, V. F., Taylor, R. G., and Zalewska, T. (1992) Is calpain activity regulated by membranes and autolysis or by calcium and calpastatin? *BioEssays* **14,** 549–556.
2. Johnson, G.V.W. and Guttmann, R.P. (1997) Calpains: intact and active? *BioEssays* **19,** 1011–1018.
3. Melloni, E., Michetti, M., Salamino, F., and Pontremoli, S. (1998) Molecular and functional properties of a calpain activator protein specific for mu-isoforms. *J. Biol. Chem.* **273,** 12,827–12,831.
4. Guttmann, R. P., Elce, J. S., Bell, P. D., Isbell, J. C., and Johnson, G. V. W. (1997) Oxidation inhibits substrate proteolysis by calpain I but not autolysis. *J. Biol. Chem.* **272,** 2005–2012.
5. Guttmann, R. P. and Johnson, G. V. W. (1998) Oxidative stress inhibits calpain activity in situ. *J. Biol. Chem.* **273,** 13,331–13,338.
6. DiLisa, F., De Tullio, R., Salamino, F., Barabato, R., Melloni, E., Siliprandi, N., Schiaffino, S., and Pontremoli, S. (1995) Specific degradation of troponin T and I by mu-calpain and its modulation by substrate phosphorylation. *Biochem. J.* **308,** 57–61.
7. Litersky, J. M. and Johnson, G. V. W. (1992) Phosphorylation by cAMP-dependent protein kinase inhibits degradation by calpain. *J. Biol. Chem.* **267,** 1563–1568.
8. Chen, M. and Stracher, A. (1989) In situ phosphorylation of platelet actin-binding protein by cAMP-dependent protein kinase stabilizes it against proteolysis by calpain. *J. Biol. Chem.* **264,** 14,282–14,289.
9. Sasaki, T., Kikuchi, T., Yumoto, N., Yoshimura, N., and Murachi, T. (1984) Comparative specificity and kinetic studies on porcine calpain I and calpain II with naturally occurring peptides and synthetic fluorogenic substrates. *J. Biol. Chem.* **259,** 12,489–12,494.
10. Elamrani, N., Balcerzak, D., Soriano, M., Brustis, J. J., Cottin, P., Poussard, S., and Ducastaing, A. (1993) Evidence for fibronectin degradation by calpain II. *Biochimie* **75,** 849–853.
11. Huang, C., Tandon, N. N., Greco, N. J., Ni, Y., Wang, T., and Zhan, X. (1997) Proteolysis of platelet cortactin by calpain. *J. Biol. Chem.* **272,** 19,248–19,252.
12. Kavita, U. and Mizel, S. B. (1995) Differential sensitivity of interleukin-1 alpha and-beta precursor proteins to cleavage by calpain, a calcium-dependent protease. *J. Biol. Chem.* **270,** 27,758–27,765.
13. Grynkiewicz, G., Poenie, M., and Tsien, R. T. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260,** 3440–3450.
14. Johnson, G. V. W., Jope, R. S., and Binder, L. I. (1989) Proteolysis of tau by calpain. *Biochem. Biophys. Res. Commun.* **163,** 1505–1511.

15. Atsma, D. E., Bastiaanse, E. M., Jerzewski, A., Van der Valk, L. J., and Van der Laarse, A. (1995) Role of calcium-activated neutral protease (calpain) in cell death in cultured neonatal rat cardiomyocytes during metabolic inhibition. *Circ. Res*. **76,** 1071–1078.
16. Rosser, B. G., Powers, S. P., and Gores, G. J. (1993) Calpain activity increases in hepatocytes following addition of ATP: Demonstration by a novel fluorescent approach. *J. Biol. Chem*. **268,** 23,593–23,600.
17. Wang, K. K. W., Nath, R., Raser, K. J., and Hajimohammadreza, I. (1996) Maitotoxin induces calpain activation in SH-SY5Y neuroblastoma cells and cerebrocortical cultures. *Arch. Biochem. Biophys.* **331,** 208–214.
18. Tsubuki, S., Saito, Y., Tomioka, M., Ito, H., and Kawashima, S. (1996) Differential inhibition of calpain and proteasome activities by peptidyl aldehydes of dileucine and tri-leucine. *J. Biochem*. **119,** 572–576.
19. Edelstein, C. L., Ling, H., Gengaro, P. E., Nemenoff, R. A., Bahr, B. A., and Schrier, R. W. (1997) Effect of glycine on prelethal and postlethal increases in calpain activity in rat renal proximal tubules. *Kidney Int*. **52,** 1271–1278.
20. Croall, D.E.and DeMartino, G.N. (1991) Calcium-activated neutral protease (calpain) system: Structure, function, and regulation. *Physiol. Rev.* **71,** 813–847.
21. Melloni, E. and Pontremoli, S. (1989) The calpains. *Trends Neurosci*. **12,** 438–444.
22. Saido, T. C., Sorimachi, H., and Suzuki, K. (1994) Calpain: new perspectives in molecular diversity and physiological-pathological involvement. *FASEB J*. **8,** 814–822.
23. Mehdi, S., Angelastro, M. R., Wiseman, J. S., and Bey, P. (1988) Inhibition of the proteolysis of rat erythrocyte membrane proteins by a synthetic inhibitor of calpain [published erratum appears in *Biochem. Biophys. Res. Commun*. (1989) **159,** 371]. *Biochem. Biophys. Res. Commun*. **157,** 1117–1123.
24. Ariyoshi, H., Shiba, E., Kambayashi, J., Sakon, M., Tsujinaka, T., Uemura, Y., and Mori, T. (1991) Characteristics of various synthetic peptide calpain inhibitors and their application for the analysis of platelet reaction. *Biochem. Int*. **23,** 1019–1033.
25. Siman, R., Baudry, M., and Lynch, G. (1984) Brain fodrin: substrate for calpain I, an endogenous calcium-activated protease. *Proc. Natl. Acad. Sci. U.S.A*. **81,** 3572–3576.
26. Johnson, G. V. W., Litersky, J. M., and Jope, R. S. (1991) Degradation of microtubule-associated protein 2 and brain spectrin by calpain: A comparative study. *J. Neurochem*. **56,** 1630–1638.
27. Xie, H. Q. and Johnson, G. V. W. (1998) Calcineurin inhibition prevents calpain-mediated proteolysis of tau in differentiated PC12 cells. *J. Neurosci. Res*. **53,** 153–164.

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Localization of Calpain by Immunofluorescence in Adherent Cells

Sucheta Kulkarni and Joan E. B. Fox

1. Introduction

Many of the early studies on the localization of calpain described a cytosolic distribution. However, it is becoming clear that calpain can play an essential role in regulating signal transduction across members of the integrin family of adhesion receptors and that in order to do this it is recruited to sites of ligand-occupied integrin.

Signaling across integrins plays a critical role in mediating numerous cellular events including migration, differentiation, gene regulation, and anchorage-dependent cell growth ***(1–3)***. Investigation of the mechanisms involved in integrin-induced signaling have revealed that several members of the Rho family of guanosine triphosphatases (GTPases) are activated ***(4–6)***. Initially, activation of Cdc42 and Rac1 leads to the formation of structures known as focal complexes. These are small complexes of integrin, cytoskeletal proteins, and signaling molecules that are assumed to provide anchorage sites for extending cell membranes. They are very dynamic structures that constantly form and break down as the membrane extends. After the initial cell spreading, RhoA is activated; this induces the formation of larger complexes of integrin, cytoskeletal proteins, and signaling molecules that are known as focal adhesions. Focal adhesions interact with bundles of actin/myosin filaments known as stress fibers that transmit contractile forces through the focal adhesions to the extracellular matrix ***(7,8)***.

Early immunofluorescence studies revealed that calpain distributes to a submembranous location when platelets are activated ***(9,10)***. Other studies showed that m-calpain is present in focal adhesions ***(11)***. More recent immunofluorescence studies have shown that in cells spreading on an integrin substrate,

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μ–calpain is present in a submembranous location in extending lamellipodia, and it is incorporated into focal complexes as they form but is not present in focal adhesions *(Kulkarni et al., unpublished observations)*. Moreover, these studies have helped reveal that μ-calpain plays a critical role at very early stages of integrin-induced cell spreading, and that it does so by inducing activation of Rac1 and RhoA. The immunofluorescence studies on the localization of calpain were important because they helped establish a potential role of the protease in integrin-induced signal transduction. Also, by showing that μ-calpain is present in focal complexes but not in focal adhesions, these studies helped focus subsequent studies on the role of this protein in the early steps following integrin–ligand interactions. To date, calpain has been shown to regulate signaling across αIIbβ3 and α5β1 in platelets, endothelial cells, NIH-3T3 and CHO cells ***(9,12,13,17)***. The potential role of calpain in mediating signaling across additional integrins or additional families of transmembrane receptors has not been established. As with the signaling across αIIbβ3 and α5β1, it appears likely that immunofluorescence might provide a powerful tool for gaining insights into such possibilities. In addition, it appears probable that such studies could be powerful in establishing additional functions for both μ-calpain and m-calpain in a variety of different cell types.

The present chapter describes a procedure for detecting calpain by immunofluorescence. The protocol is quite standard and perhaps the most important feature is the importance of being aware that functional interactions of calpain with cytoskeletal proteins, membrane receptors, or other signaling molecules are likely to be transient. Thus, in early studies, colocalization between calpain and integrins was not detected because studies were performed on fully spread cells in which focal complexes, which contain μ-calpain, are no longer the dominant integrin-containing complexes. These studies on integrins emphasize that in future studies on the distribution of calpain, it will be important to consider factors such as the biology of the system being investigated, the dynamic nature of various adhesive interactions, or the possibility of cross-talk between different adhesion receptors. These considerations should be used to establish under what conditions of adhesion or receptor signaling the distribution of calpain is to be investigated.

2. Materials

2.1. Tissue Culture Supplies

1. Appropriate tissue culture dishes are normally obtained commercially. In order to study the distribution of calpain in response to integrin-induced signaling, cells can be grown on coverslips coated with the substrate for a specific integrin. For example, to study the distribution of calpain in which cell spreading is induced by signaling across β1-containing integrins, we have used fibronectin-coated

cover slips (Becton-Dickinson, San Jose, CA).
2. Tissue culture medium: appropriate media for cultured cells can be prepared or obtained commercially. For example, DMEM/F12 (Dulbeco's modified Eagle's medium and Ham's F12,1:1, Biowhittaker, Walkersville, MD) medium with 10% fetal bovine serum containing penicillin-streptomycin and glutamine (GIBCO-BRL, Grand Island, NY).

2.2. Fixation and Permeabilization of Adherent Cells

1. Tris-buffered saline (TBS): 50 m*M* Tris-HCl, 0.15 m*M* NaCl, pH 7.6.
2. 4 % Paraformaldehyde.
   a. Add paraformaldehyde (Sigma, St. Louis, MO) slowly while stirring to TBS pre-heated in a microwave oven to about 60°C.
   b. Add 1 *M* NaOH dropwise to the resulting white solution until the solution clears (about 25 drops per 100 m*L*). The pH is monitored during the addition of NaOH to ensure that the pH does not rise above 10.
   c. Once the solution is clear, add small amounts of 1 *M* HCL to obtain a final pH of about 7.4. and adjust the volume to reach a 4% (w/v) paraformaldehyde solution. Store aliquots at –20°C and thaw one for a single use. Gentle heating may be required to clear frozen aliquots. The pH should be tested after thawing and readjusted if needed.
3. Quench solution: 0.1% Carnation™ milk, 150 m*M* ammonium acetate in TBS.
4. Permeabilization buffer: 0.5% Triton X-100 in quench solution.
5. Serum buffer: 15 m*M* ammonium acetate, 0.1% Carnation™ milk in TBS containing 1:50 dilution of serum from the animal species in which the secondary antibody was derived.

2.3. Detection of Proteins by Immunofluorescence

1. Primary antibodies: Antibodies against calpain can be obtained commercially. We have found that an antibody that detects μ-calpain but not m-calpain (obtained from Alexis Corporation, San Diego, CA) shows good binding to μ-calpain in fixed cells. Other antibodies that we have used have been raised and characterized in various laboratories ***(10,14)***. Antibodies that specifically recognize the autolytic fragments of calpain have been raised by Saido and coworkers ***(10)*** and can be particularly useful in detecting sites of active calpain.
2. Antibodies may also be required for use as markers in dual-label immunofluorescence experiments to determine whether calpain is present at specific sites or in complexes with other projections. For example, in our experiments to determine whether calpain is involved in integrin-induced signaling we have used antibodies against integrins or against other proteins (e.g., vinculin or phosphotyrosine) that are known components of integrin signaling complexes. Such antibodies can normally be obtained commercially. For example, polyclonal anti-α5β1 antibody can be obtained from Life Technology (Grand Island, NY), polyclonal antibodies against phosphotyrosine can be obtained from Santa Cruz Biotechnology (Santa Cruz, CA), monoclonal antibodies against phosphotyrosine can be obtained from UBI (Lake Placid, NY), and monoclonal antibodies against vinculin can be

obtained from Sigma.
3. Secondary antibodies: secondary antibodies such as Texas Red-labeled anti-mouse IgG and Fluorescein-labeled anti-rabbit IgG can all be obtained from various commercial sources e.g., Amersham Life Science Inc. (Arlington Heights, IL).
4. Mounting solution: Immuno Fluore mounting medium (ICN Biochemicals, Inc., Aurora, OH) (or similar preparations from many commercial sources).
5. Amplification reagents*:* biotinylated anti-mouse Ig, biotinylated anti-rabbit immunoglobulin (Ig), and fluorescently labeled streptavidin (Amersham Life Science Inc.).
6. Fluorescently labeled phalloidin (Sigma) (*see* **Note 1**).

3. Methods

3.1. Cell Culture and Stimulation

Plating of cells **(Note 2)**. Careful consideration should be given to the conditions under which calpain may localize at specific sites in the cell, in order to allow this hypothesis to be tested. For example, in our studies we have grown bovine aortic endothelial cells on fibronectin-coated coverslips placed in 6-well plates (Becton-Dickinson) using Dulbeco's modified Eagle's medium and Ham's F12, 1:1 medium containing penicillin-streptomycin and glutamine. Serum was omitted from the medium so that we could study the distribution of calpain in cells in which signaling occurred across integrins, but additional signals mediated by stimulation of growth factor receptors were minimized. Bovine aortic endothelial cells were used between passage 6 and 15 and the distribution of calpain was determined at intervals between 20 min and 24 h after plating. This approach allowed us to show that calpain colocalized with the small integrin signaling complexes that formed soon after plating but was absent from the larger focal adhesions that formed in more fully spread cells. In other experiments, we have allowed platelets to spread on fibrinogen-coated coverslips in order to determine the distribution of calpain in cells in which spreading is induced by signaling across the platelet integrin $\alpha IIb\beta 3$. In these experiments, platelets were isolated from blood by standard techniques ***(15)***, resuspended in Tyrode's buffer and allowed to spread for 5 to 60 min.

3.2. Fixation and Permeabilization of Adherent Cells

1. Aspirate the growth media and wash the cells once with TBS (1 mL/well) for 5 min at room temperature. Any nonadherent cells are removed in this step.
   From this point, all steps are performed in a dark box that can be improvised by assembling a rack for holding slides in a dish that is wrapped and covered in tin foil to keep the light out. Tissues soaked in water are placed in the container to maintain a humid environment and care is taken not to allow the cells to be left dry at any stage of the procedures.

2. Fix the cells in 4% paraformaldehyde (1 mL/well) for 15 min at room temperature.
3. Quenching of cells: treat fixed cells twice with Quench solution (1 mL/well) for 5 min at room temperature.
4. Permeabilize fixed cells by addition of 0.5% Triton X-100 in Quench solution for 5–15 min at room temperature (*see* **Note 3**).
5. Wash the cells three times for 5 min at room temperature with Serum buffer (*see* **Notes 4** and **5**).

3.3. Immunofluorescence

1. Incubation with primary antibodies: dilute monoclonal antibodies to a working concentration of 1 µg/mL and affinity-purified polyclonal antibodies to 20 µg/mL in serum buffer (*see* **Note 6**).
2. Set up control slides incubated with a control antibody such as normal rabbit or mouse IgG.
3. Incubate the slides with the primary antibody at 4°C overnight in the dark humidified environment. They should be covered with sufficient antibody solution to ensure that they do not dry out. When using 6-well plates, 1 mL of antibody solutiton is added to each well.
4. During the above incubation, absorb the secondary antibody in serum of the species from which the cells were derived or grown, for example, fetal calf serum for cultured cells or normal human serum for human platelets. Typically 1 µL of the secondary antibody is incubated with 10 µL of serum overnight at 4°C (*see* **Notes 7–9**).
5. Incubation with secondary antibodies*:*
   a. wash the cells on the coverslips at least 5 times by immersion for 5 min in serum buffer;
   b. dilute the preabsorbed secondary antibody 1:500 with serum buffer, providing ~5 mL of secondary antibody solution;
   c. add the secondary antibody (1 mL per well in a 6-well plate) and incubate for 3 h at room temperature in the dark.
6. Mounting and examination:
   a. wash the cells at least 5 times for 5 min each in TBS or serum buffer;
   b. carefully remove the last TBS wash; place one drop of mounting solution on a glass slide, and invert the coverslips with antibody-treated cells onto the drop of mounting solution (*see* **Note 10**);
   c. press the coverslip down in the center using a Q-tip;
   d. aspirate excess mounting solution from the edges which are then sealed with nail polish (*see* **Note 11**).
7. Store slides in the dark at 4°C.
8. Examine the slides in a fluorescent microscope, ideally within a few days (*see* **Note 12**).
9. Amplification of signals. If the staining obtained by this method proves to be weak, the signal can be amplified by using a biotinylated secondary antibody followed by fluorescently labeled streptavidin. In this case, the secondary antibody will be biotinylated goat anti-rabbbit immunoglobulin G (IgG) or

biotinylated sheep anti-mouse IgG. The secondary antibody will be removed by washing as described above and then incubated with fluorescently labeled streptavidin diluted 1:500 in TBS for 1 h in the dark. Loosely bound streptavidin is removed by washing five times for 5 min each or longer depending on the level of the background. The samples are then mounted as described above.

10. Dual label immunofluorescence. In many cases, it will be desirable to perform dual-label immunofluorescence in order to demonstrate colocalization of calpain with specific receptors or structures. In this case, if a calpain monoclonal antibody is used, a polyclonal antibody against the protein of interest should be used. If a calpain polyclonal antibody is used, the antibody against the second protein should be a monoclonal. Both antibodies can be added simultaneously. Two secondary antibodies will also be needed and should be labeled with fluorescent probes that are readily detected in two separate channels in the fluorescent microscope available. In our laboratory, we use Texas red labeled anti-mouse IgG to detect the monoclonal antibody and fluorescein-labeled anti rabbit IgG to detect the polyclonal antibody. These secondary antibodies can be absorbed with serum together and subsequently added to the slides together. If there is a problem with low levels of labeling, biotinylated secondary antibody can be used and subsequently detected with fluorescently labeled streptavidin. However, only one of the two proteins can be amplified in this way.

If calpain colocalization with actin filaments is to be examined, fluorescently labeled phalloidin can be added along with the fluorescently labeled secondary antibody.

4. Notes

1. Calpain appears to play an important role in regulating the organization of the cytoskeleton, in many cases cleaving cytoskeletal proteins. Thus, in many cases it may be required to determine whether calpain colocalizes with areas of the cells containing actin filaments. Phalloidin selectively binds to filamentous actin and can be obtained commercially in fluorescent-labeled forms. Examples include tetrarhodamine isothiocyanate (TRITC)-phalloidin or fluorescein isothiocyanate (FITC)-labeled phalloidin from Sigma.
2. Problems with detecting distribution of proteins in confluent cells: for photography of stained cells it is essential that cells are not overcrowded, particularly in visualizing proteins that have a submembranous location. It is much easier to see the boundaries of cells that are not contacting each other. We have found it convenient to plate 1 mL of cells at a concentration of $1–2 \times 10^5$ cells/mL in each well of a 6-well plate. An exception to this might be a situation in which it is necessary to look at the distribution of proteins that are in contact with each other; in this case cell–cell contacts could be identified by using antibodies against known marker proteins.
3. Permeabilization allows detection of molecules that are present within cells; there have been some reports that calpain may bind to extracellular membranes under

certain conditions ***(10,16)***; the permeabilization step can be omitted to investigate such a possibility.

4. This solution contains serum from the animal species from which the secondary antibody was derived, for example, sheep serum is used for monoclonal antibodies that are detected with anti-mouse IgG raised in sheep; goat serum is used for polyclonal antibodies that are detected with anti-rabbit IgG raised in goat.
5. Problems with dislodging cells during manipulations: in our studies on the distribution of calpain in cells that adhere on integrin substrates, we have found that colocalization of μ-calpain with integrin occurs only at very early times after plating on an integrin-ligand in serum-free medium (20–60 min after plating). Because the cells are attached by only small focal complexes at these times, they are easily dislodged during the numerous washing steps required for the procedures described above. In these situations, shear forces involved in washing can be minimized by incubation of cells a fewer number of times but for longer periods with larger volumes of buffer.
6. Optimum antibody concentrations need to be determined experimentally but a good place to start might be 1 μg/mL for a monoclonal antibody and 20 μg/mL for an affinity purified polyclonal antibody.
7. If the secondary antibody is fluorescently labeled (an exception might be if a biotinylated secondary antibody is used) it is especially important to carry this out in the dark.
8. When a polyclonal antibody has been used as primary antibody, the secondary antibody will normally be Texas red-labeled or fluorescein-labeled goat anti-rabbit IgG. When a monoclonal primary antibody is used, the secondary antibody will be fluorescently labeled sheep anti-mouse IgG.
9. Problems with fluorescent aggregates: all antibodies and fluorescent-labeled detection reagents should be centrifuged briefly before use. This avoids the possibility of aggregates of fluorescent-labeled proteins either within the cells or all over the background. We have found that this sort of aggregate can appear as the secondary antibodies age, even if we take the precaution of centrifuging. Thus, it is useful to obtain new vials of secondary reagents on a regular basis.
10. High background labeling: if there are problems with a high background, the washing procedures can be modified. After incubation with both primary and secondary antibodies, the length of time that each wash is carried out can be increased. It is often helpful, in both cases, to extend the washes so that they are carried out over as much as 16 h at 4°C, since this helps to dislodge loosely nonspecifically bound primary or secondary antibodies.
11. Mounting problems: sideways movement of the coverslip during mounting should be avoided as this can dislodge fixed cells. If this proves to be a problem, it may be easier to anchor the coverslip at the corners with nail polish before pressing down with the Q tip. Excess mounting solution will be pushed out at the sides of the cover slips, it can be aspirated, and the sides of the cover slips subsequently sealed.
12. Cardboard slide holders for this purpose are available commercially (Micro Slide

Tray, VWR Scientific, Cleveland, OH). Ideally, slides should be examined within a few days but depending on the intensity of the fluorescence, they can often be stored successfully for several months. Most microscopes can be fitted with cameras so that images can be recorded photographically. Each frame should be recorded at a number of different exposures in order to obtain an appropriate image. However, with experience it usually becomes possible to obtain suitable photographic images with minimal bracketing.

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References

1. Meredith, J. E., Jr., Winitz, S., Lewis, J. M., Hess, S., Ren, X. -D., Renshaw, M. W., and Schwarts, M. A. (1996) The regulation of growth and intracellular signaling by integrins. *Endocr. Rev.* **17,** 207–220.
2. Lafrenie, R. M. and Yamada, K. M. (1996) Integrin-dependent signal transduction. *J. Cell. Biochem.* **61,** 543–553.
3. Hynes, R. O. (1992) Integrins: Versatility, modulation, and signaling cell adhesion. *Cell* **69,** 11–25.
4. Price, L. S., Leng, J., Schwartz, M. A., and Bokoch, G. M. (1998) Activation of Rac and Cdc42 by integrin mediates cell spreading. *Mol. Biol. Cell* **9,** 1863–1871.
5. Clark, E. A., King, W. G., Brugge, J. S., Symons, M., and Hynes, R. O. (1998) Integrin-mediated signals regulated by members of the Rho family of GTPases. *J. Cell. Biol.* **142,** 573–586.
6. Ren, X. -D., Kiosses, W. B., and Schwartz, M. A. (1999) Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. *EMBO J.* **18,** 101–108.
7. Chrzanowska-Wodnicka, M. and Burridge, K. (1996) Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. *J. Cell. Biol.* **133,** 1403–1415.
8. Burridge, K. and Chrzanowska-Wodnicka, M. (1996) Focal adhesions, contractility, and signaling. *Annu. Rev. Cell Div. Biol.* **12,** 463–519.
9. Fox, J. E. B., Taylor, R. G., Taffarel, M., Boyles, J. K., and Goll, D. E. (1993) Evidence that activation of platelet calpain is induced as a consequence of binding of adhesive ligand to the integrin, glycoprotein IIb-IIIa. *J. Cell Biol.* **120,** 1501–1507.
10. Saido, T. C., Suzuki, H., Yamazaki, H., Tanoue, K., and Suzuki, K. (1993) In situ capture of μ-calpain activation in platelets. *J. Biol. Chem.* **268,** 7422–7426.
11. Beckerle, M. C., Burridge, K., DeMartino, G. N., and Croall, D. E. (1987) Colocalization of calcium-dependent protease II and one of its substrates at sites of cell adhesion. *Cell* **51,** 569–577.
12. Huttenlocher, A., Palecek, S. P., Lu, Q., Zhang, W., Mellgren, R. L., Lauffenburger, D. A, Ginsberg, M. H., and Horwitz, A. F. (1997) Regulation of cell migration by the calcium-dependent protease calpain. *J. Biol. Chem.* **272,** 32,719–32,722.
13. Potter, D. A., Tirnauer, J. S., Janssen, R., Croall, D. E., Hughes, C. N., Fiacco,

K. A., Mier, J. W., Maki, M., and Herman, I. M. (1998) Calpain regulates actin remodeling during cell spreading. *J. Cell Biol*. **141,** 1–16.
14. Lane, R. D., Allan, D. M., and Mellgren, R. L. (1992) A comparison of the intracellular distribution of μ-calpain, m-calpain, and calpastatin in proliferating human A431 cells. *Exp. Cell Res*. **14,** 549–556.
15. Phillips, D. R. and Agin, P. P. (1977) Platelet membrane defects in Glanzmann's thrombasthenia. *J. Clin. Invest*. **60,** 535–545.
16. Schmaier, A. H., Bradford, H. N., Lundberg, D., Farber, A., and Colman, R. W. (1990) Membrane expression of platelet calpain. *Blood* **75,** 1273–1281.
17. Kulkarni, S., Saido, T. C., Suzuki, K., and Fox, J. E. B. (1999) Calpain mediates integrin-induced signaling at a point upstream of Rho family members. *J. Biol. Chem.* **274,** 21,265–21,275.

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A Radioimmunologic Technique for Assessing Calpain Activation in Cells

Ronald L. Mellgren

1. Introduction

A major area of calpain research involves the development of techniques to assess calpain activation in cells. Knowing physiologic conditions that allow the various calpain isoforms in cells to become activated should provide valuable information about their roles in cell functions. Several approaches have been tried, only a few of which will be discussed in this brief introduction.

In assessing calpain activation, one might consider simply exposing cells to conditions thought to activate calpains, and assaying subsequently prepared cell lysates for calpain activity. The difficulty here is that most calpain assays measure total activity, rather than detecting some fraction of the total which may have been previously activated in some way. While there are indications that calpains undergo some irreversible modifications that can result in activation (e.g., autoproteolysis, as discussed below), it is also possible that some physiologically important form(s) of intracellular activation may be reversible and occur only transiently, and would therefore not be detected in cell lysates.

Cell-permeant fluorogenic substrates have been employed to detect calpain activation in cells ***(1,2)***. A drawback with these studies is the lack of specificity, since there is no low molecular weight substrate, to my knowledge, that has been shown to be hydrolyzed only by calpains.

Some studies have utilized protein immunoblotting to assess calpain activation by detection of autolytic calpain fragments ***(3–5)***. It should be noted, however, that the only reported study of calpain metabolic stability in cultured

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cells indicated that the two major calpain isoforms, μ-and m-calpain, are very long-lived, with half-lives of approximately 5 days for each isozyme *(6)*. If calpains are normally activated by autoproteolysis, it is counterintuitive that they should display such remarkable stability. Moreover, recent studies suggest that calpains may be active in the native, nonautolyzed, state *(7)*.

An alternative method for detecting calpain activation in cells relies on the ability of benzyloxycarbonyl-Leu-Leu-Tyr-diazomethylketone (ZLLY-DMK), to permeate cells and covalently modify the active site cysteine residue of calpains *(8,9)*. ZLLY-DMK can be readily radioiodinated on the tyrosine residue, a modification which has little influence on its inhibitory properties, or on its ability to diffuse into cells *(8,9)*. ZLLY-DMK is relatively selective for calpain, and the large catalytic subunit of μ-calpain appears to be a major target covalently modified by the radioiodinated inhibitor, at least in platelets *(8,9)*. Reaction of [^{125}I]ZLLY-DMK with calpain (alkylation) is absolutely dependent on calcium **(Fig. 1)**, and alkylation is greatly decreased if the calpain active site cysteine residue is masked by binding with the inhibitor protein calpastatin **(Fig. 1,** lane 6**)**. Thus, this reagent should label only those calpain molecules in cells which are activated but not bound to calpastatin.

The usefulness and specificity of [^{125}I]ZLLY-DMK as an agent for detecting calpain activation can be greatly enhanced by coupling *in situ* labeling with immunoprecipitation of calpain from subsequently prepared cell lysates. This allows selection of activated calpains by two criteria: (i) labeling of the large, catalytic subunit by a calpain-selective reagent, and (ii) immunoprecipitation using an antibody selective for the small, noncatalytic calpain subunit **(Fig. 2)**.

While the techniques described should be applicable to cell culture studies in general, the author's laboratory has utilized them largely to study calpain activation in mitogen-stimulated human fibroblasts. The protocols are therefore described with specific reference to this system.

2. Materials

1. 2 m*M* ZLLY-DMK (Enzyme System Products, Dublin, CA) in ethanol, prepared on the day of the experiment **(***see* **Note 1)**.
2. P-1 monoclonal antibody to the calpain small subunit (Chemicon, El Segundo, CA, Catalog number MAB3083) **(***see* **Note 2)**.
3. Cell lysis buffer: 50 m*M* imidazole-HCl, 70 m*M* NaCl, 1 m*M* EGTA, 0.1% Triton X-100, 10 μ*M* pepstatin A, 200 μ*M* PMSF, 100 μ*M* sodium orthovanadate, pH 7.4 when measured at ambient temperature (23°C). A solution containing all of the components listed above, except for the protease inhibitors pepstatin A and PMSF, can be stored in the refrigerator for several weeks. The inhibitors are added on the day of use from 100 × concentrated stock solutions in dimethylsulfoxide (for PMSF) or methanol (for pepstatin A).

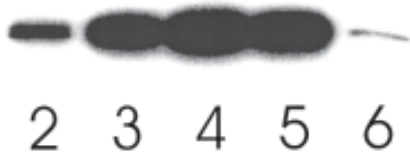

Fig. 1. The influence of [Ca^{2+}] on labeling of μ-calpain with [^{125}I]ZLLY-DMK. Purified human μ-calpain was incubated for 20 min in the presence of 7 μ*M* radioiodinated ZLLY-DMK and 2 m*M* EGTA *(lane 1)*, 50 μ*M* Ca^{2+} *(lane 2)*, 150 μ*M* Ca^{2+} *(lane 3)*, 450 μ*M* Ca^{2+} *(lane 4)*, 2 m*M* Ca^{2+} *(lane 5)*, or 2 m*M* Ca^{2+} plus 10 μg/mL calpastatin *(lane 6)*. 25 μL of each sample were electrophoresed, blotted, and subjected to autoradiography.

4. Antibody gel first wash buffer: cell lysis buffer with 0.15 *M* NaCl.
5. Antibody gel second wash buffer: 50 m*M* imidazole-HCl, 0.2 m*M* EGTA, 150 m*M* NaCl, pH 7.4. May be stored refrigerated for several weeks.

3. Methods

3.1. Radioiodination of ZLLY-DMK (See ref. 8 and 9, and Note 3.)

1. Add 22.5 μL of 22 m*M* sodium phosphate, pH 7.5, and 12.5 μL of 2 m*M* ZLLY-DMK in ethanol to a glass vial with a V-shaped bottom containing 1 mCi of carrier-free ^{125}I (ICN, Costa Mesa, CA, Catalog number 63034) in a volume of 10 μL as supplied.
2. Place the mixture on ice, and add an Iodo-Bead (Pierce, Rockford, IL). Mix gently in the hood about every 2 min for a total of 10 min.
3. Terminate iodination by diluting the reaction mixture with 450 μL of 10 m*M* sodium phosphate, pH 7.5, and subsequently withdrawing the solution from the vial, leaving the Iodo-Bead behind.
4. Wash a 0.5 mL sample of AG 501-X8 mixed-bed ion exchange resin (20–50 mesh, Bio-Rad) several times with deionized water, and add it to the radioiodinated ZLLY-DMK solution (*see* **Note 4**).
5. Resuspend the mixture briefly about once every 2 min for at least 10 min, and permit the resin to settle by gravity.
6. Remove the supernatant immediately and use it in calpain labeling studies (*see* **Note 5**).

3.2. Preparation of P-1 Antibody Gel

Immobilization of P-1 antibody and subsequent washing steps are carried out at 4°C.

1. Add a sample of 0.5–1 mg of P-1 antibody in storage buffer (50 m*M* imidazole-HCl, 50% glycerol, pH 7.4) to a 1:1 slurry of Protein A/G Agarose (Pierce) equilibrated in antibody gel second wash buffer, at an antibody to gel ratio of 1 mg/mL (*see* **Note 6**).

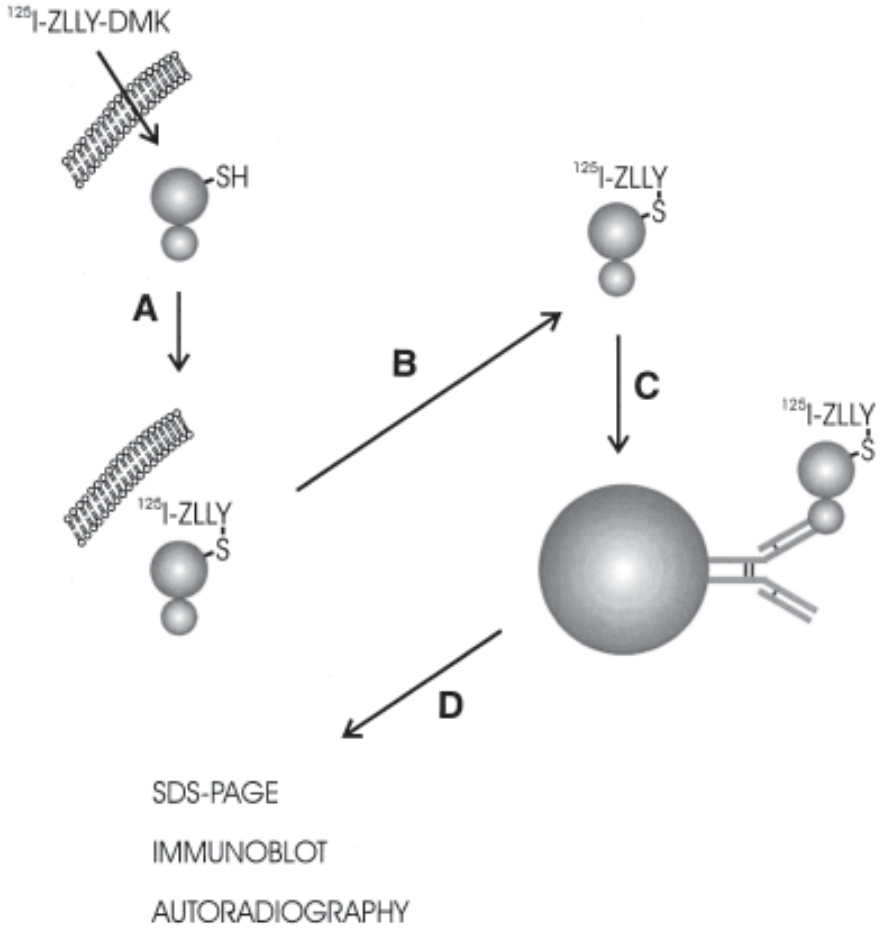

Fig. 2. Scheme depicting isolation of active site-labeled calpains from cells incubated with [^{125}I]ZLLY-DMK. Radioiodinated ZLLY-DMK is added to the culture medium and penetrates the plasmalemma. Activated calpains become covalently labeled with the inhibitor (**A**). Cells are lysed and a cell lysate supernatant is prepared (**B**). The calpain is immunoprecipitated by the addition of P-1 antibody gel (**C**), and the immunoprecipitate is extensively washed (**D**) before analysis.

2. Mix the sample for at least 2 h in a 1.5–2.0 mL microcentrifuge tube in a rotating mixing apparatus.
3. Wash the gel at least four times, using 1 mL of antibody gel second wash buffer per wash, by gentle mixing followed by centrifugation for 1 to 2 min at 2000*g*.
4. Suspend the gel as a 1:1 slurry in this buffer (*see* **Note 7**).
5. The washed P-1 antibody gel can be stored at refrigerator temperatures for at least a month before use provided that 0.04% sodium azide is added to prevent microbial growth.

3.3. Labeling of Calpains in Cultured Fibroblasts With [^{125}I]ZLLY-DMK

1. Grow WI-38 diploid human fibroblasts in 25 cm^2 flasks in a 5% CO_2 atmosphere at 37°C. The culture medium is Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum.
2. Make the cells quiescent by serum depletion for 3 to 4 d.
3. Stimulate the cells to reenter the cell cycle by addition of 10% fetal bovine serum, or individual growth factors.
4. At various times after the mitogenic signal, add [^{125}I]ZLLY-DMK to the culture medium to a final concentration of 0.1 to 0.2 µ*M* (*see* **Note 8**).
5. After 4 h of further incubation, wash the cells two times with 5 mL of 37°C Hanks buffered salts solution. A large proportion of the total radioactivity is removed by this treatment, and care should be taken to prevent contamination of the experimenter or the environment.

3.4. Immunoprecipitation

1. Place the flasks containing the washed cells on ice, remove the final wash buffer, and add 0.5 ml of cold cell lysis buffer. All subsequent steps are performed at 4°C or on ice.
2. Harvest the cells by scraping into the lysis buffer, transfer the lysates to plastic microcentrifuge tubes, and homogenize them using a small plastic pestle.
3. Centrifuge the samples at 7000*g* for 5 min, and mix each supernatant with 40 µL of a 1:1 slurry of P-1 antibody gel **(Subheading 3.2.)** in "dolphin nose" microcentrifuge tubes (*see* **Note 9**).
4. Incubate the 7000*g* supernatants with the P-1 antibody gel over the course of 3 to 4 h with constant mixing by sample inversion on a rotating mixer.
5. Wash the antibody gel once with antibody gel first wash buffer, and at least twice more with antibody gel second wash buffer (*see* **Note 10**).
6. Carefully aspirate the final wash buffer, add 20 µL each of water and SDS gel sample buffer to each gel sample, and heat the mixtures at 100°C for 3 min.
7. Centrifuge the samples at 10,000*g* for 2 min, and remove the supernatants for SDS-PAGE, immunoblotting, and autoradiography.
8. Generally, a protein blot is prepared and stained by a colorimetric immunochemical method for calpain. The blot is then subjected to autoradiography, and the immunostain and autoradiogram directly compared to show comigration of calpain immunoreactivity and radioactivity (*see* **Note 11**).

4. Notes

1. ZLLY-DMK can be stored desiccated in the dark at ambient temperature for at least 6 mo without losing activity.
2. The P-1 monoclonal antibody to the calpain small subunit was derived from Balb/C mice inoculated with purified bovine myocardial m-calpain. It does not recognize calpain large subunits ***(10)***, but recognizes bovine, human, and chinese hamster calpain small subunits on protein immunoblots. It is assumed therefore that it

will immunoprecipitate both μ-and m-calpains with equal efficiency. It will immunoprecipitate calpains from human or hamster cell lines, and presumably from bovine cell lines, although this has not been tested. It will immunoprecipitate purified bovine m-calpain ***(11)***. Calpains remain active in the P-1 immunocomplex so that their activity can be directly measured by the standard ^{14}C-casein assay ***(11,12)***.

3. Because significant quantities of ^{125}I are utilized, the labeling procedure should be performed in a laboratory hood that has been certified for radioiodine use by the institutional radiation safety office, and all applicable safety measures required by law for the handling of isotopes should be adopted.
4. The resin desalts the solution, thus decreasing the potential for nonspecific labeling of proteins by residual ^{125}I iodide in the radiolabeled ZLLY-DMK preparation.
5. The radiolabeled ZLLY-DMK should be used within 1–2 hours after preparation for *in situ* labeling studies. Samples stored at –20°C for 1–2 wk were still capable of radiolabeling purified calpain, but longer storage appears to result in loss of labeling potential.
6. Generally 0.5–1 mL of P-1 antibody gel is prepared at a time. The final concentration of glycerol carried over from the stock antibody solution is <10 %, and under these conditions the glycerol does not seem to affect binding of antibody to the gel.
7. The first supernatant contains unbound P-1 antibody, and can be saved for subsequent P-1 gel preparation, or for other uses.
8. There is significant inhibition of cell proliferation by 1 to 5 μ*M* ZLLY-DMK in culture medium ***(13,14)***. To minimize effects on cell growth, it is probably wise to utilize <1 μ*M* ^{125}I-labeled ZLLY-DMK when radiolabeling calpain in cell cultures.
9. When prepared as described, a 20-μL sample of packed P-1 gel is sufficient to immunoprecipitate almost all calpains present in confluent 25 cm^2 flasks of human fibroblasts. Lesser quantities of P-1 gel may be sufficient, but technical problems in handling smaller gel samples greatly increase the variability observed in replicate samples. To aid in pipeting the P-1 gel slurry, ~2 mm is trimmed from standard pipet tips (for 2–100 μL pipettors, "yellow tips"). To ensure uniformity of gel transfer to replicates, the same trimmed tip is used for all samples. Before pipetting into sample tubes, the tip is precoated with gel by pipetting once and redispensing into the stock gel slurry. The slurry is remixed by gently flicking the bottom of the stock gel tube by hand before dispensing each sample. The "dolphin nose" type of microcentrifuge tubes (Dot Scientific, Burton, MI, Catalog number RN2005-GMT) is useful for visualizing the small gel pellet, and reducing the risk of pellet loss during the wash procedures.
10. The initial wash with antibody gel first wash buffer reduces the amount of ^{125}I-labeled proteins nonspecifically bound to the P-1 gel. This may be attributable to the Triton X-100 in the buffer, because a similar wash step with buffer without the detergent did not appear to decrease contaminants effectively **(Fig. 3)**. Note that including nonradioactive calpain in the cell lysate prior to adding P-1 gel

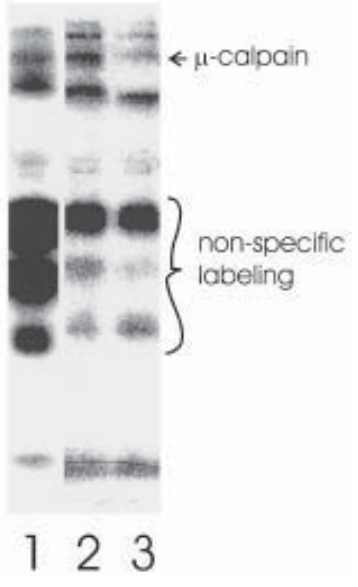

Fig. 3. Immunoprecipitation of radiolabeled μ-calpain by the P-1 antibody gel. Calpains in late G_1 WI-38 fibroblasts were labeled with [^{125}I]ZLLY-DMK by the procedure described in **Subheading 3.** Immunoprecipitates were prepared, washed, and subjected to SDS-PAGE, blotting, and autoradiography. The immunoprecipitate in lane 2 was washed as described in **Subheading 3.** The sample in lane 1 was washed the same number of times as lane 2, except that Triton X-100 was omitted from the antibody gel first wash buffer. Note the abundance of nonspecific labeling bands. The sample in lane 3 was treated the same as that in lane 2, except that 20 μg of purified calpain was added to the cell lysate before addition of P-1 antibody gel. Note that addition of the nonlabeled calpain greatly decreased the immunoprecipitation of labeled μ-calpain.

greatly decreased the intensity of the μ-calpain band, demonstrating the specificity of immunoprecipitation **(Fig. 3,** lane 3). The intensity of the lower molecular weight bands was not altered, indicating that they are nonspecifically bound to the gel.

11. Because calpains are not present in large quantities in cells, and the protocol employed is not designed to achieve maximum labeling, autoradiographic exposure for at least 1 d is required to detect label. Exposure for 1 or 2 wk is sometimes necessary to produce publication-quality data. The study depicted in **Fig. 4** demonstrates the utility of the procedure for detecting activated calpains at various times in G_1 phase of the WI-38 fibroblast cell cycle. Note the paucity of labeling in early G_1 fibroblasts **(Fig. 4,** lane 1). This is consistent with other studies indicating that inhibition of calpains influences late G_1 events, but has little effect in early G_1 ***(13)***.

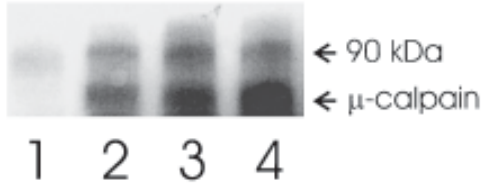

Fig. 4. Activation of μ-calpain during progression through G_1 phase. Serum-depleted (quiescent) WI-38 fibroblasts were released from growth inhibition by addition of fetal bovine serum. At various times thereafter, [^{125}I]ZLLY-DMK was added to the medium, and the samples were processed for analysis of calpain activation as described in the Methods section. *Lane 1*, early G_1 (0–4 h); *lane 2*, 6–10 h after serum addition; *lane 3*, 14–18 h after serum (late G_1); lane 4, 22 to 26 h after serum addition (early S-phase). Note that activation of calpain was first evident at 6 to 10 h after serum addition, and was more pronounced at late G_1 and early S-phase. Note also that a 90 kDa immunoprecipitated band was labeled in the same fashion. Unlike lower molecular weight bands, the 90 kDa protein seems to bind specifically to the P-1 gel (**Fig. 3,** compare lanes 2 and 3), and may be a novel calpain form.

Acknowledgments

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References

1. Rosser, B. G., Powers, S. P., and Gores, G. J. (1993) Calpain activity increases in hepatocytes following addition of ATP. *J. Biol. Chem.* **268,** 23,593–23,600.
2. Edelstein, C. L., Wieder, E. D., Yaqoob, M. M., Gengaro, P. E., Burke, T. J., Nemenoff, R. A., and Schrier, R. W. (1995) The role of cysteine proteases in hypoxia-induced rat renal proximal tubular injury. *Proc. Natl. Acad. Sci., U.S.A.* **92,** 7662–7666.
3. Saido, T. C., Nagao, S., Shiramine, M., Tsukaguchi, M., Sorimachi, H., Murofushi, H., Tsuchiya, T., Ito, H., and Suzuki, K. (1992) Autolytic transition of mu-calpain upon activation as resolved by antibodies distinguishing between the pre- and post-autolysis forms. *J. Biochem. (Tokyo)* **111,** 81–86.
4. Croall, D. E., Slaughter, C. A., Wortham, H. S., Skelly, C. M., DeOgny, L., and Moomaw, C. R. (1992) Polyclonal antisera specific for the proenzyme form of each calpain. *Biochim. Biophys. Acta* **1121,** 47–53.
5. Schoenwaelder, S. M., Kulkarni, S., Salem, H. H., Imajoh-Ohmi, S., Yamao-Harigaya, W., Saido, T. C., and Jackson, S. P. (1997) Distinct substrate specificities and functional roles for the 78-and 76-kDa forms of mu-calpain in human platelets. *J. Biol. Chem.* **272,** 24,876–24,884.

6. Zhang, W., Lane, R. D., and Mellgren, R. L. (1996) The major calpain isozymes are long-lived proteins. *J. Biol. Chem.* **271,** 18,825–18,830.
7. Molinari, M., Anagli, J., and Carafoli, E. (1994) Ca^{2+}-activated neutral protease is active in the erythrocyte membrane in its nonautolyzed 80-kDa form. *J. Biol. Chem.* **269,** 27,992–27,995.
8. Anagli, J., Hagmann, J., and Shaw, E. (1991) Investigation of the role of calpain as a stimulus-response mediator in human platelets using new synthetic inhibitors. *Biochem. J.* **274,** 497–502.
9. Anagli, J., Hagmann, J., and Shaw, E. (1993) Affinity labelling of the Ca^{2+}-activated neutral proteinase (calpain) in intact human platelets. *Biochem. J.* **289,** 93–99.
10. Mellgren, R. L. and Lane, R. D. (1988) Myocardial calpain 2 is inhibited by monoclonal antibodies specific for the small, noncatalytic subunit. *Biochim. Biophys. Acta* **954,** 154–160.
11. Zhang, W. and Mellgren, R. L. (1996) Calpain subunits remain associated during catalysis. *Biochem. Biophys. Res. Commun.*, **227,** 890–896.
12. Mellgren, R. L., Repetti, A., Muck, T. C., and Easly, J. (1982) Rabbit skeletal muscle calcium-dependent protease requiring millimolar Ca^{2+}. *J. Biol. Chem.* **257,** 7203–7209.
13. Zhang, W., Lu, Q., Xie, Z. -J., and Mellgren, R. L. (1996) Inhibition of the growth of WI-38 fibroblasts by benzyloxycarbonyl-Leu-Leu-Tyr diazomethyl ketone: evidence that cleavage of p53 by a calpain-like protease is necessary for G_1 to S-phase transition. *Oncogene* **14,** 255–263.
14. Mellgren, R. L. (1997) Evidence for participation of a calpain-like cysteine protease in cell cycle progression through late G_1 phase. *Biochem. Biophys. Res. Commun.* **236,** 555–558.

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Calpains and Myogenesis

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1. Introduction

There is now a considerable literature suggesting that the calpain/calpastatin system may be involved in myogenesis ***(1–7)***. Primary myoblasts in culture at first proliferate, but then usually cease to divide, in response to complex signals. The cells then migrate, align, and fuse to form the multinucleated syncytium or myotube, a process that clearly involves major remodeling of the cell membrane ***(1)***. Many factors such as growth factors, hormones, calpain antibodies, and inhibitors may be added to the culture system to investigate the mechanisms of cell fusion. The emphasis in this chapter is, however, on the use of antisense oligonucleotides based on calpain or calpastatin cDNA sequences to investigate the role of this protease system in the cell fusion process ***(1,3)***. At the present time, this appears to represent the only reported use of antisense methods in the calpain field. We describe here protocols for working with myoblast cultures that permit investigation of the roles of calpain and calpastatin in myogenesis, in carefully controlled and highly reproducible conditions. The use of reverse transcriptase-polymerase chain reaction (RT/PCR) methods to quantify mRNA levels of members of the calpain system in these myoblast cultures has been described elsewhere ***(1)***.

2. Materials

1. Growth medium: 9 g/L minium essential medium (MEM) with Earle's salts and L-glutamine, 3 g/L medium 199, 2.5 g/L $NaHCO_3$, 10,000 U/L penicillin, 10 mg/L streptomycin (Eurobio, France), 10% (v/v) horse serum (Boehringer, Gagny, France).
2. Serum-free medium: 9 g/lL MEM with Earle's salts and L-glutamine, 3 g/L medium 199, 2.5 g/L $NaHCO_3$, 10,000 U/L penicillin, 10 mg/L streptomycin, 10 µg/mL

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insulin, 1% bovine serum albumin (BSA).
3. Calpain inhibitor II (*N*-acetyl-leucyl-leucyl-methioninal), insulin (Boehringer).
4. Insulin-like growth factor-1, transforming growth factor-beta (IGF-1, TGF-β), (GIBCO-BRL).
5. Creatine kinase Merckotest kit (Merck, Nogent sur Marne, France).
6. [*methyl*-^{3}H]Thymidine (82 Ci/mmol, Amersham, Aylesbury,U.K.).
7. Vimentin antibodies (Sigma).
8. Purified oligodeoxyribonucleotides were purchased from Eurogentec (Belgium).
9. Four-well plates (Nunc) and 35-mm culture dishes (Falcon, Los Angeles, CA) (Poly-Labo, France).
10. Hansen's hemalun is prepared as follows: dissolve 1 g of hematoxylin in 100 mL of boiling water; dissolve 20 g of potassium aluminum sulfate ($KAl(SO_4)_2.12H_2O$) in 200 mL of boiling water; leave at room temperature for 24 h, mix both solutions and add 0.177 g of $KMnO_4$; boil the mixture for 1 min; cool and filter.

3. Methods

3.1. Cell Culture

3.1.1. Preparation of Cells From Muscle

1. Dissect the hind limb muscles from 18-d-old rat embryos (Wistar), and permit to autolyze for 1 h at 4°C.
2. Mince the muscles with scissors, and disrupt the tissue by aspiration through a pasteur pipet.
3. Filter dissociated cells through a 70 µm nylon filter and suspend the cells in growth medium.
4. Preplating is carried out in order to isolate myoblasts: leave the cell suspension on gelatin-coated dishes (1%) for 1 h at 37°C.
5. After 1 h, seed the unattached cells at a density of 5×10^5 viable cells /35 mm culture dish in growth medium.
6. Maintain the cells at 37°C under a continuous 9% CO_2 stream for 8 d to obtain differentiating myotubes. Replace the growth medium on day 4 of culture.
7. Assess contamination by fibroblasts (*see* **Note 1**) by:
   a. light microscopy after Giemsa staining which permits differentiation of myoblasts from fibroblasts on the basis of morphology;
   b. immunohistochemistry with a specific marker of myoblasts, such as desmin antibody ***(8)***.
8. Usually on days 6 and 8 of cell culture, remove the large muscle mass mechanically from the thin layer of underlying fibroblasts. This tissue is used to quantify µ-and m-calpain activity (**Subheadings 3.4.** and **3.5.**).

3.1.2. Measurement of Cell Fusion

1. Rinse cultured cells in PBS and fix in 4% paraformaldehyde in PBS for 15 min.
2. Stain in Hansen's hemalun.

3. In 10–12 randomly selected fields in each well, count total nuclei in single myoblasts and in myotubes, and count nuclei in myotubes alone. Perform the count in four separate wells, and in three culture plates.
4. Fusion (%) is expressed as (number of nuclei in myotubes/total number of nuclei in single myoblasts and in myotubes) × 100 (*see* **Note 2**).
5. Fusion inhibition is expressed as (1–[% fusion in treated cultures /% fusion in control cultures]) × 100.

3.1.3. Measurement of Cell Proliferation

1. Add [*methyl*-^{3}H]thymidine (0.1 µCi/mL) to the growth medium at the time of seeding.
2. At appropriate times, wash cells in PBS and solubilize them in 0.5 mL of 0.5 *M* NaOH per 35-mm dish.
3. Measure protein concentration with the Bradford assay, with BSA as standard.
4. Determine the radioactivity in the sample by liquid scintillation counting.
5. Express the results in disintegrations per minute ^{3}H / mg protein.

The progress of cell proliferation and fusion in cultures seeded at 5×10^5 cells/35 mm dish under our culture conditions is shown in **Fig. l** ***(2)***. In these conditions, the majority of the cells initially exhibit the bipolar morphology characteristic of presumptive myoblasts: cell alignment is extensive at 40–44 h and fusion is complete after 120 h.

3.1.4. Cell Differentiation

1. Wash the cells in PBS, resuspend them in 50 m*M* Tris-HCl, pH 7.5, 3 m*M* diadenosine 5'-pentaphosphate, 1m*M* PMSF, and sonicate.
2. Measure the increase in creatine kinase activity with a Merckotest kit ***(9)***.

3.1.5. Immunocytochemistry and Cytological Staining

1. Grow cells for immunocytochemistry on glass cover slips coated with 1% gelatin in 35 mm culture dishes.
2. Fix cultured cells with 4% paraformaldehyde in PBS solution for 15 min at room temperature, rinse the cells three times with PBS solution and permeabilize them with 0.2% Triton X-100 in PBS for 15 min.
3. Wash in PBS, and incubate overnight at 4°C with monospecific antibodies directed against specific rat muscle antigens diluted in phosphate buffer containing 1% BSA.
4. Rinse for 5 min in PBS solution and incubate with peroxidase-labeled anti-IgG (diluted 1:200), or with FITC-conjugated anti-IgG (diluted 1:50) for 1 h at room temperature.
5. Wash in PBS and immerse the samples in 0.05% diaminobenzidine in Tris-HCl, pH 7.5, containing 0.02% H_2O_2. Monitor the reaction, wash the cells, and mount them for light-microscopy. When using FITC-labeled antibodies, the preparations are examined with an epiilluminated fluorescence microscope.

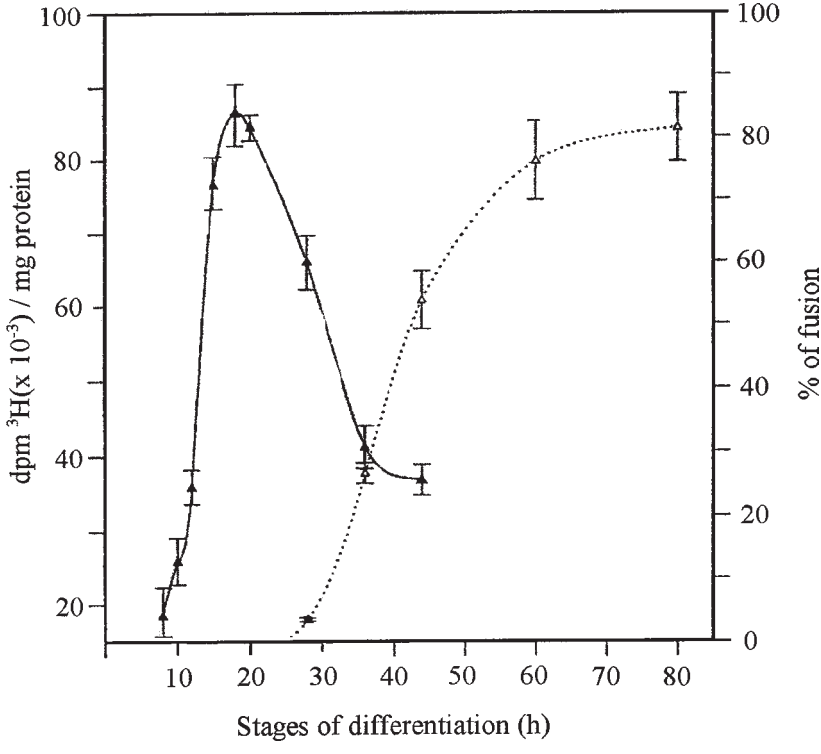

Fig. l. Proliferation and fusion of cultured primary rat myoblasts. Primary myoblasts were grown in growth medium. Cell fusion (△) was measured as in **Subheading 3.1., step 2**, and cell proliferation (▲) as in **Subheading 3.1., step 3.** Values are means ± SEM (n = 3 different cultures).

6. To follow cell differentiation by morphology in control and treated cultures, muscle cells are fixed as described above, stained with Hansen's hemalun for 8 min and observed under a light microscope.

3.2. Culture With Added Factors

At 20 hours after the initial myoblast plating, remove the growth medium and replace with serum-free medium containing insulin (10 µg/mL). This medium enhances myoblast fusion (*see* **Note 3**). Two types of experiments can be conducted. In the first case, control and treated cultures are fixed and stained after a 24-h period of treatment to examine the effects of different concentrations of added factors on the rate and extent of fusion. In the second case, in order to determine the fusion kinetics of myoblasts, cultures are treated with selected concentrations of added factors and are washed and fixed at different stages of early myogenesis (28, 36, 44, and 60 h after initial plating) to assess the rate of fusion ***(2)***.

3.2.1. Specific Factors

In each case, control cultures are set up in identical conditions, but without the added factor.

1. Leupeptin, 15, 30, and 60 µg/mL.
2. Anti-m-calpain IgG, 0.25, 0.5, 1.0, and 1.5 µg/mL. In addition to controls without any antibodies, controls with antibodies to vimentin (0.05, 5 µg/mL) or antibodies to µ-calpain are included **(Note 4)**.
3. Calpain inhibitor II, (*N*-acetyl-leucyl-leucyl-methioninal), 1.25, 2.5, 5, and 10 µg/mL.
4. TGF-β, 5 ng/mL.
5. Measure the extent of myoblast fusion.

3.2.2. Reversibility Test

1. 24 h after addition of specific factors, wash the cultures with prewarmed PBS solution for 1 min.
2. Replace the buffer with serum-free medium and grow for 24 h at 37°C.
3. Measure the extent of myoblast fusion.

3.3. Treatment With Antisense Oligonucleotides

Oligonucleotides are added at 15 h after initial plating of the cells, in concentrations from 5–40 µ*M*, and incubated for 20–40 h. Proliferation of the cells can be checked by incubation simultaneously with [*methyl*-^{3}H]thymidine (0.1 µCi/mL), and the progress of fusion is assessed as in **Subheading 3.1., step 2.** (*see* **Note 5**). The oligonucleotides that have been used in our work are listed in **Tables 1** and **2**.

3.4. Quantification of µ-and m-Calpain by Immunoblotting

1. Solubilize the contents of one or two dishes in 150 µL of SDS gel sample buffer.
2. Run ~100 µg of protein per well on 1.5 mm 4% stacking- and 10%-resolving-SDS polyacrylamide gels and transfer the proteins to nitrocellulose membranes.
3. Incubate the blots with IgG monospecific for µ-or m-calpain (*see* **Note 6**).
4. Wash, and incubate the blots with horseradish peroxidase-conjugated rabbit anti-goat IgG.
5. Develop the blots with enhanced chemiluminescence (Highlights-Amersham kit).
6. The densities of the resultant bands are estimated by scanning (λ = 555 nm) with a densitometer (CAMAG-TLC) connected to an integrator-recorder (CAMAG-SP 4400).

3.5. Quantification of µ-and m-Calpain Activity by Enzyme Assay After Ion-Exchange Separation

Calpain activities are assayed after separation on an ion-exchange column, using FITC-casein as a substrate and measuring the fluorescence of a 2.5% trichloroacetic acid supernatant.

Table 1
Synthetic μ- and m-Calpain Oligonucleotides

Abbreviation	Sequence (5'–3')	Target
m-Calpain–antisense	TGC TGA GGT GGA TGT TGG	Exon 12 of rat m-calpain
m-Calpain–sense	CCA ACA TCC ACC TCA GCA	Exon 12 of rat m-calpain
μ-calpain–antisense	ATT GTG CCC GAG AAG CAT	Exon 12 of rat μ-calpain
p94–antisense.	CGC GCA ATC AAT TCC CCC	IS I region of rat p94

See **ref. *10–12.***

Table 2
Synthetic Calpastatin Oligonucleotides

GGC AGG AGC GGT TTT CCA	Antisense	Calpastatin–domain III
TGG AAA ACC GCT CCT GCC	Sense	Calpastatin–domain III
CCC TTC CTT TGG CTT TCC	Antisense	Calpastatin–L domain
GGA AAG CCA AAG GAA GGG	Sense	Calpastatin–L domain

See **ref. *13,*** with permission.

1. Detach cells in 50 m*M*-Tris-HCl, pH 7.5, 2 m*M* EDTA, 2 m*M* EGTA, 4 m*M* DTT, 1 m*M* NaN_3. About 20 to 40 dishes are required, according to the progress of differentiation, to obtain about 5 mg of protein.
2. Sonicate with an Ultra-Turrax, for 3 × 15 s.
3. Centrifuge for 20 min at 6000 g.
4. Adjust the supernatant to 1 *M* NaCl and pH 7.5 with Tris base, and filter through gauze.
5. Apply the supernatant to a phenyl-Sepharose column (0.5 × 3 cm).
6. Calpastatin is not retained, and may be assayed in the nonretained fractions; μ-and m-calpain are eluted with 25 m*M* Tris-HCl, pH 7.5, 2 m*M* EDTA, 4 m*M* DTT.
7. Load the fractions containing μ-and m-calpain onto a diethylaminoethyl (DEAE-TSK) (Merck) column (0.5 × 2.5 cm; μ-and m-calpain are recovered at ~0.15 and ~0.25 *M* NaCl, respectively.
8. Add 50 μL of enzyme to 50 μL of substrate (FITC-casein 6.75 mg/mL, 250 m*M* KCl, 250 m*M* Tris-acetate, pH 7.5, 8 m*M* 2-ME) and start the reaction by addition of 25 μL of 25 m*M* $CaCl_2$. Run control assays without added calcium.
9. After incubation at 25°C for 30 min, add 125 μL of 2.5% (w/v) TCA.
10. Centrifuge, and dilute 200 μL of the TCA-supernatant with 800 μL of 0.3 *M* phosphate buffer, pH = 8.5.
11. Measure the fluorescence intensity (λ_{Em} = 518 nm, λ_{Exc} = 490 nm).
12. One fluorescence unit (FU) of calpain activity corresponds to the amount of enzyme required to increase the fluorescence of the 2.5% trichloroacetic acid-supernatant by 1 unit in the given conditions, compared to a control incubation without calcium.

3.6. Calpastatin Activity Quantification

The nonretained fractions from the phenyl-Sepharose CL-4B column are collected, pooled, concentrated 10-fold by ultrafiltration on YM 10 membrane (Diaflo Amicon, Epernon, France), and dialyzed against the extraction buffer.

1. Mix 50 µL of the dialyzed solution with 50 µL of standard m-calpain solution (40×10^{-3} FU/ µL) and 50 µL of FITC-casein (6.75 mg/mL).
2. Set up control assays lacking the calpastatin solution.
3. Start reaction by addition of 25 µL of 35 m*M* $CaCl_2$.
4. Measure the residual calpain activity as described above and compare it to the control assay.
5. One calpastatin fluorescence unit is defined as the amount of calpastatin that inhibits 1 FU of m-calpain.

4. Notes

1. Fibroblasts represent only a minor population of cells at the beginning of the myoblast culture.
2. In each culture well, 10 to 12 randomly selected fields are counted, (a total of ~2000 nuclei), and four separate wells are evaluated, in three separate cultures, for a total of ~24,000 nuclei.
3. All treatments are performed 20 h after initial plating because, at this stage, the treatments will not affect myoblast proliferation, and because it is certain that myoblast fusion will not have started at this time.
4. An antibody such as that against vimentin is used to control whether added IgG affects myoblast fusion. Antibodies to µ-calpain are used as a control since µ-calpain is not detected in fusing myoblasts (***2,5–7***).
5. The oligonucleotides used had sequence homologies below 40% when compared to mRNAs of other members of the calpain family. No identical sequences were found in GenBank when searched at the time of the experiments. To avoid degradation of the oligonucleotides which may occur in the presence of horse serum, treatments are carried out in serum-free medium. In our hands, maximum inhibition of fusion of 67% was reached at 30 µ*M* concentration of the m-calpain antisense oligonucleotide ***(3)***. The calpastatin antisense oligonucleotide gave equal effects at 5, 15, and 30 µ*M* ***(1)***.
6. Antisera against highly purified rabbit m-and µ-calpains were obtained from goats, and purified to minimize cross-reaction with the other calpain isoform by two successive steps of affinity chromatography on columns of purified µ-and m-calpain coupled to cyanogen bromide (CNBr)-activated Sepharose 4B.

References

1. Balcerzak, D., Cottin, P., Poussard, S., Cucuron, A., Brustis, J. J., and Ducastaing, A. (1998) Calpastatin-modulation of m-calpain activity is required for myoblast fusion. *Eur. J. Cell Biol.* **75,** 247–253.

2. Cottin, P., Brustis, J. J., Poussard, S., Elamrani, N., Broncard, S., and Ducastaing, A. (1994) Ca^{2+}-dependent protease (calpains) and muscle cell differentiation. *Biochim. Biophys. Acta* **1223,** 170–178.
3. Balcerzak, D., Poussard, S., Brustis, J. J., Elamrani, N., Soriano, M., Cottin, P., and Ducastaing, A. (1995) An antisense oligodeoxynucleotide to m-calpain mRNA inhibits myoblast fusion. *J. Cell. Sci*. **108,** 2077–2082.
4. Dourdin, N., Brustis, J. J., Balcerzak, D., Elamrani, N., Poussard, S., Cottin, P., and Ducastaing, A. (1997) Myoblast fusion requires fibronectin degradation by exteriorized m-calpain. *Exp. Cell Res*. **235,** 385–394.
5. Kwak, K. B., Chung, S. S., Kim, O. M., Kang, M. S., Ha, D. B., and Chung, C. (1993) Increase in the level of m-calpain correlates with the elevated cleavage of filamin during myogenic differentiation of embryonic muscle cells. *Biochim. Biophys. Acta* **1175,** 243–249.
6. Kumar, A., Shafiq, S., Wagdaonkar, R., and Stracher, A. (1992) The effects of protease inhibitors, leupeptin and E64d, on differentiation of C2C12 myoblasts in tissue culture. *Cell Mol. Biol*. **38,** 477–483.
7. Schollmeyer, J. E. (1986) Role of Ca^{2+} and the Ca^{2+}-activated protease in myoblast fusion. *Exp. Cell Res.* **162,** 411–422.
8. Debus, E., Weber, K., and Osborn, M. (1983) Monoclonal antibodies to desmin, the muscle intermediate filament protein. *EMBO J*. **12,** 2305–2312.
9. Alterio, J., Courtois, X., Bechet, D., and Martelly, I. (1990) Acidic and fibroblast growth factor mRNA are expressed by skeletal muscle satellite cells. *Biochem. Biophys. Res. Commun*. **166,** 1205–1212.
10. Deluca, C. I., Davies, P. L., Samis, J. A., and Elce, J. S. (1993) Molecular cloning and bacterial expression of cDNA for rat calpain II 80 kDa subunit. *Biochim. Biophys. Acta* **1216,** 81–93.
11. Deluca, C. I., Samis, J. A., Graham, E. J., Back, D. W., and Elce J. S. (1991) Partial cDNA and genomic clones for the large subunit of rat calpains I and II. *FASEB J*. **5,** A 830.
12. Sorimachi, H., Imajoh-Ohmi, S., Emori, Y., Kawasaki, H., Ohno, S., Minami, Y., and Suzuki, K. (1989) Molecular cloning of a novel mammalian calcium-dependent protease distinct from both m-and mu-types: specific expression of the mRNA in skeletal muscle. *J. Biol. Chem*. **264,** 20,106–20,111.
13. Ishida, S., Emori, Y., and Suzuki, K (1991) Rat calpastatin has diverged primary sequence from other mammalian calpastatins but retains functionally important sequences. *Biochim. Biophys. Acta* **1088,** 436–438.

22

Calpastatin (the Endogenous Calpain Inhibitor) and Membrane Protein Degradation in Cell Fusion

Nechama S. Kosower and Sivia Barnoy

1. Introduction

Cell membrane fusion is a ubiquitous cellular process, mediating such phenomena as fertilization, muscle development, certain viral infections, and giant cell formation from macrophages. For membrane fusion to occur, the cell membranes must attach to each other, usually by means of interactions between membrane receptors. Increased cellular Ca^{2+} is required for the various fusion events, and changes occur in the cell membrane and cytoskeleton. Fusion of membranes occurs between the two phospholipid bilayers in regions that are poor in proteins (fusion-potent domains). It has been proposed that membrane protein degradation contributes to the disorganization of cell membrane and cytoskeleton in preparation for the fusion of the cell membranes ***(1–3)***.

Calpain isozymes are present in the cytoplasm of most cells, along with their endogenous specific inhibitor, calpastatin, and the calpain:calpastatin ratio varies among different tissues and species ***(4)***. We have shown that limited degradation by calpain of some cellular and membrane proteins occurs in cells undergoing fusion and is required for the fusion. We have found that the calpain:calpastatin ratio determines cell fusibility, using as experimental models both freshly prepared erythrocytes and myoblast cell lines grown in culture.

1.1. Erythrocyte Fusion

Fusion can be induced in erythrocytes by certain viruses and chemical agents ***(3,5,6)***. We used the membrane mobility agent A_2C (a reagent that promotes movement of cell membrane components, altered phospholipid organization,

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and Ca^{2+} entry into cells), to induce erythrocyte fusion ***(6)***. Erythrocytes of some avian and mammalian species fused easily when treated with A_2C and Ca^{2+}, whereas those of others did not. The A_2C-induced erythrocyte fusion required Ca^{2+} and was accompanied by limited membrane protein degradation; inhibition of protein degradation prevented erythrocyte fusion ***(7)***. Calpain was shown to be responsible for this protein degradation, and a correlation was found between erythrocyte fusion and the ratio of calpain to calpastatin **(Fig. 1)** ***(8)***. Rat erythrocytes, which fuse easily, contain an excess of calpain to calpastatin, whereas human erythrocytes, which do not fuse, contain an excess of calpastatin. These studies provided evidence that calpain-induced degradation of certain membrane proteins is required for membrane fusion and that the fusibility of cell membranes depends on the ratio of calpain to calpastatin.

1.2. Myoblast Fusion

The formation of skeletal muscle during embryonic development involves the differentiation and fusion of myoblasts to form multinucleated muscle cells, and these events can be followed in myoblasts grown in primary culture or as cultures of myoblast cell lines. Myoblast fusion is Ca^{2+}-dependent. We studied the calpain–calpastatin system during myoblast differentiation and fusion, using a line of myoblast cells (L8) that fuse, and a line of mutant cells (L87) that do not fuse.

In L8 cells, we found that the levels of μ-and m-calpain did not change significantly during myoblast differentiation and fusion, while the level of calpastatin diminished significantly before fusion and increased following fusion. Calpain activity was not detected in crude extracts of dividing myoblasts because of the higher levels of calpastatin, but was detected in myoblasts at the stage when the level of calpastatin was low ***(9)***. Both μ- and m-calpain activities could, however, be detected at any stage of growth and differentiation provided they were separated from calpastatin (as shown by casein zymography). Consistent with these results is our finding that calpain is associated with calpastatin in dividing myoblasts and in myoblasts at the beginning of differentiation. Such an association would prevent calpain activation. As a result of the diminution in calpastatin that occurs prior to fusion, calpain is no longer inhibited and can be activated. Myoblast fusion was inhibited when the L8 cells were treated with fusion inhibitors (transforming growth factor beta [TGFβ], EGTA), with cysteine protease inhibitors (calpeptin, E64d) and with the specific calpain inhibitor, calpastatin **(Fig. 2)** ***(10)***.

Calpain and calpastatin were also studied in mutant myoblasts that did not express myogenin and had lost their ability to fuse (line L87). The levels of μ-and m-calpain did not change in the mutant cells cultured in differentiation medium (a behavior similar to calpain behavior in the control line, L8). Calpastatin lev-

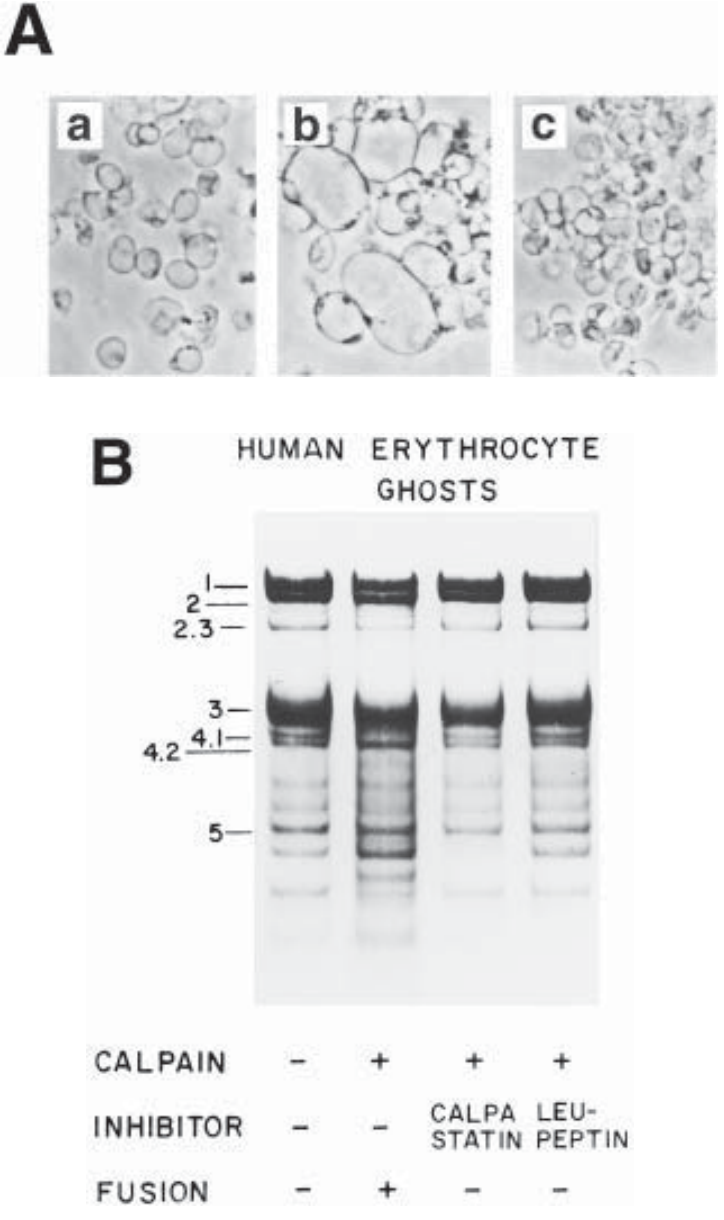

Fig. 1. Ca^{2+} and A_2C-induced fusion of human erythrocyte ghosts. Ghost suspensions were incubated with Ca^{2+}, with and without calpain, and with or without calpastatin, centrifuged, and reincubated with A_2C. **(A)** Micrographs showing fusing and nonfusing ghosts: **a**, without calpain; **b**, with calpain; **c**, with calpain and calpastatin. **(B)** SDS-PAGE of membrane proteins of fusing and nonfusing ghosts. (From **ref. *17***, with permission from Elsevier Science.)

els however also *did not change* in the nonfusing, mutant L87 cells (a behavior *different* from the fall in calpastatin observed in the fusing L8 cells) **(Fig. 3)**. These results indicate that the fall in calpastatin level before myoblast fusion is part of the myogenic program and depends on the expression of transcription factors that trigger the biochemical differentiation.

We observed a selective, limited degradation of some membrane and cytoskeletal proteins in fusing cells, but not in the nonfusing, mutant cells, nor when fusion was prevented by calpain and fusion inhibitors ***(11)***. Thus, as is the case in the erythrocyte fusion model, myoblast fusion appears to require calpain-induced proteolysis and to be dependent on the ratio of calpain : calpastatin. Overall, our results point to a central role of calpastatin in differentiation and fusion of myoblasts. These results may be relevant to other fusion events, as well as to cell membrane repair processes.

2. Materials

All materials are available from Sigma Chemical Co., St. Louis, MO, unless otherwise noted.

2.1. Erythrocyte Fusion

1. Heparinized blood from mammalian species (e.g., human, rat, mouse, rabbit, guinea pig).
2. 150 m*M* NaCl.
3. Acetate buffer: 75 m*M* sodium acetate, 70 m*M* NaCl, pH 5.6.
4. Erythrocyte fusion buffer: acetate buffer, 1 m*M* $CaCl_2$, 100 mg/mL dextran (T-70, Pharmacia, Uppsala, Sweden).
5. Ghost fusion buffer: 10 m*M* Tris-HCl, pH 7.4, 135 m*M* NaCl, 0.1 m*M* EGTA, 0.5 m*M* DTT, 1 m*M* $CaCl_2$, 40 mg/mL dextran.
6. 100 m*M* $CaCl_2$ (stored at 4°C).
7. 2 m*M* ionophore A23187 in acetonitrile (stored in small aliquots at –20°C).
8. 200 m*M* sodium phosphate buffer, pH 8, made by titrating 93 vol of 200 m*M* Na_2HPO_4 to pH 8 with about 7 vol of 200 m*M* NaH_2PO_4.
9. 5 m*M* phosphate buffer, pH 8.0 (5p8), diluted before use from 200 m*M* sodium phosphate.
10. 100 m*M* DTT (stored in small aliquots at –20°C).
11. Stock solutions of 200 m*M* EDTA and 200 m*M* EGTA, titrated to pH 7.4 and stored at 4°C.
12. A_2C suspension: place 1–3 µL A_2C in a glass tube and add 150 m*M* NaCl to give 0.5 µL of A_2C/mL 150 m*M* NaCl; sonicate the mixture for about 30 s with a micro tip (*see* **Note 1**).
13. Inhibitors, (stock solutions kept at –20°C): 100 m*M* leupeptin; 100 m*M* pepstatin; 100 m*M* *N*-tosyl-L-lysylchloromethylketone (TLCK); 200 m*M* 1,10-phenanthroline/50% ethanol; 100 m*M* *p*-aminoethyl™benzenesulfonyl fluoride (AEBSF);

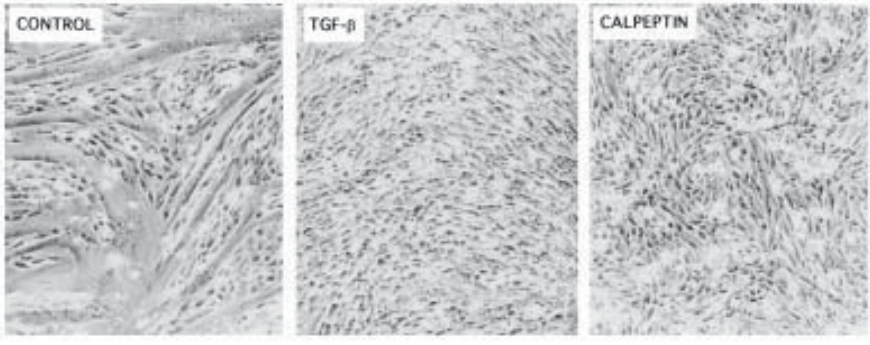

Fig. 2. Phase-contrast micrographs of rat L8 myoblasts. Myoblasts shown at 120 hours after change of growth medium to differentiation medium, without and with the addition of 0.4 n*M* TGFβ or 20 μ*M* calpeptin. (From **ref. *10***, with permission from Elsevier Science.)

100 m*M* monobromobimane (mBBr) in acetonitrile (*see* **Note 2**); 200 m*M* EGTA; 10 m*M* calpeptin in DMSO.

2.2. Myoblast Fusion

1. Myoblast cells (rat L8, mutant L87) (cell lines obtained from D. Yaffe, the Weizmann Institute of Science, Rehovot, Israel).
2. 100-mm plastic tissue culture Petri dishes, coated with 0.1% gelatin.
3. Growth medium: Waymouth medium, 15% (v/v) fetal calf serum, 1% (w/v) glutamine, 1% (v/v) antibiotics (diluted from a stock solution containing: 10,000 units of penicillin, 10 mg of streptomycin sulfate, 1250 U of nystatin/mL) (Biological Industries, Beit Haemek, Israel).
4. Trypsin solution (Ca^{2+}, Mg^{2+}-free, and without EDTA) (Biological Industries).
5. Differentiation medium: Dulbecco's modified Eagle's medium (DMEM), 2% (v/v) horse serum, 1% (w/v) glutamine, 1% (v/v) antibiotics (Biological Industries), 4 U insulin/mL (Actrapid HM, Novo Nordsik, Denmark).
6. Inhibitors (stock solutions kept at –20°C): 1 μ*M* TGFβ 1, 200 m*M* EGTA; 10 m*M* calpeptin in DMSO; 15 m*M* E64d in DMSO; human recombinant calpastatin, M_r 14,000 (Calbiochem, La Jolla, CA).
7. Isotonic phosphate buffer (PBS): 8.2 m*M* Na_2HPO_4, 1.8 m*M* KH_2PO_4, pH 7.4, 135 m*M* NaCl, 2.7 m*M* KCl.
8. Giemsa solution, diluted 1:10 in water from the commercial solution.
9. Lysis buffer: 20 m*M* Tris-HCl, pH 7.4, 150 m*M* NaCl, 1% Triton X-100, 2.5 m*M* EDTA, 2.5 m*M* EGTA, 0.1 m*M* AEBSF, 10 μg aprotonin/mL.
10. Immunoprecipitation buffer (IP buffer): 20 m*M* Tris-HCl, pH 7.4, 450 m*M* NaCl, 24% (v/v) glycerol, 0.2 m*M* EDTA, 0.2 m*M* AEBSF, 0.1 m*M* leupeptin, 0.02 m*M* pepstatin, 0.1 m*M* TLCK, 10 μg aprotonin/mL.
11. Protein A-Sepharose 4 FF (fast flow) (Pharmacia Biotech, Uppsala, Sweden).

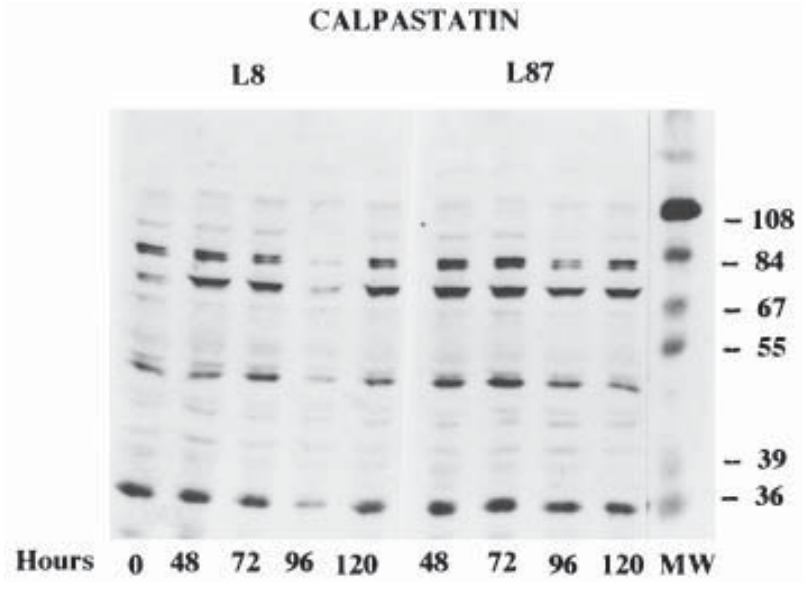

Fig. 3. Detection of calpastatin by immunoblotting. Fusing L8 and nonfusing L87 myoblasts were grown in differentiation medium.

2.3. Calpain, Calpastatin, Antibodies, Electrophoresis, and Immunoblot Materials

1. μ-Calpain, purified from human erythrocytes; m-calpain, purified from rabbit muscle; calpastatin, purified from human erythrocytes (*see* **Note 3**).
2. Calpain buffer ×10 (stock): 100 m*M* Tris-HCl, pH 7.4, 1 m*M* EGTA, 5 m*M* DTT.
3. Primary antibodies: monoclonal anti-μ-calpain antibody (anti-μ–calpain), that recognizes the large subunit of μ-calpain; goat anti-m-calpain antibody (anti-m-calpain), that recognizes the small and large subunits of m-calpain; rabbit anticalpain antibody ([anticalpain], raised against human erythrocyte μ-calpain), that recognizes the large subunits of both μ-and m-calpain; rabbit anticalpastatin antibody ([anticalpastatin], raised against human erythrocyte calpastatin). These antibodies have been prepared by us. Antibodies to calpain and calpastatin, and to various membrane and cytoskeletal proteins (e.g., talin, spectrin, β-integrin, actin, tubulin, tropomyosin) are available from commercial sources.
4. Secondary antibodies: rabbit anti-mouse IgG antibody for detection of primary mouse monoclonal antibodies; goat anti-rabbit IgG antibody for detection of primary rabbit antibodies; rabbit anti-goat IgG antibody for the detection of the primary goat antibody. All secondary antibodies are peroxidase-conjugated and available from commercial sources.
5. Materials for the estimation of calpain and calpastatin activities in solution:
   a. substrate solution A: 0.4% casein, 5 m*M* cysteine, 50 m*M* imidazole-HCl buffer, pH 7.5, 0.5 m*M* DTT, 2 m*M* $CaCl_2$;

b. Solution B: 375 m*M* Na_2CO_3, 0.1 *M* NaOH;
c. Solution C: 2 *M* sodium iodoacetate (freshly prepared before use by dissolving iodoacetic acid in 2 *M* NaOH);
d. Solution D: a mixture freshly made before use of: 100 vol of 280 m*M* Na_2CO_3, 0.1 *M* NaOH; 1 vol of 2% (w/v) $CuSO_4$,$5H_2O$; 1 vol of 4% (w/v) sodium potassium tartrate;
e. Solution E: Folin reagent, diluted 1:1 with water.

3. Methods

3.1. Erythrocyte Fusion

All procedures were carried out on ice or at 4°C, unless otherwise stated.

3.1.1. Preparation of erythrocyte suspensions:
1. centrifuge 1–5 mL of heparinized blood in a clinical centrifuge, at 1800*g* for 5 min;
2. remove the plasma and buffy coat;
3. wash the erythrocytes three times in 150 m*M* NaCl by gentle resuspension and centrifugation;
4. wash the erythrocytes once in acetate buffer and resuspend them in acetate buffer at room temperature to a hematocrit of 8% (*see* **Note 4**).

3.1.2. Preparation of erythrocyte membranes:
1. centrifuge erythrocyte suspensions and mix 1 vol of the sedimented cells with approx 40 vol of 5p8 buffer;
2. centrifuge at 24,000*g* for 10 min, then wash the pellet of erythrocyte ghosts three times with 40 vol of 5p8 buffer, and once with 40 vol of 10 m*M* NaCl (each time followed by the same centrifugation), to obtain hemoglobin-free erythrocyte membranes (white ghosts).

3.1.3. Preparation of membrane-free cytosol: centrifuge erythrocyte suspensions, mix the sedimented cells with approx 5 vol of 10 mM Tris-HCl buffer, pH 7.4, 1 mM EDTA, and centrifuge at 24,000g for 10 min to obtain the supernatant membrane-free cytosol (*see* **Note 5**).

3.1.4. Effects of Ca^{2+} on erythrocytes (*see* **Note 6**):
1. incubate erythrocyte suspensions in acetate buffer at 37°C for 30 min without (control) or with 25–50 μ*M* calpeptin, or with one of the following reagents at 1 m*M*: AEBSF, leupeptin, EDTA, pepstatin, TLCK, mBBr, 1,10-phenanthroline;
2. centrifuge control and reagent-treated erythrocyte suspensions and resuspend the pellets in acetate buffer at room temperature to a hematocrit of 4%;
3. add 0.5 m*M* $CaCl_2$ and 10 μ*M* ionophore (final concentrations) and incubate further at 37°C for 30 min;
4. stop reaction by adding EDTA (final concentration 5 m*M*), and place the erythrocyte suspensions on ice;
5. prepare erythrocyte membranes, solubilize in SDS gel sample buffer, and analyze by electrophoresis and immunoblotting.

3.1.5. Effects of A_2C on erythrocytes:
1. prepare control and reagent-treated erythrocyte suspensions, as described in

Subheading 3.1.4., step 1

2. centrifuge and resuspend the erythrocytes to a hematocrit of 8% in erythrocyte fusion buffer (*see* **Note 7**);
3. mix the cell suspensions with an equal volume of freshly prepared A_2C suspension (*see* **Note 8**), and incubate at 37°C for 10–60 min, with gentle mixing every 5–10 min;
4. remove aliquots at intervals for observation by light microscopy and photography, to estimate the degree of erythrocyte fusion;
5. stop reaction by addition of 5 m*M* EDTA and place the erythrocyte suspensions on ice;
6. prepare erythrocyte membranes, solubilize in SDS gel sample buffer, and analyze by electrophoresis and immunoblotting;
7. run two gels, one to be stained with Coomassie brilliant blue, the other for immunoblotting, to identify calpain and calpain autolytic products ***(13)*** (*see* **Note 9**).

3.1.6. Effect of Ca^{2+} and A_2C on reconstituted erythrocyte ghosts (*see* **Note 10**):

1. suspend erythrocyte white ghosts (**Subheading 3.1.2., step 2**) in 4 vol of ghost fusion buffer to yield a 10% ghost suspension containing 0.5 mg protein/mL, and place on ice (*see* **Note 11**);
2. preincubate a solution of calpain, or calpain and calpastatin in calpain buffer × 1 at room temperature for 15 min, and add it to the ghost suspension (final concentrations, 1–2 units of calpain, with or without 2–4 units of calpastatin/ml of 8% ghost suspension);
3. keep the mixtures on ice for 15 min, then incubate at 37°C for 30 min;
4. centrifuge the mixtures and resuspend the membranes in ghost fusion buffer to yield an 8–10% ghost suspension;
5. mix 1 vol of this ghost suspension **(step 4)** with 1 vol of A_2C suspension, and incubate at 37°C for 10–60 min (*see* **Note 12**);
6. examine aliquots microscopically for fusion;
7. stop reaction by addition of 5 mM EDTA and centrifuge the suspensions at 24,000*g* for 10 min;
8. solubilize the pelleted membranes and analyze by SDS-PAGE) **(Fig. 1B)**.

3.2. Myoblast Fusion (See Note 13.)

3.2.1. Myoblast growth (myoblast stocks are kept at –180°C in vials containing 1×10^6 cells/vial):

1. thaw myoblasts at 37°C, mix them with 10 mL of prewarmed growth medium, and centrifuge at 1,200*g* for 10 min;
2. resuspend the cells and place in gelatin-coated 10 cm Petri dishes in a total of 10 mL growth medium;
3. culture the cells at 37°C in air and 5% CO_2;
4. change the growth medium after 24 h;
5. when the number of myoblasts reaches about $4\text{-}6 \times 10^6$/dish, (usually at 48–72 h of culture for the L8 rat myoblast cell line, before confluency), remove the medium;

6. add 5 mL of 0.25% trypsin (without EDTA) per dish, and incubate for about 1 min at room temperature (*see* **Note 14**), remove the trypsin solution by aspiration.
7. release the cells by gentle tapping or agitation (if the cells are not released, incubate the plates for 2-3 min at 37°C, and agitate again by hand).
8. add fresh medium, count the cells in an aliquot, and divide the cells into new dishes. Aliquots of cells may be refrozen in DMSO. It is important to minimize the passage number.

3.2.2. Myoblast differentiation and fusion:

1. distribute L8 myoblasts from several Petri dishes into new Petri dishes ($0.5–1 \times 10^6$ cells per dish) and culture in growth medium until they are about 50% confluent (about $4–6 \times 10^6$/dish, at about 48–72 h);
2. change the medium to differentiation medium (the medium contains 1.8 m*M* $CaCl_2$); time 0 is defined as the time at which the medium is changed to differentiation medium; the medium is renewed every 48 h (*see* **Note 15**).

3.2.3. To study the effects of inhibitors, add inhibitors to the cultures at time 0, and replenish every 48 h, together with fresh differentiation medium. Final concentrations of the inhibitors are: 0.4 n*M* TGF-β, 1.5 m*M* EGTA, 20 μ*M* calpeptin, 30 μ*M* E-64d, 50 μg/mL calpastatin. To study certain aspects of differentiation, inhibitors can also be added at times later than time 0 (*see* **Note 16**).

3.2.4. To evaluate the extent of myoblast fusion, wash one dish every 24 h with PBS, examine by phase microscopy, and photograph the cells (**Fig. 2**).

3.2.5. Estimate the extent of fusion after fixing the cells in methanol (precooled to –20°C) and staining them with Giemsa. The percent of fused cells is estimated by counting nuclei in myotubes/total of 400–500 nuclei.

3.2.6. Preparation of myoblast extracts for the analysis of calpain, calpastatin, and membrane proteins:

1. wash one dish (two at time 0) three times with PBS;
2. treat the cell monolayer with 10 mL of 5 m*M* EDTA in PBS to release the cells;
3. transfer the cells to a 50 mL test tube, dilute with PBS to a final volume of 50 mL (diluting the EDTA to 1 m*M*), centrifuge, resuspend the cells in 40 μL of lysis buffer/1×10^6 cells, transfer the samples to 1.5-mL Eppendorf tubes, and leave on ice for 30 min (*see* **Note 17**);
4. centrifuge the samples at 8000*g* at 4°C (refrigerated Eppendorf), and measure the protein concentration in the lysate supernatant by standard methods (usual concentration is about 1 mg/mL); use aliquots for the various analyses.

3.2.7. Immunoprecipitation of calpain and calpastatin (for the study of calpain–calpastatin association in myoblasts) (*see* **Note 18**):

1. wash myoblasts in Petri dishes (four for time 0, two for 48 h) twice with PBS at room temperature, then scrape them off the dishes with a rubber policeman;
2. wash the cells once more with PBS and centrifuge them at 1200*g*;
3. suspend the cells in 15 μL of IP buffer/1×10^6 cells (usually 300 μL of IP buffer is added to ~ 20×10^6 cells);
4. transfer the cells to Eppendorf tubes and lyse them by quick freezing in dry

ice/ethanol and thawing at 30°C, repeated three times;
5. centrifuge lysed samples at 4°C for 15 min at 14,000*g*;
6. dilute the cell lysates to 150 m*M* NaCl, 8% glycerol, by adding two parts of IP buffer (without NaCl and glycerol) to one part of the cell lysate;
7. preclear aliquots of the diluted lysate (usually 3 aliquots of 300 µL each) by incubation for one hour at 4°C in the presence of 5 µL of normal rabbit serum and 15 µL of Protein A Sepharose per aliquot;
8. centrifuge at 4°C for 5 min at 14,000*g*;
9. incubate the recovered supernatants (~300 µL) with 5 to 10 µL of anticalpain or anticalpastatin, or preimmune serum for controls, by rotation at 4°C for 4 h;
10. add 30 µL of protein A Sepharose and incubate overnight at 4°C;
11. centrifuge at 14,000*g* for 10 min, wash the precipitates three times with IP buffer containing 150 m*M* NaCl (without glycerol) and resuspend them in 100 µL of the same buffer;
12. solubilize the immunoprecipitates in SDS gel sample buffer for gel electrophoresis.

3.2.8. Effects of calpain and calpastatin on myoblast proteins:
1. mix aliquots of myoblast lysates containing 50 µg protein with a buffer solution of 20 m*M* Tris-HCl, pH 7.4, 150 m*M* NaCl, 0.1 m*M* AEBSF, to a final volume of 200 µL;
2. incubate the samples for 15 min at 30°C in the presence of 2 m*M* $CaCl_2$, 0.5 m*M* DTT, with and without added calpain and with or without added calpastatin (0.5–1 unit of calpain and 1–2 units of calpastatin);
3. mix the samples with SDS gel sample buffer, heat, and analyze by gel electrophoresis.

3.3. Analysis of Calpain, Calpastatin, and Cell Membrane Proteins

1. SDS-PAGE: samples are solubilized in Laemmli sample buffer (one part of Laemmli's buffer to four parts of samples) and heated to 100°C for 3 min. Aliquots containing 10–30 µg of protein are used for electrophoresis. Electrophoresis is carried out on 10% polyacrylamide minislab gels by standard procedures. It is advisable to run two gels for each set of samples, so that one gel can be stained with Coomassie brilliant blue to visualize the protein bands, and the other one used for immunoblotting.
2. Immunoblotting is carried out by standard procedures. The working dilutions for each antibody have to be determined in each case. Detection of bands is carried out with the enhanced chemiluminescence (ECL) immunoblotting detection system (Amersham).
3. Casein zymography for detecting activities of µ-and m-calpain ***(12)*** (*see* Chapter 13). Aliquots of myoblast extracts are mixed with DTT (final concentration of 1 m*M* DTT) and with Laemmli's sample buffer lacking SDS (one volume of sample buffer to four volumes of cell extract). Aliquots (30–40 µL

containing 25–30 µg protein) are loaded onto the casein gel. Following electrophoresis, gels are incubated overnight in calpain activation buffer ***(12)*** containing 0.2 U calpastatin/mL, and then stained in Coomassie brilliant blue (*see* **Note 19**).

3.4. Estimation of Calpain Activity

For erythrocytes, calpain activity is estimated in cytosol samples partially purified by column chromatography to remove hemoglobin and calpastatin. For myoblasts, calpain is determined in lysates prepared in lysis buffer, and in extracts partially purified by column chromatography. The reagents used are described as a–e in **Subheading 2.3, step 5**:

1. Mix samples of 25–50 µL with 200 µL of substrate solution a to a final volume of 250 µL, and incubate at 30°C for 30 min; control samples are run with 1 mM EDTA in place of Ca^{2+}.
2. Stop the reaction by adding an equal volume of 5% TCA, vortex the samples, leave on ice for 10 min, and centrifuge.
3. Use the clear supernatants for analysis of acid-soluble, casein proteolytic products, by the method of Ross and Schatz ***(14)*** (*see* **Note 20**).
4. Mix 200 µL of the sample supernatant with 200 µL of solution b and 100 µL of solution c.
5. Incubate at room temperature for 30 min, add 1 mL of solution D, and incubate further for 10 min.
6. Add 0.1 mL of solution e, permit color to develop for 15 min, and record the absorption at 750 nm. One unit of calpain proteolytic activity is defined as that giving 1.0 unit of absorption at 750 nm.

3.5 Estimation of Calpastatin Activity

1. Heat membrane-free erythrocyte cytosol samples and myoblast extracts in lysis buffer at 100°C for 5 min to destroy calpain activity.
2. Centrifuge samples at 10,000*g* to remove denatured proteins.
3. Mix aliquots of the supernatants (10-40 µL), with 0.2–0.25 units of calpain and 5 µL of calpain buffer × 10 in a final volume of 50 µL buffer (final concentrations, 10 m*M* Tris-HCl, pH 7.4, 0.1 m*M* EDTA, 0.1 m*M* EGTA, 0.5 m*M* DTT); control samples contain no cell extract.
4. Incubate the mixtures for 10 min at room temperature, then estimate the activity of calpain, as described above; 1 U of calpastatin is defined as the amount that inhibits 1 U of calpain (*see* **Note 21**).

4. Notes

1. A_2C is an oil that can be readily dispersed in water and in the usual salt or buffer solutions. This suspension is prepared just before use, and consists of oil droplets about 0.5–1.0 µm in diameter.
2. mBBr is used for alkylation and fluorescent labeling of reactive thiols; a stock

solution of 100 m*M* mBBr/acetonitrile is kept at 4°C in the dark; the solution is stable for at least 3 mo.

3. The calpain and calpastatin listed here have been prepared by us; they are also available from commercial sources.
4. Acetate buffer, pH 5.6, has been used for studies of erythrocyte fusion ***(5,6)***, so that it is convenient to use the same buffer for erythrocyte preparation. In cases where only the effects of Ca^{2+} on erythrocytes are studied, other isotonic buffers can be used (e.g., 20 m*M* Tris-HCl, pH 7.4, 135 m*M* NaCl).
5. Rat hemoglobin tends to precipitate at pH 6–8 so that some modification of established methods are advisable for the preparation of rat erythrocyte ghosts and hemolysate. For the preparation of rat erythrocyte white ghosts, 5 m*M* phosphate buffer at pH 8.2–8.4 is used, instead of the usual buffer at pH 8.0, followed by wash with 10 m*M* NaCl **(Subheading 3.1. 2.)**. For the preparation of hemolysate (when used in reconstitution of ghosts in fusion experiments), 5 mM acetate buffer at pH 5.6 is used, followed by centrifugation to remove membranes and any precipitated hemoglobin.
6. This procedure allows the study of effects of increased cell Ca^{2+} in erythrocytes of various species, independent of fusion. For example, in human erythrocytes, Ca^{2+} induced the formation of bands of high molecular weight, due to activation of transglutaminase, with little proteolysis; in rat cells, significant membrane proteolysis was observed under the same conditions. Proteolysis was inhibited by cysteine protease inhibitors and by thiol alkylation, but not by metalloprotease or serine protease inhibitors ***(7)***.
7. Dextran in the erythrocyte fusion buffer promotes erythrocyte adhesion that is necessary for fusion to occur between cells that are fusible.
8. A_2C suspension tends to coalesce in time, and should be sonicated just before use.
9. Erythrocyte membrane protein degradation associated with fusion is visualized by Coomassie staining of the gel. It can also be analyzed by immunoblotting, using appropriate antibodies (e.g., antispectrin antibody, antiband 3 antibody; antiband 4.1 antibody). Immunoblotting is used to detect autolysis of μ-calpain that accompanies calpain activation (erythrocytes contain only μ-calpain) ***(13)***.
10. Reconstitution of erythrocyte ghosts with calpain/calpastatin allows evaluation of the fusibility of cell membranes, independent of the original calpain/calpastatin ratio in the cells. Cytosol and partially purified cytosol fractions can be added to the ghost reconstitution incubations to evaluate their contribution to fusion.
11. Less dextran is used for ghost fusion than for erythrocyte fusion, to avoid excessive clumping of ghosts.
12. Some types of ghosts (e.g., mouse erythrocyte ghosts) are sensitive to A_2C/Ca^{2+} and may disintegrate to small vesicles. The number of ghosts, and concentrations of Ca^{2+}, A_2C, and calpain, may have to be modified to achieve adequate ghost fusion.
13. Myoblast cell lines have some advantage over primary cell cultures, being a defined cell type that can be studied under reproducible conditions. However cell lines may differ in their growth and differentiation medium requirements, in their response to inhibitors, and in their rate of fusion. For example, mouse C2 myo-

blasts do not require insulin for differentiation and fuse within 48 h. Cells that usually fuse reproducibly, may lose their ability to fuse efficiently. The reasons for the change are not clear but may include too high a passage number, or changes in insulin requirements, and in batches of media and sera.

14. Trypsin without EDTA is used to release the myoblasts, since EDTA may cause aggregation in some cell types and interfere with subsequent cell counting.
15. Cell division initially continues in this medium and cells reach confluency at about 48 h; morphological changes usually become apparent at 72–96 h, with fusion and myotube formation at 96–144 h.
16. Several points concerning the use of inhibitors should be considered. Some inhibitors (TGF-β, EGTA) inhibit myoblast fusion by interfering with mRNA transcription (or stability) or translation of muscle-specific proteins ***(15)***. The cell-penetrating cysteine protease inhibitors (calpeptin, which is reversible, and E-64d, which is nonreversible), are assumed to act on cellular proteases including calpain; added calpastatin is assumed to enter cells as fragments. The inhibitors are normally added at time 0 (when growth medium is changed to differentiation medium), and replenished every 48 h. Variations on their use include addition only at later time points and/or removing the inhibitors, to study the differentiation stage at which a reagent inhibits myoblast fusion and reversibility.
17. Myoblast extracts can be prepared by means other than the use of Triton X-100, such as milder detergents, homogenization, or freezing and thawing, but with possibly lower yields. Extracts that are not used immediately should be stored at –90°C. Myoblasts can be solubilized directly in SDS gel sample buffer for gel analysis but not for immunoprecipitation or activity assays.
18. For immunoprecipitation studies of calpain–calpastatin association, the cells are removed from the dishes with a rubber policeman, rather than with EDTA solution, since EDTA may disrupt calpain–calpastatin association, and the extracts are prepared by freezing and thawing, to avoid possible detergent-induced dissociation. The IP buffer contains 0.2 m*M* EDTA as a compromise between the need to avoid EDTA and the necessity of protecting calpain from autolysis. Myoblast m-calpain is more easily detected in immunoprecipitates than μ-calpain, which may be less stable to the long incubations.
19. Since the activation of calpain in the casein gel involves an overnight incubation in the presence of DTT and Ca^{2+}, there may be a problem with stability of the isozymes. Myoblast m-calpain appears to be stable, whereas myoblast μ-calpain is unstable under these conditions. The presence of calpastatin in the Ca^{2+} activation buffer appears to stabilize the μ-calpain without preventing it from clearing a zone in the gel. We interpret these results as indicating that calpastatin protects an active μ-calpain intermediate in the gel from further autolysis.
20. This method allows the determination of acid soluble proteolysis products in the presence of SH groups.
21. It is advisable to use aliquots containing various amounts of the heated extracts for the estimation of calpastatin activity, since a sigmoidal curve is obtained. Calpastatin activity can then be estimated from the straight part of the titration curve ***(16)***.

References

1. Yanagimachi, R. (1988) Sperm-egg fusion. *Curr. Top. Membr. Transp*. **32,** 3–43.
2. Wakelam, M. J. O. (1988) Myoblast fusion: a mechanistic analysis. *Curr. Top. Membr. Transp*. **32,** 87–112.
3. Okada, Y. (1988) Sendai virus-mediated cell fusion. *Curr. Top. Membr. Transp*. **32,** 297–336.
4. Murachi, T. (1984) Calcium-dependent proteinase and specific inhibitors: Calpain and calpastatin. *Biochem. Soc. Symp*. **49,** 149–167.
5. Ahkong, Q. F., Fisher, D., Tampion, W., and Lucy, J. A. (1975) Mechanisms of cell fusion. *Nature* **253,** 194–195.
6. Kosower, E. M., Kosower, N. S., and Wegman, P. (1977) Membrane Mobility agents. V: The mechanism of particle-cell and cell-cell fusion. *Biochim. Biophys. Acta* **471,** 311–329.
7. Kosower, N. S., Glaser, T., and Kosower, E. M. (1983) Membrane-mobility agent-promoted fusion of erythrocyte: Fusibility is correlated with attack by calcium-activated cytoplasmic proteases on membrane proteins. *Proc. Natl. Acad. Sci. U.S.A.* **80,** 7542–7546.
8. Kosower, N. S. and Glaser, T. (1990) The calpain-calpastatin system and membrane fusion, in Intracellular Calcium-Dependent Proteolysis (Mellgren, R. L. and Murachi, T., eds.) CRC Press, Boca Raton, FL. pp. 163–180.
9. Barnoy, S., Glaser, T., and Kosower, N.S. (1996) The role of calpastatin (the specifc calpain inhibitor) in myoblast differentiation and fusion. *Biochem. Biophys. Res. Commun*. **220,** 933–938.
10. Barnoy, S., Glaser, T., and Kosower, N. S. (1997) Calpain and calpastatin in myoblast differentiation and fusion: Effects of inhibitors. *Biochim. Biophys. Acta* **1358,** 181–188.
11. Barnoy, S., Glaser, T., and Kosower, N. S. (1998) The calpain-calpastatin system and protein degradation in fusing myoblasts. *Biochim. Biophys. Acta* **1402,** 52–60.
12. Raser, K. J., Posner, A., and Wang, K. K. W. (1995) Casein zymography: A method to study μ- calpain, m-calpain and their inhibitory agents. *Arch. Biochem. Biophys*. **319,** 211–216.
13. Hayashi, M., Saito,Y., and Kawashima, S. (1992) Calpain activation is essential for membrane fusion of erythrocytes in the presence of exogenous Ca^{2+}. *Biochem. Biophys. Res. Commun*. **182,** 939–946.
14. Ross, E. and Schatz, G. (1973) Assay of protein in the presence of high concentrations of sulfhydryl compounds. *Anal. Biochem*. **54,** 304–306.
15. Massague, J., Cheifetz, S., Endo, T., and Nadal-Ginard, B. (1986) Type β transforming growth factor is an inhibitor of myogenic differentiation. *Proc. Natl. Acad. Sci. U.S.A.* **83,** 8206–8210.
16. Schwarz-Benmeir, N., Glaser, T., Barnoy, S., and Kosower, N.S. (1994) Calpastatin in erythrocytes of young and old individuals. *Biochem. J*. **304,** 365–370.
17. Glaser, T. and Kosower, N.S. (1986) Calpain–calpastatin and fusion: Fusibility of erythrocytes is determined by a protease-protease inhibitor (calpain-calpastatin) balance *FEBS Lett.* **206,** 115–120.

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The Role of Calpain in Neurofilament Protein Degradation Associated With Spinal Cord Injury

Naren L. Banik and Donald C. Shields

1. Introduction

Calpain is a calcium-activated neutral proteinase present in all mammalian tissues thus far studied *(1,2)*. Ubiquitous calpain exists as μ-calpain and m-calpain isoforms which require μ*M* and m*M* calcium levels for activation, respectively. In the central nervous system (CNS) μ-calpain is predominantly cytosolic while m-calpain is localized in both cytosolic and membrane fractions *(2)*. Although μ-calpain is largely neuronal and m-calpain is present predominantly in glial cells, both calpain isoforms share similar substrate specificities and are inhibited by an endogenous inhibitor, calpastatin *(2)*. A broad range of proteins in the CNS, including cytoskeletal and myelin proteins such as neurofilament proteins (NFPs), vimentin, microtubule-associated protein (MAP), and myelin basic protein (MBP) are known to be calpain substrates. The μ-and m-calpains also cleave protein kinase C (PKC), hormones, receptors, and histones *(3)*.

Calpain has been implicated in tissue destruction associated with CNS trauma, Alzheimer's disease, ischemia, Parkinson's disease, apoptotic cell death, multiple sclerosis (MS) and the corresponding animal model, experimental allergic encephalomyelitis (EAE) *(4–11)*. Increased calpain activity and expression together with cytoskeletal protein degradation are believed to participate in the cell death mechanism *(12–14)*. Previous ultrastructural studies revealed granular degeneration of axonal filaments and microtubules, vesiculation of the myelin sheath, and accumulation of calcium hydroxyapatite crystals in lesioned spinal cords *(4)*. These findings were correlated with increased calcium levels and neutral proteinase activity in spinal cord lesions which suggested the involvement of calpain in the degradation of neurofilament, microtubule, and myelin proteins *(4)*.

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In order to demonstrate that the loss of neurofilament and microtubule proteins may lead to axonal degeneration, spinal cords from injured animals were analyzed by gradient SDS-PAGE. The gel pattern of whole lesioned spinal cord homogenates revealed losses of NFP compared to controls ***(4)***. The degradation of cytoskeletal (NFPs) and myelin proteins concomitant with progressively increased calpain immunoreactivity in spinal cord lesions ***(12,15)*** suggested an important role for calpain in SCI pathogenesis. The increased degradation of calpain substrates (68 kDa and 200 kDa NFPs) and prevention of their breakdown by calpain inhibitors in vivo further implies a critical role for calpain in axon and myelin degeneration associated with spinal cord injury ***(4,5,12)*** **(Fig. 1)**.

Since cytoskeletal proteins (vimentin, microtubule associated proteins, NFPs) and MBP among others are substrates of calpain, calcium-mediated degradation of these proteins was examined in spinal cord and optic nerve samples by SDS-PAGE ***(3,4,16)***. The incubation of these tissues with calcium revealed a significant loss of neurofilament and myelin proteins suggesting calpain involvement. This finding was confirmed by investigating the effects of purified calpain on isolated NFPs, MBP, and microtubule-associated protein (MAP) in vitro ***(4,17,18)***. Degradation of the above proteins is also inhibited by calpain inhibitors. Thus, the degradation of these proteins in neuropathophysiological situations may be used as an indirect measure of calpain activity.

2. Materials

2.1. Induction of Spinal Cord Injury

1. Ketamine (Ketalar, Parke-Davis, Morris Plains, NJ).
2. Xylazine (Rompun, Bayer, Shawnee Mission, KS).
3. Betadine.
4. Sodium pentobarbital (Nembutal, Abbott Laboratories, North Chicago, IL).

2.2. Processing of Spinal Cord Tissue

1. Homogenizing buffer: 50mMTris-HCl, pH 7.4, 5 m*M* EGTA, 1 m*M* PMSF. Store at 2–8°C. Add PMSF immediately before use (*see* **Note 1**).
2. Coomassie Plus Reagent (Pierce, Rockford, IL). Store at room temperature.
3. Bovine serum albumin (BSA), 1 mg/mL in dH$_2$O. Store at –20°C.
4. SDS gel sample buffer: 62.5 m*M* Tris-HCl, pH 6.8, 2% (w/v) SDS, 5 m*M* 2-ME), 10% (v/v) glycerol, 0.004% bromphenol blue. Store at room temperature.
5. Tris-buffered saline (TBS)-Tween: 20 m*M* Tris-HCl, pH 7.6, 137 m*M* NaCl, 1% Tween-20. Store at room temperature.
6. Primary antibodies against 68 kDa and 200 kDa NFP (Sigma Chemical Company, St. Louis, MO) (Sternberger Monoclonals, Baltimore, MD). Store as 20-µL aliquots at –20°C.

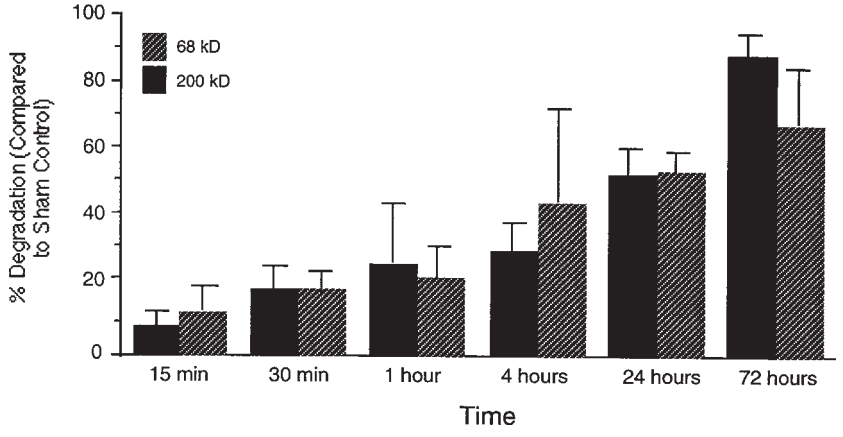

Fig. 1 The extent of degradation of 68-kDa and 200-kDa neurofilament proteins in spinal cord injury. Proteins of lesion cord and sham cord were separated by SDS-PAGE, immunoblotted in association with ECL using 68-kDa and 200-kDa NFP antibodies. The extent of protein loss was quantitated from gel scan using PDI software. Results are expressed as percent loss compared to control from 3 to 7 experiments + SEM. Experimental details are described in the text. (Reprinted from Banik et al., **ref. 7** 1997, 301–306, with permission from Elsevier Science.)

7. Peroxidase-labeled secondary antibody. Store as 20 µL aliquots at –20°C.
8. Enhanced chemiluminescence system (Amersham, Buckinghamshire, UK). Store at 2–8°C.

2.3. Incubation of Homogenates for In Vitro Studies

1. 500 m*M* Tris-HCl buffer, pH 7.6. Store at 2–8°C.
2. 20 m*M* 2-ME. Store at 2–8°C.
3. 100 m*M* KCl. Store at 2–8°C.
4. 50 m*M* $CaCl_2$. Store at 2–8°C.
5. 50 m*M* EGTA. Store at 2–8°C.

3. Methods

3.1. Induction of Spinal Cord Injury

Spinal cord injury is induced in adult Sprague-Dawley rats by the weight-drop technique ***(4)***.

1. Anesthetize the rats by intraperitoneal injection of ketamine (80 mg/kg body weight) and xylazine (10 mg/kg body weight).
2. Shave the skin and prepare with a Betadine solution.
3. Perform laminectomies under aseptic conditions to expose the spinal cord.

4. Drop a 5.0 g weight from a height of 8 cm (40 g-cm force) on the dura-invested spinal cord, which has been stabilized on a stereotactic device. Rats with laminectomy alone (no injury) are used as controls.
5. Sacrifice animals by means of an overdose of sodium-pentobarbital at specific time points following trauma and remove the injured spinal cord for SDS-PAGE analysis.

3.2. Processing of Spinal Cord Tissue for Gel Electrophoresis

1. Homogenize tissues from lesion and sham animals (10% homogenate, w/v) in cold homogenizing buffer with a Polytron homogenizer **(***see* **Note 2)**.
2. Determine total protein content using the Coomassie Plus Reagent (Pierce, Rockford, IL), with BSA standards in the 5–25 µg protein range **(***see* **Note 3)**.
3. To compare in vivo enzyme activity, dilute homogenates 1:1 with SDS gel sample buffer, place in a boiling water bath for 5 min, and spin for 10 min in a table top centrifuge. Store supernatants at –20°C or run immediately on gels.

3.3. Incubation of Homogenates for In Vitro Studies

Homogenates of normal spinal cord may be incubated to test the effect of various inhibitory/stimulatory factors on the endogenous substrate degradation and enzyme activity in vitro.

1. Homogenize tissue and determine protein content as described above.
2. Incubate an aliquot of the homogenate for 1–6 hours in 50m*M* Tris-HCl, pH 7.6, 0.2 m*M* 2-ME, 10 m*M* KCl, 5 m*M* $CaCl_2$. Incubate samples with and without the calpain inhibitors or stimulators being tested. Replace the Ca^{2+} with 5 m*M* EGTA for the controls **(***see* **Note 4)**.
3. After incubation, lyophilyze, and delipidate samples with ether:ethanol (3:2, v/v).
4. Dilute samples with SDS gel sample buffer to a final concentration of 1 mg/mL. Store supernatants at –20°C or run immediately on gels.

3.4. Separation of Proteins by SDS-PAGE, and Immunoblot Analysis

Proteins in the spinal cord preparations are separated by standard methods on 4–20% polyacrylamide gradient gels, and immunoblotted **(Note 5)**.

1. Transfer the proteins to a membrane such as Immobilon-P (Millipore, Bedford, MA).
2. Block the immunoblot using 5% (w/v) nonfat dry milk in TBS-Tween.
3. Incubate the blot with appropriate dilutions of 68 kDa (1:400) and 200 kDa (1:2000) NFP antibodies in TBS-Tween for 1 h.
4. Wash the blot and incubate it with peroxidase-labeled secondary antibody (1:2000) for 1 h.
5. Develop the blot with the enhanced chemiluminescence system (ECL) (Amersham). After exposure of the immunoblot to the ECL chemicals (1 mL A + 1 mL B) for 1 min, immediately expose the blots to X-ray film (Kodak X-OMAT XAR-2)

for 15 s to 1 min. The extent of protein degradation may be evaluated by scanning the ECL autoradiograms and measuring the band densities (*see* **Note 6**). The amount of protein loss may be calculated as percent degraded compared to controls. The ranges of protein loading, antibody dilutions, and incubation times must be calibrated in advance to permit reasonable quantification. A standard curve consisting of 200 kDa or 68 kDa NFP should be run with each set of samples.

The degradation of NFP in spinal cord injury as quantified by these methods is shown in **Fig. 1**.

4. Notes

1. To aid with solubilization, PMSF may be dissolved in 1 mL of ethanol shortly before adding it to the homogenizing buffer.
2. Some tissue samples may require the addition of a detergent such as 0.1% Triton X-100 for solubilization.
3. If a sample requires higher concentrations of detergent for solubilization (e.g., SDS > 0.05%, Triton X-100 > 0.5%), the Coomassie Plus Reagent may not be used. Instead, use the Bicinchoninic Acid (BCA) assay (Pierce, Rockford, IL).
4. The final incubation volume and amount of protein will depend on the amount of tissue available. For example, incubate 0.5 mg of whole spinal cord homogenate in a final volume of 1 mL. Add the stock solutions in proportion to reach the correct final concentrations, using $d\mathrm{H_2O}$ to reach the correct volume. The incubation time will vary, depending on the enzyme activity in the tissue and the effect of the inhibitory/stimulatory factors.
5. Commercially prepared SDS-PAGE gels such as the 4–20% gradient polyacrylamide gels (Bio-Rad, Rockville Centre, NY) have the advantages of uniformity, saving time, and avoiding exposure to the toxic acrylamide. Other acrylamide percentages may give better separation for some applications.
6. ECL membranes may be stripped of bound antibodies and reprobed to clarify or confirm results. Store the membrane at 2–8°C, and keep the membrane damp until ready to strip. Incubate the membrane with 100 m*M* β-mercaptoethanol, 2% SDS, 62.5 m*M* Tris-HCl, pH 6.7 in a sealed container for 30 min at 50°C. Wash the membrane twice for 10 minutes in large volumes of TBS-Tween. The membrane is now ready to reprobe.

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References

1. Suzuki, K., Sorimachi, H., Yoshizawa, T., Kinbara, K., and Ishiura, S. (1995) Calpain: Novel family members, activation, and physiological function. *Biol. Chem.* **376,** 523–529.
2. Banik, N. L., Chakrabarti, A. K., and Hogan, E. L. (1992) Calcium-activated neutral proteinase in myelin: Its role and function, in *Myelin, Biology and Chemistry* (Martenson, R., ed.), CRC Press, Boca Raton, FL. pp. 571–598.
3. Takahashi, K. (1990) Calpain substrate specificity, in *Intracellular Calcium-Dependent Proteolysis* (Mellgren, R. L. and Murachi, T., eds.), CRC Press, Boca Raton, FL, pp. 55–74.
4. Banik, N. L., Matzelle, D., Gantt-Wilford, G., Osborne, A., and Hogan, E. L. (1997) Increased calpain activity and content in spinal cord injury: Progressive degradation of 68kD and 200kD neurofilament protein in trauma. *Brain Res*. **752,** 301–306.
5. Springer, J. E, Azbill, R. D., Kennedy, S. E., George, J., and Geddes, J. W. (1997) Rapid calpain I activation and cytoskeletal protein degradation following traumatic spinal cord injury: Attenuation with Raluzole pretreatment. *J. Neurochem.* **69,** 1592–1600.
6. Posmantur, R., Hayes, R. L., Dixon, C. D., and Taft, W. C. (1994) Neurofilament 68 and neurofilament 200 protein decrease after traumatic brain injury. *J. Neurotrauma* **11,** 533–545.
7. Bartus, R. T. (1997) The calpain hypothesis of neurodegeneration: Evidence for a common cytotoxic pathway. *Neuroscientist* **3,** 314–327.
8. Nixon, R. A., Saito, K. I., Grynspan, F., Griffin, W., Katayama, S., Honda, T., Mohan, P., Shea, T., and Beermann, M. (1994) Calcium-activated neutral proteinase (calpain) system in aging and Alzheimer's disease. *Ann. N. Y. Acad. Sci.* **747,** 77–91.
9. Shields, D. C., and Banik, N. L. (1998) Upregulation of calpain activity and expression in experimental allergic encephalomyelitis (EAE): A putative role for calpain in demyelination. *Brain Res.* **794,** 68–74.
10. Saito, K., Elce, J. S., Hamos, J. E., and Nixon, R. A. (1993) Widespread activation of calcium-activated neutral proteinase (calpain) in the brain in Alzheimer disease: A potential molecular basis for neuronal degeneration. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 2628–2632.
11. Mouatt-Prigent, A., Karlsson, J. O., Agid, Y., and Hirsch, E. C. (1996) Increased m-calpain expression in the mesencephalon of patients with Parkinson's disease but not in other neurodegenerative disorders involving the mesencephalon: A role in nerve cell death? *Neuroscience* **73,** 979–987.
12. Ray, S., Shields, D. C., Davis, B., Matzelle, D., Wilford, G., Hogan, E., and Banik, N. (1998) Role of Calpain in spinal cord injury: effects of calpain and free radical inhibitors. *Ann N.Y. Acad. Sci.,* **844,** 131–137.
13. Squier, M. K. and Cohen, J. J. (1997) Calpain, an upstream regulator of thymocyte apoptosis. *J. Immunol*. **158,** 3690–3697.
14. Nath, R., Raser, K. J., Stafford, D., Hajimohammadreza, I., Posner, A., Allen, H., Talanian, R., Yuen, P., Gilbertsen, R. B., and Wang, K. K. W. (1996) Non-erythroid α-spectrin breakdown by calpain and interleukin 1β-converting enzyme-like

protease(s) in apoptotic cells: Contributory roles of both protease families in neuronal apoptosis. *Biochem. J.* **319,** 683–690.

15. Li, Z., Hogan, E. L., and Banik, N. L. (1995) Role of calpain in spinal cord injury: Increased calpain immunoreactivity in compression injury in the rat. *Neurochem. Int.* **27,** 425–432.
16. Schlaepfer, W. W. and Zimmerman, U. J. P. (1990) The degradation of neurofilaments by calpains, in *Intracellular Calcium-Dependent Proteolysis* (Mellgren, R. L. and Murachi, T., eds.), CRC Press, Boca Raton, FL, pp. 241–250.
17. Johnson, G. V. and Foley, V. G. (1993) Calpain-mediated proteolysis of microtubule-associated protein 2 (MAP-2) is inhibited by phosphorylation by cAMP-dependent protein kinase, but not by Ca^{2+}/calmodulin-dependent protein kinase II. *J. Neurosci. Res*. **34,** 642–647.
18. Fischer, I., Romano-Clarke, G., and Grynspan, F. (1991) Calpain-mediated proteolysis of microtubule-associated proteins MAP_1 and MAP_2 in developing brain. *Neurochem. Res*. **16,** 891–898.

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Concurrent Assessment of Calpain and Caspase-3 Activity by Means of Western Blots of Protease-Specific Spectrin Breakdown Products

Jennifer K. Newcomb, Brian R. Pike, Xiurong Zhao, and Ronald L. Hayes

1. Introduction

The calpains are found ubiquitously in mammalian cells and are activated following various central nervous system (CNS) insults, including ischemia *(1)*, spinal cord injury *(2)* and traumatic brain injury (TBI) *(3–5)*. Nonerythroid α-spectrin is a submembrane cytoskeletal protein and preferred calpain substrate *(6)*. Calpain overactivation following TBI leads to rapid spectrin proteolysis that precedes and is thought to contribute to cellular dysfunction and cell death *(3)*. In addition to cleavage by calpain, α-spectrin is also cleaved by caspase-3, a cysteine protease believed to be a key executioner in apoptosis *(7)*. While both the calpains and caspase-3 produce an initial fragment of nearly identical size (150 kDa), these proteases also produce distinct α-spectrin breakdown products; a 145-kDa fragment generated by calpain and a 120-kDa fragment generated by caspase-3 *(7)*.

We describe here the use of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting techniques to assess calpain and caspase-3 activation by measuring the density of particular bands that represent specific α-spectrin breakdown products. Importantly, this technique allows concurrent temporal and regional analyses of calpain and caspase-3 activation **(Fig. 1)**. It is fairly inexpensive to perform since minigels can usually be employed, therefore decreasing the amount of sample and primary antibody required. In addition, commercially available anti-α-spectrin monoclonal antibodies (Affiniti, UK; Chemicon, Temecula, CA) cross-react with brain α-spectrin from several species. While cleavage of poly(ADP-ribose) poly-

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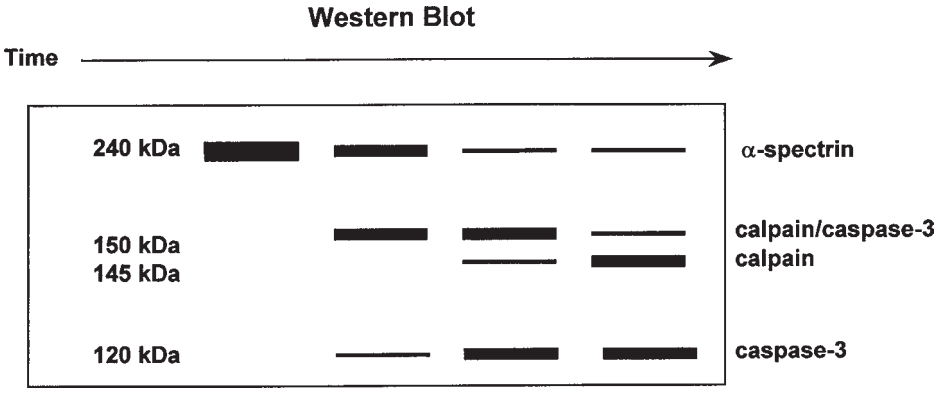

Fig. 1. The diagram illustrates a typical Western blot labeled with an anti-α-spectrin antibody using samples collected from uninjured animals *(lane 1)* or animals subjected to in vivo TBI *(lanes 2–4)* and sacrificed at progressively later time points after injury. Note that in the uninjured animal *(lane 1)*, α-spectrin is present in the intact form only and no proteolytic fragments are detected. Following injury *(lane 2)*, intact α-spectrin begins to degrade into a 150-kDa (calpain and caspase-3-mediated) and a 120-kDa (caspase-3-mediated) fragment. As the time following injury progresses *(lanes 3* and *4)*, intact α-spectrin is further degraded and a 145-kDa fragment (calpain-mediated) is detected along with increases in the 120-kDa fragment.

merase (PARP) can also be used as an indicator of calpain and caspase-3 activity, commercially available antibodies fail to detect rat or mouse PARP or do so only weakly ***(8)***.

This technique has been successfully used to characterize calpain and caspase-3 activation in both in vitro ***(8–10)*** and in vivo ***(11)*** models of CNS insult.

2. Materials

1. Anti-α-spectrin monoclonal antibody (clone AA6; catalog no. FG 6090) (Affiniti Research Products Limited, UK).
2. Horseradish peroxidase (HRP)-conjugated secondary antibody (Cappel, West Chester, PA).
3. Enhanced chemiluminescence (ECL) kit and Hyperfilm-ECL (Amersham, Arlington Heights, IL).
4. Homogenization buffer: 20 m*M* PIPES, pH 7.6, 1 m*M* EDTA, 2 m*M* EGTA, 1 m*M* DTT, 0.5 m*M* PMSF, 50 µg/mL leupeptin and 10 µg/mL each of 4-(2-aminoethyl)benzenesulfonylflouride (AEBSF), aprotinin, pepstatin, *N*α-*p*-tosyl-L-lysine chloromethylketone (TLCK); *N*-tosyl-L-phenylalanine chloromethylketone (TPCK).
5. Tris-Tween buffer (TTB): 20 m*M* Tris-HCl, 0.15 *M* NaCl, 0.005% Tween-20.

3. Methods

3.1. Sample Preparation

1. Anesthetize animals with pentobarbital (100 mg/kg, intraperitoneally) and decapitate at the loss of the toe-pinch reflex.
2. Dissect appropriate brain tissue (10–100 mg) and quickly freeze in liquid N_2. Tissue can be stored at –70°C until homogenization.
3. Homogenize tissue in 1 mL of ice-cold homogenization buffer per 100 mg at 4°C with 15 strokes in a glass/Teflon homogenizer. Cell lysis can be facilitated by a variety of techniques. We typically use 15–20 passes in a 1-mL syringe fitted with a 25-gauge needle.
4. For in vitro samples from cell culture, add 100 µL of homogenization buffer to each well (24-well plate). Collect cells and shear using a 1-mL syringe with a 25-gauge needle by 15–20 passes (*see* **Note 1**).
5. Determine protein concentrations with a bicinchoninic acid protein assay with albumin standards (Pierce, Rockford, IL).

3.2. Gel Electrophoresis and Immunoblotting

1. Run 6.5% SDS-PAGE mini gels using an electrophoresis buffer consisting of 0.025 *M* Tris, 0.1 *M* glycine and 0.1% SDS at 4°C (*see* **Note 2**).
2. Transfer proteins to a nitrocellulose or PVDF membrane using a transfer buffer consisting of 0.192 *M* glycine, 0.025 *M* Tris, pH 8.3, with 10% methanol at 4°C.
3. Block membrane in 5% nonfat milk in TTB for 1–2 h at room temperature.
4. Incubate membrane in anti-α-spectrin monoclonal antibody diluted 1:4,000 in 1% BSA in TTB for 2 h at room temperature or overnight at 4°C.
5. Rinse membrane for 5 min in TTB.
6. Incubate membrane in HRP-conjugated sheep anti-mouse secondary antibody diluted 1:10,000 in 1% non-fat milk in TTB for 45 min at room temperature.
7. Rinse membrane in TTB for 1–1.5 h at room temperature, changing buffer every 15 min.
8. Use an enhanced chemiluminescence kit (ECL) to visualize immunolabeling on high performance chemiluminescence film (Hyperfilm-ECL) (*see* **Note 3**).

4. Notes

1. Positive controls, such as maitotoxin (activates voltage and receptor-mediated Ca^{2+} channels) or staurosporine (a general protein kinase inhibitor), can be used to induce calpain activation only, or both calpain and caspase-3 activation, respectively ***(9,10)***.
2. If α-spectrin breakdown products are not detected initially, increase the amounts of protein loaded in each lane of the minigel, or consider using a larger gel electrophoresis apparatus.
3. Due to its lower molecular weight, the 120-kDa band (caspase-3 mediated) is easily distinguished from other α-spectrin breakdown products. However, detecting both the 150-kDa (calpain and caspase-3 mediated) and 145-kDa

(calpain-mediated only) bands may be difficult. Experimenting with different gel concentrations (5–7.5%) can aid in optimizing the separation of these bands. In addition, overexposure of chemiluminescence film or overloading of protein can cause difficulties in detecting two separate bands.

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References

1. Roberts-Lewis, J. M., Savage, M. J., Marcy, V. R., Pinsker L. R., and Siman, R. (1994) Immunolocalization of μ-calpain mediated spectrin degradation to vulnerable neurons in ischemic gerbil brain. *J. Neurosci.* **14,** 3934–3944.
2. Banik, N. L., Matzelle, D. C., Gantt-Wilford, G., Osborne, A., and Hogan, E. L. (1997) Increased calpain content and progressive degradation of neurofilament protein in spinal cord injury. *Brain Res.* **752,** 301–306.
3. Newcomb, J. K., Kampfl, A., Posmantur, R. M., Zhao, X., Pike, B. R., Liu, S. J., Clifton, G. L. and Hayes, R. L. (1997) Immunohistochemical study of calpain-mediated breakdown products to α-spectrin following controlled cortical impact injury in the rat. *J. Neurotrauma* **14,** 369–383.
4. Posmantur, R. M., Kampfl, A., Siman, R., Liu, S. J., Zhao, X., Clifton, G. L., and Hayes, R. L. (1997) A calpain inhibitor attenuates cortical cytoskeletal protein loss after experimental brain injury in the rat. *Neuroscience* **77,** 875–888.
5. Saatman, K. E., Bozyczko-Coyne, D., Marcy, V., Siman, R., and McIntosh, T. K. (1996) Prolonged calpain-mediated spectrin breakdown occurs regionally following experimental brain injury in the rat. *J. Neuropathol. Exp. Neurol*. **55,** 850–860.
6. Wang, K. K., Villalobo, A., and Roufogalis, B. D. (1989) Calmodulin-binding proteins as calpain substrates. *Biochem. J.* **262,** 693–706.
7. Nath, R., Raser, K. J., Stafford, D., Hajimohammadreza, I., Posner, A., Allen, H., Talanian, R. V., Yuen, P., Gilbertsen, R. B., and Wang, K. K. (1996) Non-erythroid alpha-spectrin breakdown by calpain and interleukin 1 beta-converting-enzyme-like protease(s) in apoptotic cells: Contributory roles of both protease families in neuronal apoptosis. *Biochem. J.* **319,** 683–690.
8. Nath, R., Probert, A. Jr., McGinnis, K. M., and Wang, K. K. (1998) Evidence for activation of caspase-3-like protease in excitotoxin- and hypoxia/hypoglycemia-injured neurons. *J. Neurochem*. **71,** 186–195.
9. Pike, B. R., Zhao, X., Newcomb, J. K., Wang, K. K., Posmantur, R. M., and Hayes, R. L. (1998) Temporal relationships between de novo protein synthesis, calpain and caspase 3-like protease activation, and DNA fragmentation during apoptosis in septo-hippocampal cultures. *J. Neurosci. Res.* **52,** 505–520.
10. Zhao, X., Pike, B. R., Newcomb, J. K., Wang, K. K. W., Posmantur, R. M., and Hayes, R. L. (1999) Maitotoxin induces calpain but not caspase-3 activation and necrotic cell death in primary septo-hippocampal cultures. *Neurochem. Res.*, **24,** 371–382.

11. Pike, B. R., Zhao, X., Newcomb, J. K., Posmantur, R. M., Wang, K. K. W., and Hayes, R. L. (1998) Regional calpain and caspase-3 proteolysis of alpha-spectrin after traumatic brain injury. *Neuroreport* **9,** 2437–2442.

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Rat Renal Proximal Tubules, Hypoxia, Ionomycin, and Calpain

Charles L. Edelstein

1. Introduction

The preparation of freshly isolated rat renal proximal tubules in suspension has been utilized by our laboratory for more than 10 yr and reported in numerous publications ***(1–10)***. This method of tubule isolation by collagenase digestion and Percoll centrifugation has recently been used by us for the study of calpain activity in this tissue ***(11–13)***. Briefly, the kidneys of two male Sprague-Dawley rats are perfused, and the cortices are digested with collagenase and hyaluronidase. After washing and filtering, proximal tubules are separated from other nephron segments by centrifugation on a Percoll gradient. The preparation has a purity of more than 95% proximal tubules. The tubule suspension is aliquotted into Erlenmeyer flasks and gassed with 95% O_2 / 5% CO_2. At this time, release of lactate dehydrogenase (LDH) activity into the incubation medium, which is used as an index of cell membrane integrity, consistently averages 10% or less of the total LDH activity of the whole preparation, and remains at this level under oxygenated conditions for the duration of a typical experiment (15–20 min). Hypoxia is achieved by gassing the suspension with 95% N_2 / 5% CO_2, which results in a 2-3-fold increase in LDH release over 15 min. We have demonstrated that hypoxia results in a prelethal increase in free cytosolic Ca^{2+} in this model ***(14)***. Further, the Ca^{2+} ionophore, ionomycin, added to normoxic tubules, elicits a dose-dependent increase in free cytosolic Ca^{2+} ***(14)***, which permits the study of proximal tubules with a known free cytosolic [Ca^{2+}]. Thus, hypoxia and ionomycin-induced proximal tubular injury provide excellent models in which to observe the effects of increased free cytosolic Ca^{2+} on membrane damage as well as the effects of cytoprotec-

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tive agents on Ca^{2+}-mediated events. In this model of rat renal proximal tubules, we have demonstrated that calpain inhibitors attenuate the increase in calpain activity during both hypoxia and ionomycin treatment and protect against cell membrane injury ***(11).***

2. Materials

1. Buffer A (prepare 5L, the pH is not adjusted): 112 m*M* NaCl, 25 m*M* $NaHCO_3$, 10 m*M* mannitol, 5 m*M* KCl, 2 m*M* NaH_2PO_4, 5 m*M* glucose, 2.5 m*M* HEPES, 1.2 m*M* $MgSO_4$, 1.6 m*M* $CaCl_2$ (*see* **Note 1**).
   Nutrients for buffer A must be added on day of use: these are 1 m*M* glutamine, 1 m*M* sodium butyrate, 1 m*M* sodium lactate.
2. Percoll diluent (prepare 0.2 L): 1.1 *M* NaCl, 0.2 *M* $NaHCO_3$.
3. Buffer B (prepare 50 mL): 110 m*M* NaCl, 20 m*M* $NaHCO_3$ (i.e., 10-fold dilution of Percoll diluent).
4. Buffer C (prepare 0.5 L): 106 m*M* NaCl, 18 m*M* $NaHCO_3$, 5 m*M* KCl, 2 m*M* NaH_2PO_4, 5 m*M* glucose, 2.5 m*M* HEPES, 1 m*M* $MgSO_4$, 1 m*M* $CaCl_2$ (for pH of this buffer, *see* **Subheading 3.5**.).
   Nutrients for buffer C must be added on the day of use: these are 2 m*M* glutamine 2, 10 m*M* sodium butyrate, 4 m*M* sodium lactate.
5. Perfusion solution: 60 mL of buffer A, 0.8 mL of heparin (1000 U/mL).
6. C and H solution: 60 mL of buffer A, 15 mg collagenase (*see* **Note 2**), 15–20 mg hyaluronidase (hyaluronidase type III from sheep testes, Sigma, St. Louis, MO).
7. Collagenase solution: 60 mL of buffer A, 40 mg collagenase, 10 mg hyaluronidase.
8. Bovine serum albumin (BSA) solution: 30 mL of buffer A, 1 g of BSA (Sigma), pH should be between 6.9 and 7.4.
9. 100% Percoll: 6 mL of Percoll diluent, 54 mL of 100% Percoll (Sigma).
10. 45% Percoll: 33 mL of buffer B, 27 mL of 100% Percoll. The pH of the 45% Percoll should be between 6.9 and 7.4.
11. Instruments needed for rat surgery: 30 mL plastic syringes; 21G (gauge) butterfly infusion set (cut off the sharp tip of the needle); 2 cm crocodile clamp; large and small surgical scissors; curved forceps; 4.0 black silk suture; umbilical tape; gauze sponges.
12. Other materials needed: tissue slicer blades; tea strainer; 95% O_2 / 5% CO_2 tank; 95% N_2 / 5% CO_2 tank; 125-mL Erlenmeyer flask and rubber stopper with 2 holes; 25 ml Erlenmeyer flasks and rubber stoppers with 1 hole.
13. Reagents for LDH measurement:
    a. LDH buffer: 85 m*M* K_2HPO_4, 11 m*M* KH_2PO_4.
    b. Sodium pyruvate solution: 22.7 m*M* sodium pyruvate in 0.1 *M* phosphate buffer (Sigma).
    c. Working solution (make fresh each day): 2.74 mL of sodium pyruvate solution, 47.26 mL of water, 50 mL of LDH buffer.
    d. Reduced nicotinamide adenine dinucleotide (NADH) solution: reconstitute a 25-mg vial of β-NADH with 2.68 mL of 1% (w/v) $NaHCO_3$.

14. Tubule Lowry protein method:
    a. Solution I: 2% (w/v) Na_2CO_3 in 0.1 *M* NaOH (100 vol), 1% (w/v) $CuSO_4$ (1 vol), 2% (w/v) sodium potassium tartrate (1 vol).
    b. Solution II: phenol reagent diluted 1:1 with water.

3. Method

3.1. Rat Surgery

1. Prepare 1 L of buffer A with nutrients: place 800 ml of this buffer in a 1-L flask and put on ice; gas with oxygen at 1 L/min for 10 min before and during use.
2. Gas the perfusion solution, the C and H solution, the collagenase solution, and the BSA solution, on ice with oxygen at 1 L/min **(Note 3)**.
3. Proximal tubules are isolated from the kidney cortex of two male Sprague-Dawley rats (200–300 g body weight), as follows:
    a. anesthetize with pentobarbital sodium (60 mg/kg body weight, i.p.);
    b. perform laparotomy;
    c. tie off coeliac artery;
    d. put umbilical tape tie around aorta above renal artery but do not tighten;
    e. tease the aorta apart from the vena cava below the renal artery;
    f. put suture around the aorta but do not tighten;
    g. fill syringe with 30 mL of cold gassed perfusion solution;
    h. put crocodile clamp on the aorta below the renal artery;
    i. cannulate the aorta with a butterfly catheter at least 1 cm below the crocodile clamp;
    j. tie a suture around the catheter;
    k. remove the crocodile clip;
    l. perfuse the kidneys with the 30 mL of perfusion solution over 1 min;
    m. cut the vena cava after 5 mL of perfusion solution have been infused, and continue the perfusion;
    n. remove the syringe; refill it with 30 mL cold gassed C and H solution, reattach the syringe, and continue to perfuse the kidneys;
    o. clip out the perfused kidneys, decapsulate them, and place them into cold gassed buffer A;
    p. transfer the kidneys to a bed of ice in a Petri-dish lid, and remove the kidney cortices with blade;
    q. mince kidney cortices with the same blade.

3.2. Washing Procedure

All procedures are performed on ice or at 4°C unless otherwise stated. Wash the minced tissue three times with cold gassed buffer A as follows:

1. place tissue in a 50 mL plastic tube with 40 mL of buffer A, and mix with the transfer pipette;
2. centrifuge at 60*g* for 30 s;

3. pour off buffer A;
4. repeat wash in 40 mL of cold gassed buffer A twice more.

3.3. Collagenase Digestion

1. Transfer washed cortex into 55 mL of cold gassed collagenase solution in a 125-mL Erlenmeyer flask.
2. Digest at 37°C in a shaking water bath with continuous gassing with 95% O_2 / 5% CO_2.
3. Start timer.
4. Prepare buffer B and start gassing it with 1 L/min 95% O_2 / 5% CO_2 on ice.
5. At 15, 20, 25, and 35 min, remove the Erlenmeyer containing suspended digesting tubules from the water bath and carry out the following procedure:
   a. squirt up and down 10–15 times with plastic transfer pipette; the tip of the pipette is cut to make it wider for the 15-and 20-min digestions;
   b. allow pieces of cortex to settle for 30 s, and pour solution above the tubules into a 50-mL centrifuge tube; centrifuge at 60*g* for 30 s;
   c. return supernatant to the Erlenmeyer flask;
   d. transfer pellet into a beaker with cold gassed BSA solution.
6. At 35 min, squirt the mixture up and down, and transfer the whole suspension into two 50-mL centrifuge tubes.
7. Centrifuge at 60*g* for 30 s.
8. Discard the supernatants.
9. Resuspend each pellet with 30 mL of BSA solution.
10. Keep the digested tubules gassed in BSA solution on ice for 10 min (*see* **Note 4**).

3.4. Percoll Centrifugation

1. Make the 45% Percoll solution and gas it with 95% O_2 / 5% CO_2 on ice.
2. After 10 min in BSA solution (**Subheading 3.3., step 10**.), filter the tubule suspension through a tea strainer into 40 mL of cold gassed buffer A.
3. Wash 3 times with buffer A as described in **Subheading 3.2**.
4. Place 30 mL of 45% Percoll into each of 2 clean 50 ml centrifuge tubes.
5. Carefully layer 10 mL of 100% Percoll under the 45% Percoll.
6. Resuspend the washed tubules in 5 mL of 45% Percoll.
7. Carefully layer the tubules in 45% Percoll on top of the Percoll in the centrifuge tube.
8. Centrifuge at 15,000*g* for 10 min in a Sorvall RB-4 centrifuge at 4°C.

3.5. Retrieving the Proximal Tubules

1. Add nutrients to 60 ml buffer C; start gassing on ice at 4 L/min, and continue until pH is between 6.8 and 7.0.
2. Recover proximal tubules from lowest band after Percoll centrifugation; this band contains 95% proximal tubules and no glomeruli.
3. Wash twice with buffer A as above, to remove the Percoll.
4. Resuspend tubules in 40–50 mL of buffer C depending on the protein concentration desired (**Note 5**).

5. Mix tubules carefully with buffer C, and place 6-mL aliquots in siliconized 25-mL Erlenmeyer flasks on ice (*see* **Note 6**).
6. Gas on ice with 95% O_2 / 5% CO_2 at 1 L/min for 5 min and then seal the flasks with rubber stoppers.
7. Tubules then undergo a "recovery" period and are gradually warmed from 4°C to 37°C over 15 min as follows:
   a. incubate the flasks at room temperature for 5 min;
   b. place the flasks in a shaking water bath at 37°C for 10 min, and check that the stoppers do not "pop" off during this time.
8. Keep flasks in the shaking water bath to perform subsequent experiments.

3.6. Hypoxia-Induced Damage

As a rule, added agents such as inhibitors are preincubated with the proximal tubules for 10 min before the induction of hypoxia or ionomycin exposure. Experiments in normoxic control suspensions are always performed in parallel to hypoxia studies.

Hypoxia is achieved by gassing the suspension with 95% N_2 / 5% CO_2 (3 L/min) for 5 min and stoppering the vial, thereby reducing the pO_2 to approximately 30 mm Hg ***(15,16)***. Following this 5-min gassing procedure, the effects of hypoxia are studied at 7.5 and 15 min. The effects of cytoprotective agents and enzyme inhibitors are determined at 7.5 min and at 15 min of hypoxia.

Cell membrane damage is assessed by measuring the percentage LDH release into the media of the tubules (*see* **Note 7**).

3.7. Ionomycin-Induced Damage

To evaluate the effects of increased cytoplasmic free Ca^{2+}, the Ca^{2+} ionophore, ionomycin, is used. Ionomycin added to normoxic tubules at 1, 3, and 5 µ*M* elicits a dose-dependent increase in free cytosolic Ca^{2+} in rat proximal tubules ***(14,17)***. The increase in free cytosolic Ca^{2+} occurs at 30 s when measured in the video imaging system ***(14)*** and the degree of cell membrane damage depends on the time period of exposure of tubules to ionomycin ***(17)*** (*see* **Note 8**).

3.8. Prelethal Injury Model

Glycine protects tissue against hypoxia and ionomycin-induced cell membrane damage, and can therefore be used as a tool to study prelethal hypoxic injury. Tubules continuously exposed to 2 m*M* glycine do not show an increase in LDH release even after 60 min hypoxia ***(17,18)***. This allows the study of hypoxic events that occur before the loss of cell membrane integrity and the resultant massive influx of extracellular Ca^{2+} or leakage of intracellular enzymes.

3.9. Measurement of LDH Release

Percent LDH release into the suspension media is used as an index of lethal membrane injury ***(19)***. The percentage LDH released from tubules is calculated by determining the ratio of LDH in the supernatant compared to the sum of LDH in the lysed tubule pellet plus LDH in the supernatant.

1. Take 1 mL of tubule suspension at end of experiment.
2. Centrifuge immediately in a 75 × 12 mm glass tube by accelerating up to 1000*g* and immediately decelerating.
3. Pour off supernatant into another 5 mL glass tube.
4. Invert and drain pellet.
5. Resuspend pellet with 1 mL of 1.5% Triton X-100 in water.
6. Prepare test tubes with 3-mL aliquots of working solution (**Subheading 2.1.3.**).
7. Add 100 µL samples of tubule supernatant or resuspended pellet to 3 ml of working solution.
8. Add 50 µL NADH solution.
9. Vortex for a few seconds, and immediately measure the decrease in absorbance at 340 nm at 15-s intervals for 2 min.
10. % LDH release equals:

$$\triangle(\text{absorbance supernatant}) \times 100 / \{ \triangle(\text{absorbance pellet}) + \triangle(\text{absorbance supernatant})\}$$

3.10. Measurement of Protein

Protein in the proximal tubule suspension is measured by the Lowry method using bovine serum albumin as standard ***(20)***.

1. Standards: prepare blank, 10, 20, 40, 80, and 120 µg standards from a 1 mg/mL BSA stock solution, using water to equalize final volumes.
2. Add 50 µL buffer C to the blank and standard tubes.
3. Add 100 µL of 1.2% (w/v) SDS in 0.1 *M* NaOH to all tubes.
4. Add 2 mL of solution I to all tubes, vortex, and wait 10 min (**Subheading 2.1.4.**).
5. Add 0.2 mL of solution II, vortex immediately and wait 30–60 min.
6. Read absorbance at 550 nm.

4. Notes

1. Use deionized water for all solutions.
2. Collagenase type B (lot number OFAA133) (Boehringer Mannheim, Indianapolis, IN). The activity of collagenase varies with the lot number, and the amount of collagenase may have to be adjusted to obtain optimal digestion.
3. Do not bubble oxygen directly through the BSA solution, since this denatures the protein. Instead gas indirectly through a hole in the container lid.
4. Do not bubble oxygen through the tubules. Instead gas indirectly through hole in container lid as in **Note 3**.
5. Adjust the pH of buffer C to exactly 7.1. This is a crucial step since the characteristics of the proximal tubules depend on the pH of the buffer in which the experiments are done.

6. The normal yield of tubules obtained from four kidneys and suspended in 48 mL of buffer C is approximately 1–2 mg/mL protein.
7. In this model, there is no increase in LDH release and there is also no increase in propidium iodide uptake, another index of cell membrane damage, at 7.5 min hypoxia compared to normoxic controls ***(16)***. At 7.5 min hypoxia the cell membrane is grossly intact, eliminating the possibility of leakage either into or out of the cell of enzymes/ions that may affect the results, while at 15 min of hypoxia, there is a significant increase in LDH release into the media. Therefore, changes observed at 7.5 min are thought to precede gross destruction of the cell membrane and thus may play a potentially important role in the mediation of the later membrane damage.
8. At 2 min ionomycin incubation there is generally no increase in LDH release compared to controls. At 7.5 min ionomycin incubation there is a doubling in LDH release compared to controls. These doses of ionomycin do not decrease ATP levels in isolated tubules ***(17)***. Thus, ionomycin-induced proximal tubule injury provides an excellent model to observe the effects of controlled elevated free cytosolic Ca^{2+} and subsequent calpain activity on membrane damage.

References

1. Almeida, A. R., Bunnachak, D., Burnier, M., Wetzels, J. F. M., Burke, T. J., and Schrier, R. W. (1992) Time-dependent protective effects of calcium channel blockers on anoxia and hypoxia-induced proximal tubule injury. *J. Pharmacol. Exp. Ther.* **260,** 526–532.
2. Almeida, A. R., Wetzels, J. F. M., Bunnachak, D., Burke, T. J., Chaimovitz, C., Hammond, W. S., and Schrier, R. W. (1992) Acute phosphate depletion and in vitro rat proximal tubule injury: Protection by glycine and acidosis. *Kidney Int.* **41,** 1494–1500.
3. Burnier, M., Van Putten, V. J., Schieppati, A., and Schrier, R. W. (1988) Effect of extracellular acidosis on ^{45}Ca uptake in isolated hypoxic proximal tubules. *Am. J. Physiol.* **254,** C839–C846.
4. Wetzels, J. F. M., Yu, L., Wang, X., Kribben, A., Burke, T. J., and Schrier, R. W. (1993) Calcium modulation and cell injury in isolated rat proximal tubules. *J. Pharmacol. Exp. Ther.* **267,** 176–180.
5. Wetzels, J. F. M., Wang, X., Gengaro, P. E., Nemenoff, R. A., Burke, T. J., and Schrier, R. W. (1993) Glycine protection against hypoxic but not phospholipase A_2-induced injury in rat proximal tubules. *Am. J. Physiol.* **264,** F94–F99.
6. Bunnachak, D., Almeida, A. R., Wetzels, J. F. M., Gengaro, P. E., Nemenoff, R. A., Burke, T. J., and Schrier, R. W. (1994) Ca^{2+} uptake, fatty acid, and LDH release during proximal tubule hypoxia: Effects of mepacrine and dibucaine. *Am. J. Physiol.* **266,** F196–F201.
7. Yu, L., Gengaro, P. E., Niederberger, M., Burke, T. J., and Schrier, R. W. (1994) Nitric oxide: A mediator in rat tubular hypoxia/reoxygenation injury. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 1691–1695.

8. Yaqoob, M. M., Edelstein, C. L., Wieder, E. D., Alkhunaizi, A., Gengaro, P. E., and Schrier, R. W. (1996) Nitric oxide kinetics during hypoxia in proximal tubules: Effects of acidosis and glycine. *Kidney Int.* **49,** 1314–1319.
9. Choi, K. H., Edelstein, C. L., Gengaro, P. E., Schrier, R. W., and Nemenoff, R. A.(1995) Hypoxia induces changes in phospholipase A_2 in rat proximal tubules: Evidence for multiple forms. *Am. J. Physiol.* **269,** F846–F853.
10. Alkhunaizi, A. M., Yaqoob, M. M., Edelstein, C. L., Gengaro, P. E., Burke, T. J., Nemenoff, R. A., and Schrier, R. W.(1996) Arachidonic acid protects against hypoxic injury in rat proximal tubules. *Kidney Int.* **49,** 620–625.
11. Edelstein, C. L., Wieder, E. D., Yaqoob, M. M., Gengaro, P. E., Burke, T. J., and Schrier, R. W. (1995) The role of cysteine proteases in hypoxia-induced renal proximal tubular injury. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 7662–7666.
12. Edelstein, C. L., Yaqoob, M. M., Alkhunaizi, A., Gengaro, P. E., Nemenoff, R. A., Wang, K. K. W., and Schrier, R. W. (1996) Modulation of hypoxia-induced calpain activity in rat renal proximal tubules. *Kidney Int.* **50,** 1150–1157.
13. Edelstein ,C. L., Ling, H., Gengaro, P. E., Nemenoff, R. A., Bahr, B. A., and Schrier, R. W. (1997) Effect of glycine on prelethal and postlethal increases in calpain activity in rat renal proximal tubules. *Kidney Int.* **52,** 1271–1278.
14. Kribben, A., Wieder, E. D., Wetzels, J. F. M., Yu, L., Gengaro, P. E., Burke, T. J., and Schrier, R. W. (1994) Evidence for role of cytosolic free calcium in hypoxia-induced proximal tubule injury. *J. Clin. Invest.* **93,** 1922–1929.
15. Joseph, J. K., Bunnachak, D., Burke, T. J., and Schrier, R. W. (1990) A novel method of inducing and assuring total anoxia during in vitro studies of O_2 deprivation injury. *J. Am. Soc. Nephrol.* **1,** 837–840.
16. Kribben, A., Wetzels, J. F. M., Wieder, E. D., Burke, T. J., and Schrier, R. W. (1993) New technique to assess hypoxia-induced cell injury in individual isolated renal tubules. *Kidney Int.* **43,** 464–469.
17. Weinberg, J. M., Davis, J. A., Roeser, N. F., and Venkatachalam, M. A. (1991) Role of increased cytosolic free calcium in the pathogenesis of rabbit proximal tubule cell injury and protection by glycine or acidosis. *J. Clin. Invest.* **87,** 581–590.
18. Garza-Quintero, R., Weinberg, J. M., Ortega-Lopez, J., Davis, J. A., and Venkatachalam, M. A. (1993) Conservation of structure in ATP-depleted proximal tubules: Role of calcium, polyphosphoinositides, and glycine. *Am. J. Physiol*. **265,** F605–F623.
19. Bergmeyer, H. U. (1974) *Methods in Enzymatic Analysis*, 2nd ed. Academic Press, New York p. 574.
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–275.

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Calpain Activity in Rat Renal Proximal Tubules

An In Vitro Assay

Charles L. Edelstein

1. Introduction

The general assay for proteolytic activity using casein as a substrate was described in 1947. A modification using azocasein has been described, in which digestion by calpain results in colored peptides soluble in trichloroacetic acid (TCA), so that the intensity of the color in the supernatant from TCA precipitation is a function of proteolytic activity. The assay is simple, reliable, and sensitive, but not specific, although the absolute requirement of calpain for Ca^{2+} is used to impart some degree of specificity ***(1)***.

There have been some recent advances in protease assay using synthetic substrates which involve a leaving group whose fluorescence changes after hydrolysis ***(2,3)***. Fluorescent detector groups include 2-naphthylamine (2-NA), 4-methoxy-2-naphthylamine (MNA), 7-amido-4-methylcoumarin (AMC) and 7-amino-trifluoromethylcoumarin (AFC), linked to the C-terminal carboxylic acid group of N-terminally blocked or unblocked peptides. They do not fluoresce when covalently bound, but only after cleavage from the peptide by a protease. In addition, there are some peptide substrates that are preferred by calpain in comparison to other proteases ***(3)***. Under the optimal pH and calcium concentration for calpain activity, the amount of fluorescence derived from the AMC-conjugated substrate should indicate the amount of calpain activity.

The calpain assay in freshly isolated rat renal proximal tubules is based on that described by Sasaki et al. ***(3)*** for purified porcine kidney calpain. *N*-Succinyl-leucyl-tyrosyl-AMC (Succ-LY-AMC) was used as a substrate for calpain in our early manuscript ***(4)*** and is still used for assay of purified calpains. We now use *N*-succinyl-leucyl-leucyl-valyl-tyrosine-AMC (Succ-LLVY-AMC)

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(3) as a substrate for measurement of calpain in extracts of proximal tubules, as it was found to be more sensitive *(5,6)*.

These substrates have been shown to be proteolyzed in vitro by some cathepsins *(3)*. To enhance the calpain selectivity of our assay and to exclude any possible effect of lysosomal leakage of cathepsins, the following measures were taken: (i) digitonin was used for cell lysis, since it is known to release cytoplasmic contents while keeping lysosomes intact, thus limiting release of lysosomal cathepsins *(7,8)* (*see* **Note 1**); (ii) the assay was performed at pH 7.3 at which cathepsins are known to be inactivated *(9)*; (iii) calpain activity was defined and measured as Ca^{2+}-dependent activity, thus excluding cathepsin activity, which is strictly Ca^{2+}-independent *(9)*.

We have demonstrated with Succ-LY-AMC that the Ca^{2+} ionophore ionomycin induces a dose-dependent increase in calpain activity in proximal tubules that occurs before cell membrane damage as assessed by LDH release **(Fig. 1)**. With Succ-LLVY-AMC, we have shown that hypoxia induces an increase in calpain activity in proximal tubules *(5,6)* and that inhibition of calpain by the specific inhibitor, (2)-3-(4-iodophenyl)-2-mercapto-2-propenoic acid (PD150606) (*see* **Note 2**) *(11)*, protects against cell membrane damage **(Fig. 2)**.

2. Materials

1. Imidazole buffer: 63.2 m*M* imidazole-HCl, pH 7.3, 10 m*M* 2-ME.
2. Imidazole-Ca^{2+}-free buffer: 63.2 m*M* imidazole-HCl, pH 7.3, 10 m*M* 2-ME, 1 m*M* EDTA, 10 m*M* EGTA.
3. Stock solutions:
   a. 10 m*M* Succ-LLVY-AMC in DMSO (stored at –20°C) (*see* **Note 3**);
   b. 10 m*M* Succ-LY-AMC (Sigma, St. Louis, MO) in DMSO (stored at –20°C);
   c. 0.5 m*M* AMC in DMSO (stored at room temperature);
   d. 500 m*M* $CaCl_2$ in water.
4. Purified calpain I (μ-calpain) (from porcine erythrocytes) and purified calpain II (m-calpain) (from porcine kidney) (Calbiochem, San Diego, CA).

3. Method

3.1. Measurement of Calpain Activity in Tubule Extracts

1. After exposure of the tubule suspension to hypoxia, to normoxia, or to ionomycin, as described in Chapter 26, remove 2-mL aliquots of the tubule suspension and recover the tubules by a brief acceleration in a glass tube up to 1000*g* and immediate deceleration.
2. Discard supernatant (*see* **Note 4**).
3. Carefully resuspend the tubule pellet in 2 mL of imidazole-Ca^{2+}-free buffer.
4. Incubate this suspension with 10 m*M* digitonin at 37°C in a shaking water bath for 5 min.

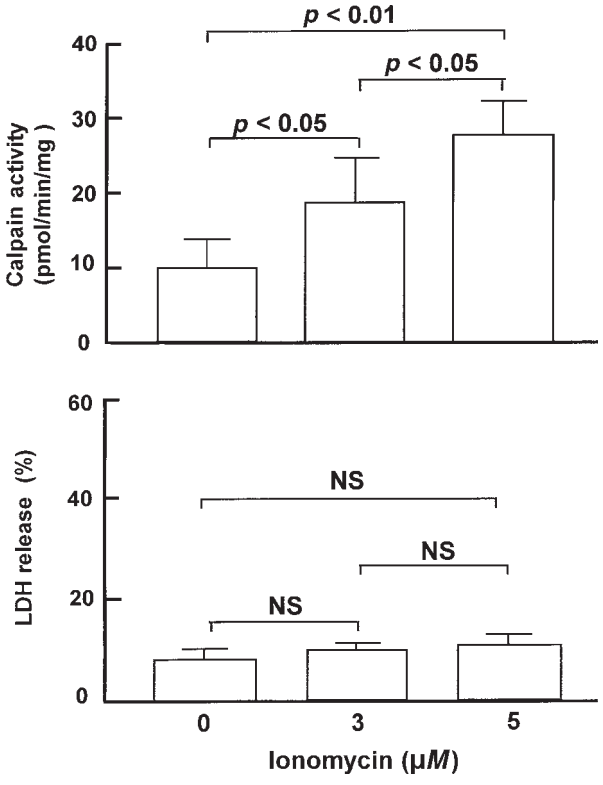

Fig. 1. Dose-dependent effect of ionomycin on calpain activity precedes cell membrane injury. **(Top)** Calpain activity after 2 min of exposure to increasing doses of ionomycin (n = 5). **(Bottom)** Membrane damage as assessed by LDH release does not increase after 2 min of incubation with increasing doses of ionomycin (n = 5). (From **ref. *4*** with permission.)

5. After incubation with digitonin, separate the tubule pellet from the supernatant (which contains released cytosolic calpain) by brief centrifugation to 1000*g* as above.
6. Measure calpain activity in this supernatant (cytosolic extract, 1–2 mg protein/mL) in the presence and absence of calcium as follows:
   a. place 1.45 mL imidazole buffer in a 12 × 75 mm glass tube and add 40 µL of 0.5 *M* $CaCl_2$;
   b. in the control tube, place 1.49 mL of imidazole-Ca^{2+}-free buffer;
   c. add 0.5 mL of cytosolic extract to both tubes;
   d. add 10 µL of 10 m*M* Succ-LLVY-AMC to both tubes;
   e. incubate for 30 min at 37°C in a shaking water bath;
   f. place the samples in ice, and read the fluorescence immediately;
   g. measure the fluorescence of the solutions at 380 nm excitation and 460 nm emission.

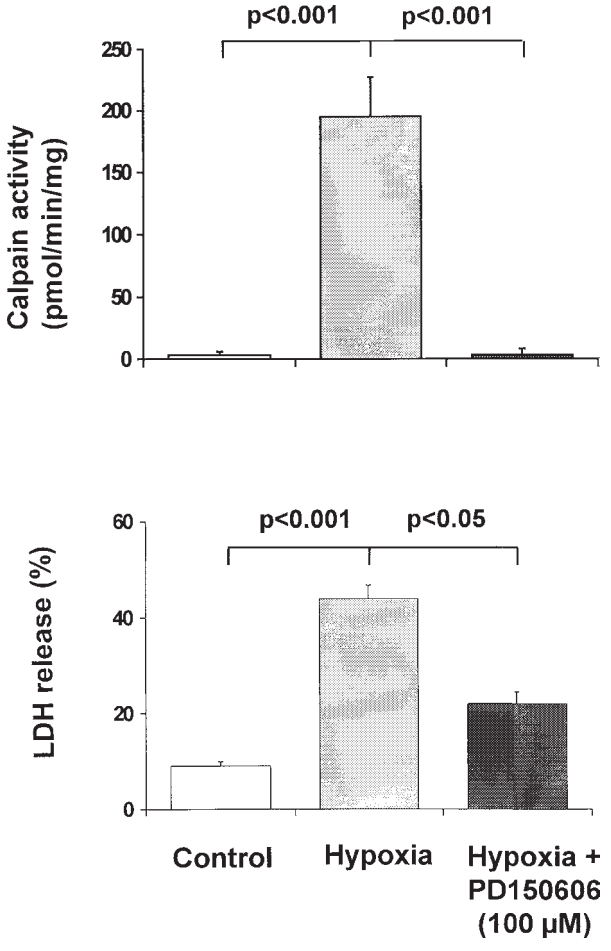

Fig. 2. Calpain activity was measured using the substrate *N*-succinyl-Leu-Leu-Val-Tyr-AMC in proximal tubules. 15 min of hypoxia induces an increase in calpain activity and LDH release. Inhibition of calpain activity with the specific calpain inhibitor PD150606 results in protection against cell membrane damage as assessed by LDH release.

7. Calpain activity is determined as the difference between the Ca^{2+}-dependent fluorescence and the Ca^{2+} independent fluorescence.
8. A standard curve of AMC concentration against fluorescence is determined for each experiment.
9. Calpain activity is expressed as p*M* AMC released/min of incubation/mg of tubule protein.

Notes

1. Digitonin at a concentration of 10 m*M* selectively permeabilizes the plasma membrane but does not destroy lysosomal or mitochondrial membranes of hepatocytes ***(8)*** or mitochondrial membranes of rat proximal tubules ***(10)***. Using the lysosomal dye Lucifer Yellow and a video imaging microscope previously described ***(7)***, it was confirmed that 10 m*M* digitonin selectively permeabilizes the plasma membrane of isolated rat proximal tubules without affecting the lysosomal membrane (unpublished observations). Thus 10 m*M* digitonin releases cytosolic enzymes, including calpain, while keeping the lysosomal membrane intact. Calpain activity in the tubule pellet after digitonin incubation was zero, confirming that all the calpain had been released into the supernatant (cytosolic extract).
2. PD150606 [(2)-3-(4-iodophenyl)-2-mercapto-2-propenoic acid] ***(11)*** (Calbiochem, La Jolla, CA).
3. *N*-succinyl-Leu-Leu-Val-Tyr-AMC, and 7-amido-4-methyl coumarin (AMC), made by Peptide Institute, Osaka, Japan, and obtained from Peptides International, Louisville, KY.
4. We have previously determined that calpain activity does not leak out of the tubules into this medium.

References

1. Moss, D. E, Gutierrez,Y. R., Perez, R. G., and Kobayashi, H. (1991) Simple spectrophotometric assay for calcium-activated neutral proteases (calpains). *Pharmacol. Biochem. Behav.* **39,** 495–497.
2. Gossrau, R., Lojda, Z., Smith, R. E., and Sinha, P. (1988) Recent advances in protease research using synthetic substrates. *Adv. Exp. Med. Biol.* **240,** 191–207.
3. Sasaki, T., Kikuchi, T., Yomuto, N., Yoshimura, N., and Murachi, T. (1984) Comparative specificity and kinetic studies on porcine calpain I and calpain II with naturally occurring peptides and synthetic fluorescent substrates. *J. Biol. Chem.* **259,** 12,489–12,494.
4. Edelstein, C. L., Wieder, E. D., Yaqoob, M. M., Gengaro, P. E., Burke, T. J., and Schrier, R. W. (1995) The role of cysteine proteases in hypoxia-induced renal proximal tubular injury. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 7662–7666.
5. Edelstein, C. L., Yaqoob, M. M., Alkhunaizi, A., Gengaro, P. E., Nemenoff, R. A., Wang, K. K. W., and Schrier, R. W. (1996) Modulation of hypoxia-induced calpain activity in rat renal proximal tubules. *Kidney Int.* **50,** 1150–1157.
6. Edelstein, C. L., Ling, H., Gengaro, P. E., Nemenoff, R. A., Bahr, B. A., and Schrier, R. W. (1997) Effect of glycine on prelethal and postlethal increases in calpain activity in rat renal proximal tubules. *Kidney Int*. **52,** 1271–1278.
7. Kribben, A., Wieder, E. D., Wetzels, J. F., Yu, L., Gengaro, P. E., Burke, T. J., and Schrier, R. W. (1994) Evidence for role of cytosolic free calcium in hypoxia-induced proximal tubule injury. *J. Clin. Invest.* **93,** 1922–1929.
8. Gores, G. J., Nieminen, A. L., Wray, B. E., Herman, B., and Lemasters, J. J. (1989) Intracellular pH during "chemical hypoxia" in cultured rat hepatocytes. Protection by intracellular acidosis against the onset of cell death. *J. Clin. Invest.* **83,** 386–396.

9. Barrett, A. J. and Kirschke, H. (1981) Cathepsin B, cathepsin H, and cathepsin L. [Review]. *Methods Enzymology* **80,** Pt C: 535–561.
10. Wetzels, J. F. M., Yu, L., Wang, X., Kribben, A., Burke, T. J., Schrier, R. W. (1993) Calcium modulation and cell injury in isolated rat proximal tubules. *J. Pharmacol. Exp. Ther.* **267,** 176–180.
11. Wang, K. K. W., Nath, R., Posner, A., Raser, K. D., Buroker-Kilgore, M., Hajimohammadreza, I., Probert, A. W., Marcoux, F. W., Ye, Q., Takano, E., Hatanaka, M., Maki, M., Caner, H., Collins, J. L., Fergus, A., Lee, K. S., Lunney, E. A., Hays, S. J., and Yuen, P. (1996) An alpha-mercaptomercaptoacrylic acid derivative is a selective non-peptide cell-permeable calpain inhibitor and is neuroprotective. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 6687–6692.

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Calpain Activity in Rat Renal Proximal Tubules

An In Situ Assay

Charles L. Edelstein

1. Introduction

Although considerable information about calpain has been obtained from studies using isolated enzyme preparations, this does not permit the dynamic assessment of intracellular protease activity during changes in intracellular Ca^{2+} and pH. Intracellular calpain activity is regulated by complex interactions with the specific inhibitory protein (calpastatin), Ca^{2+} and phospholipids, conditions which are difficult to duplicate in isolated systems. Thus a method to measure calpain activity over time in living cells would be helpful in studying its regulation and physiological roles.

Formation of calpain-specific breakdown products (BDP) of spectrin has been established as a useful measure of calpain activation in intact cells ***(1–3)***. These studies complement the direct in vitro measurements of calpain activity in extracts of proximal tubules described in Chapter 27. Spectrin hydrolysis is assessed by immunoblotting extracts of proximal tubules with antibodies that recognize the 147-kDa calpain-specific spectrin BDP. Specifically, we have used rabbit polyclonal antibodies developed against the amino-terminal peptide (BDPn) and the carboxy-terminal peptide (BDPc) at the calpain cleavage site in α-spectrin. These antibodies were developed by Dr. Ben Bahr ***(1)***.

We have demonstrated that the amount of the calpain-specific spectrin BDPn is increased in Triton X-100-soluble extracts from hypoxic proximal tubules when compared to normoxic proximal tubules **(Fig. 1)**. SBPn is also increased in cytosolic extracts of hypoxic compared to normoxic tubules ***(4)***. Similar results have been obtained with SBPc.

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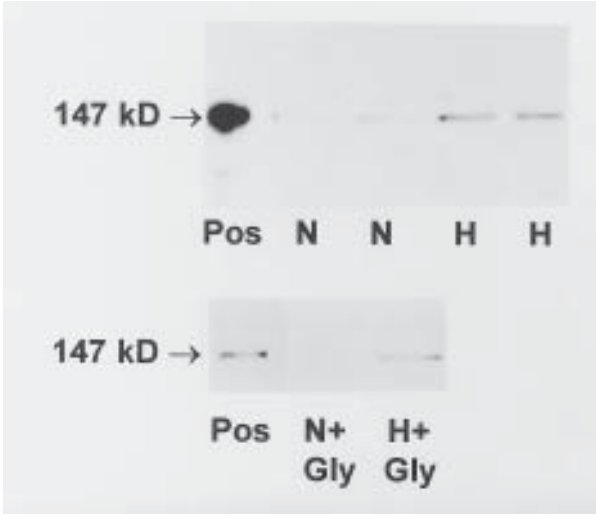

Fig. 1. Calpain specific spectrin breakdown product (147-kDa) (BDP) in Triton X-100-soluble extracts from normoxic (N) and hypoxic (H) (H;15 min) tubules. The formation of BDP increased in hypoxic tissue in the presence and absence of 2 m*M* glycine (Gly). Pos represents positive controls (calcium-treated hippocampal slices). (From **ref. *4***, with permission.)

The association of spectrin with the cytoskeleton was determined by its solubility in 0.25%Triton X-100. The cytoskeleton and its associated proteins remain insoluble in 0.25% Triton X-100 while proteins that dissociate from the cytoskeleton become Triton X-100 soluble ***(5)***. The method of preparation of Triton X-100-soluble and-insoluble fractions as well as membrane and cytosolic fractions of proximal tubules is described below.

2. Materials

1. Cell lysis buffer A: 0.5% Triton X-100, 300 m*M* sucrose, 5 m*M* Tris-HCl, pH 7.4, 2 m*M* EGTA, containing the protease inhibitors 20 µ*M* pepstatin, 20 µ*M* leupeptin, 1000 U/mL aprotonin, 1 m*M* PMSF.
2. Cell lysis buffer B: 0.5% Triton X-100, 50 m*M* 2-glycerophosphate, 100 µ*M* Na_3VO_4, 2 m*M* $MgCl_2$, 1 m*M* EGTA, 1 m*M* DTT, pH 7.2, 20 µ*M* pepstatin, 20 µ*M* leupeptin, 1000 U/mL aprotonin, 1 m*M* PMSF.
3. Lysis buffer C: 50 m*M* Tris -HCl, pH 7.3, 150 m*M* NaCl, 1 m*M* EDTA, 1% Triton X-100.
4. Homogenizing buffer: 50 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA, 2 m*M* 2-ME, 1 m*M* EDTA, 20 µ*M* pepstatin, 20 µ*M* leupeptin, 200 µ*M* PMSF.
5. SDS-PAGE buffer: 5% (w/v), 25% (w/v) sucrose, 5 m*M* Tris-HCl, 5 m*M* EDTA ***(5)***.
6. SDS gel sample buffer (5×): 0.35 *M* Tris-HCl, pH 6.5, 10% glycerol, 0.01% bromophenol blue, 15% SDS, 3.6 m*M* 2-ME.

3. Methods

3.1. Preparation of Triton-Soluble and -Insoluble Fractions of Proximal Tubules: Method 1

Triton-soluble and-insoluble fractions are prepared according to Chen et al. ***(5)***.

1. Expose freshly isolated rat proximal tubules to normoxic or hypoxic conditions for 15 min as described in Chapter 26.
2. Mix 1 mL of tubule suspension with an equal volume of ice-cold cell lysis buffer A and leave for 10 min on ice.
3. Centrifuge the suspension at 4000*g* for 10 min.
4. Separate the supernatant, which contains the 0.25% Triton X-100-soluble proteins, from the pellet, which contains the 0.25% Triton X-100-insoluble proteins.
5. Precipitate these proteins from the supernatant by immediate addition of 3 mL of ice-cold 100% methanol.
6. Centrifuge the mixture at 4000*g* for 10 min.
7. Resuspend the pellets from **step 4** (Triton-insoluble protein) and **step 6** (Triton-soluble protein) in an equal volume (300 µL) of PAGE buffer.
8. Measure the protein concentration in aliquots of both samples by the Lowry method **(Note 1)** ***(6)***.
9. Mix the samples with 75 µL of 5 × SDS gel sample buffer and store at –20°C.
10. Heat samples at 100°C for 3 min before loading onto gels. For spectrin, samples containing 100 µg protein were loaded onto the gel.

3.2. Preparation of Triton-Soluble Fractions of Proximal Tubules: Method 2

1. Freshly isolated rat proximal tubules are exposed to normoxic or hypoxic conditions for 15 min as described earlier.
2. Mix 1 mL of tubule suspension with an equal volume of ice-cold cell lysis buffer B and leave for 10 min on ice.
3. Centrifuge the suspension at 5,000*g* for 10 min.
4. Measure the protein concentration in the Triton-soluble supernatant by the Bradford method (as described in the Bio-Rad protein assay kit) with bovine serum albumin as standard.
5. Mix samples with 0.25 vol of 5 × SDS gel sample buffer and store them at –20°C.
6. Heat samples at 100°C for 3 min before loading onto gels.

3.3. Preparation of Membrane and Cytosolic (Soluble) Fractions of Proximal Tubules

Subcellular fractionation is performed as described by Michel et al. ***(7)***.

1. Collect the whole preparation, ~6 mL, of normoxic and hypoxic proximal tubules (~ 6–8 mg protein) by very brief centrifugation up to 1000*g*.
2. Resuspend the pellets in 2 mL of homogenizing buffer.
3. Disrupt the tubules by freeze-thawing three times followed by homogenization by hand in a Teflon-glass homogenizer.

4. Centrifuge the homogenate at 400*g* for 5 min.
5. Centrifuge the supernatant from the previous step at 100,000*g* at 4°C for 1 h.
6. Keep the high-speed supernatant as the cytosolic (soluble) fraction.
7. Resuspend the pellet in 1 mL of lysis buffer C (which contains 1% Triton X-100) and leave on ice for 30 min.
8. Centrifuge the suspension at 100,000*g* at 4°C for 1 h.
9. Keep the supernatant as the membrane fraction.
10. Add 1 mL of lysis buffer C to the pellet, and resuspend the pellet with the help of sonication.
11. Keep the suspension as the high speed Triton X-100-insoluble cytoskeleton fraction.
12. Mix portions of the cytosolic, membrane, and cytoskeleton fractions with 5 × SDS gel sample buffer.

3.4. Western Blot Procedure for Detection of Spectrin BDP

Western blotting is performed by means of standard protocols ***(8,9)***, using rabbit polyclonal antibodies developed to either side of the calpain cleavage site in α-spectrin (breakdown product specific antibodies, carboxy-terminal peptide [BDPc] and breakdown product specific antibodies, amino-terminal peptide [BDPn], obtained from Dr. Ben Bahr ***[1])***. The blotted proteins are probed with BDPc and BDPn antibodies diluted 1:50, overnight at 4°C or for 1 h at room temperature **(Note 2)**. The secondary antibody is horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin G, used at a dilution of 1:1000. Positive controls for BDP were run with Ca^{2+}-treated hippocampal slices (obtained from Dr. Ben Bahr).

4. Notes

1. It is important to measure the protein concentration before adding SDS gel sample buffer because the high concentration of 2-ME in the latter buffer interferes with protein measurement.
2. The primary antibody can be reused for a second blot, but is sometimes unsuccessful on a third use.

References

1. Bahr, B. A., Tiriveedhi, S., Park, G. Y., and Lynch, G. (1995) Induction of calpain-mediated spectrin fragments by pathogenic treatments in long term hippocampal slices. *J. Pharmacol. Exp. Ther.* **273,** 902–908.
2. Roberts-Lewis, J. M., Savage, M. J., Marcy, V. R., Pinsker, L. R., and Siman, R. (1994) Immunolocalization of calpain I-mediated spectrin degradation to vulnerable neurons in ischemic gerbil brain. *J. Neurosci.* **14,** 3934–3944.
3. Saido, T. C., Yakota, M., Nagao, S., Yamaura, I., Tani, E., Tsuchiya, T., Suzuki, K., and Kawashima, S. (1993) Spatial resolution of fodrin proteolysis in postischemic brain. *J. Biol. Chem.* **268,** 25,239–25,243.

4. Edelstein, C. L., Ling, H., Gengaro, P. E., Nemenoff, R. A., Bahr, B. A., and Schrier, R. W. (1997) Effect of glycine on prelethal and postlethal increases in calpain activity in rat renal proximal tubules. *Kidney Int.* **52,** 1271–1278.
5. Chen, J., Doctor, B., and Mandel, M. J. (1994) Cytoskeletal dissociation of ezrin during renal anoxia: Role in microvillar injury. *Am. J. Physiol.* **36,** C784–C795.
6. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–275.
7. Michel, T., Li, G. K., and Busconi, L. (1993) Phosphorylation and subcellular translocation of endothelial nitric oxide synthase. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 6252–6256.
8. Martin, P., Lixu, D., Niederberger, M., Weigert, A., Tsai, P., StJohn, J., Gines, P., and Schrier, R. W. (1996) Upregulation of endothelial constitutive NOS: A major role in the increased nitric oxide production in cirrhotic rats. *Am. J. Physiol.* **270,** 495–499.
9. Burnette, W. N. (1981) Western blotting: Electrophoretic transfer of proteins from SDS-polyacrylamide to unmodified nitrocellulose and radiographic detection with an antibody to radiolabelled protein A. *Anal. Biochem.* **112,** 195–203.

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Cellular In Vivo Assay of Calpain Activity Using a Fluorescent Substrate

Application to Study of Anoxic Liver Injury

Barry G. Rosser and Gregory J. Gores

1. Introduction

Calpain proteases have been extensively studied over the past 20 years but substrate specificity and activity studies have been limited to investigations using isolated calpain proteases in vitro and in the presence of calcium concentrations beyond the physiologic range found in normal cells (***1–3***). Intracellular autolytic activation, interactions with small acidic phospholipids, and interactions with the calpain inhibitor protein calpastatin all modulate physiologic and pathophysiologic calpain activity (***1–3***). Thus, the development of calpain assays for use in intact cells is critical to the study of the normal and pathological activity of these proteases.

Fluorogenic peptide substrates have proven useful for measurement of protease activity in a variety of experimental models. Formation of an amide linkage between the C-terminal carboxyl group of a peptide and the amino-group of certain fluorogenic molecules quenches their fluorescence, rendering them minimally or nonfluorescent (***4,5***). When the peptide is cleaved from this amino-group by a protease, the fluorophore is released and unquenched, resulting in the generation of fluorescence which can be measured. The increase in fluorescence with respect to time provides an assay of protease activity, with some degree of specificity provided by the peptide (***5***).

Use of previously available peptide probes to measure calpain activity in living cells is limited by the fact that these peptide probes measured both

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intracellular (low calcium environment) and extracellular (high calcium environment) calpain activity in cell systems (*4*). They were also unsuitable for measurement of calpain protease activity in single cells by microscopy or fluorescence-activated cell sorting systems (FACS), since the fluorescent products of intracellular probe hydrolysis could diffuse freely across the plasma membrane into the extracellular environment. While these probes may be used to measure protease activity in populations of cells, this results in some loss of sensitivity for subtle changes in intracellular protease activity.

In order to measure intracellular calpain protease activity in isolated hepatocytes, a substrate peptide fluorophore ideally needs to fulfill four criteria:

- The substrate peptide sequence must be favored by calpains.
- The peptide fluorophore must be membrane-permeable to enter the hepatocyte.
- The substrate should be trapped in the hepatocyte allowing only intracellular cleavage.
- The fluorescent product released by proteolysis should remain trapped within the hepatocyte.

We modified existing methods to develop a technique for measuring calpain activity in individual, isolated hepatocytes (**Fig. 1**) (*5*). The method uses the lipid-soluble calpain substrate peptide, Boc-Leu-Met, conjugated to the GSH binding fluorophore 7-amino-4-chloromethylcoumarin (CMAC), to provide a membrane-permeant calpain substrate. On entering the cell, this compound is conjugated to glutathione (GSH) by glutathione transferase (GST), and therefore trapped in the cell. This allows measurement of intracellular calpain activity, since GSH becomes linked to the coumarin moiety, without affecting its fluorescence. The probe was validated using specific calpain inhibitors, modulation of intracellular pH and calcium, confirmation that substrate levels exceeded the K_m for calpain, and identification of the CMAC-GSH conjugate as the major intracellular fluorescent product generated by hydrolysis of the probe (*5*). The probe has been used to measure the physiologic changes of calpain activity during calcium fluxes, and the pathologic changes in calpain activity associated with anoxic hepatocyte injury (*6,7*).

2. Materials

Almost all of these stock solutions are stored at –20°C and protected from light. Care should be taken that the DMSO concentration in experimental conditions does not exceed 0.5%.

1. 7-Amino-4-chloromethylcoumarin (CMAC): 5 m*M* in DMSO.
2. Boc-Leu-Met-CMAC: 5 m*M* in DMSO.
3. Fura2-acetoxymethylester (Fura-2-AM): 5 m*M* in DMSO.
4. Tetramethylrhodamine-5-dUTP (Molecular Probes; Eugene, OR).
5. Succ-Leu-Leu-Val-Tyr-AMC: 5 m*M* in DMSO (Bachem Bioscience; King of Prussia, PA).

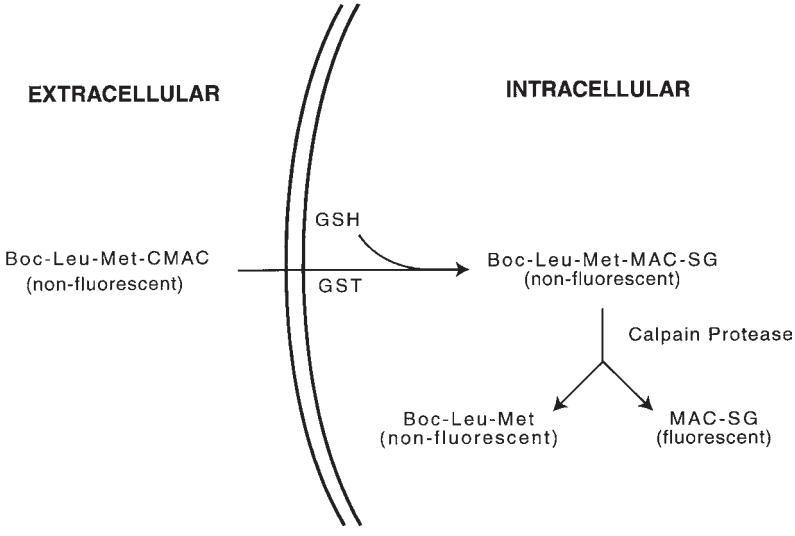

Fig. 1. Measurement of intracellular calpain protease activity using the fluorescent substrate Boc-Leu-Met-CMAC.

6. Cbz-Leu-Leu-Tyr-CHN_2: 10 m*M* in DMSO (Dr. Elliot Shaw, Friedrich Miescher Institut, Basel, Switzerland).
7. cDNA for rat m-calpain and calpastatin in a pT7 plasmid vector (Dr. J. S. Elce, Queen's University, Kingston, ONT, Canada).
8. Krebs-Ringer-HEPES (KRH) buffer: 25 m*M* HEPES, pH 7.4, 115 m*M* NaCl, 1 m*M* KH_2PO_4, 5 m*M* KCl, 1.2 m*M* $MgSO_4$, 2 m*M* $CaCl_2$.
9. The following reagents are made fresh daily in KRH and and stored at 4°C until used: 10 m*M* UTP; 10 µ*M* vasopressin; 10 m*M* phenylephrine; 100 m*M* glucagon; 100 m*M* diethylmaleate (DEM), 10 m*M* adenosine.

3. Methods

3.1. Assessment of Target Cells for Adequate Glutathione Stores

Utilization of Boc-Leu-Met-CMAC requires confirmation of adequate baseline GSH levels and subsequent monitoring for any toxic effects of GSH depletion. We demonstrated that hepatocytes had high GSH levels, allowing for adequate intracellular trapping of the peptide probe to reach its K_m for calpain (***5***). However, other cells may not have adequate GSH or GST, making CMAC conjugation rather than probe hydrolysis the rate-limiting step of fluorescence generation. The following protocol should be utilized before calpain activity assessment to confirm that Boc-Leu-Met-CMAC can be used (*see* **Note 1**).

1. Mount cultured cells of interest on digitized videofluorescence microscopy (DVFM) system stage at 37°C in 1 mL of KRH (or similar buffer solution).

2. Add CMAC (in DMSO) to a final concentration of 20 μ*M* and allow to react for 15 min.
3. Wash cells three times with fresh KRH to remove extracellular CMAC, and resuspend hepatocytes in 1 mL of KRH.
4. Adjust camera settings and measure fluorescence in individual cells using DVFM (excitation 380 nm; a dichroic cutoff of 430 nm, with emission wavelength 510 nm). Obtain the average fluorescence of 50 cells in 5 or more high- power fields.
5. Observe cells for signs of injury (blebbing, shrinkage, or lysis). If injury occurs, Boc-Leu-Met-CMAC will not be useful for this cell type due to toxic effects of GSH depletion. Consider assessment for propidium iodide exclusion if indeterminant changes are observed **(*8*)**.
6. Monitor cells by measuring fluorescence every 60 s for a minimum of 30 min to confirm the stability of intracellular fluorescence. A decrease over time suggests significant photobleaching effects or loss of the CMAC-GSH conjugate from the cell (through leakage or active transport) and may identify limitations to the use of Boc-Leu-Met-CMAC in the cell model.
7. Using the same DVFM settings, repeat the protocol in cells pretreated with 1 m*M* diethylmaleimide (DEM) for 30 min to deplete GSH before mounting on the DVFM stage. This treatment with DEM removes more than 95% of cellular GSH from hepatocytes **(***see* **Note 2)**.

3.2. Measurement of Calpain Activity in Individual Cultured Hepatocytes Using Boc-Leu-Met-CMAC

1. Isolate hepatocytes from rat liver (or obtain other cells of interest) and culture them for 2–4 h on collagen-coated coverslips **(*5*)**.
2. Mount cells on DVFM stage at 37°C in 1 mL of KRH **(Subheading 3.1., step 1.)**.
3. Measure fluorescence using DVFM (excitation 380 nM; a dichroic cutoff of 430 nm with emission wavelength 510 nm) in individual cells over 2 min before Boc-Leu-Met-CMAC exposure to obtain a baseline. Adjust camera settings such that autofluorescence of hepatocytes (or cells of interest) is barely detectable, with defined cell outlines. Include a minimum of 5 cells in the field as only cells in the initial field evaluated can be used for accurate calpain activity assessment. Once this baseline DVFM setting is established, it must remain unchanged for that day's experiments.
4. Add Boc-Leu-Met-CMAC (in DMSO) to a final concentration of 20 μ*M*.
5. Measure change in fluorescence (protease activity) over time intervals of 30–60 s to minimize photobleaching **(Fig. 2)**.

3.2.1. Method I: Comparison of Calpain Activity Between Groups of Cells Exposed to Different Experimental Manipulations

1. Calculate the average calpain activity in the cells (minimum of 5 cells). Repeat above in a minimum of two additional separate control hepatocyte isolations to obtain average baseline calpain activity, expressed as the change in fluorescence (arbitrary units) per min.

2. Repeat the protocol in hepatocytes exposed to experimental manipulation of interest, and compare mean activity between control and treated hepatocytes.

3.2.2. Method II: Comparison of Calpain Activity in Single Cells Before and After Experimental Manipulation

1. After 5 min of fluorescence measurements to allow assessment of baseline calpain activity in individual hepatocytes, add reagent of interest to hepatocytes on stage and measure change in fluorescence over the subsequent 10–15 min in the same cells.
2. Assess calpain activity postintervention as compared to baseline calpain activity in each individual cell to determine relative calpain activity associated with the experimental manipulation of interest (peak activity postintervention divided by mean activity preintervention [percent of basal calpain activity]). Take care to insure that the experimental reagent or manipulation (e.g., hormone agonist or toxin exposure) does not have fluorescent properties that will modify measured fluorescence (e.g., compounds that have UV-induced fluorescence themselves).
3. Repeat experimental protocol in a minimum of 3 separate cell isolates with a minimum of 15 cells evaluated.

3.3. Measurement of Calpain Activity Using Boc-Leu-Met-CMAC or Succ-Leu-Leu-Val-Tyr-AMC in Cell Suspensions

1. Isolate hepatocytes from rat liver (or cells of interest).
2. Incubate cells (500,000/mL) at 37°C in 1 mL of KRH.
3. Measure fluorescence in a fluorometer (excitation 380 nm; emission 430–510 nm) over 2–3 min to confirm a stable baseline.
4. Add Boc-Leu-Met-CMAC or Succ-Leu-Leu-Val-Tyr-AMC (in DMSO) to a final concentration of 20 μ*M*.
5. Wait 2 min to allow uptake of Boc-Leu-Met-CMAC or equilibration of Succ-Leu-Leu-Val-Tyr-AMC across cell membrane. Subsequently, measure change in fluorescence (protease activity) over time intervals of 30–60 s. Determine relative calpain activity by measuring the linear change in fluorescence over 5–10 min.
6. Repeat protocol in hepatocytes exposed to experimental manipulation of interest and compare to activity measured under baseline conditions.
7. Express activity as one of the following:
   a. rate of fluorescence change (activity) compared to untreated control cells, expressed as percent of baseline calpain activity;
   b. activity per μg protein;
   c. activity per 100,000 cells.

3.4. Use of Boc-Leu-Met-CMAC to Assess Calpain Activity in Hepatocyte Physiology and Pathophysiology: Utility for Study of Calcium Signaling and Anoxic Liver Injury

Based on our preliminary findings, we were satisfied that Boc-Leu-Met-CMAC could be used to measure calpain activity in isolated liver cells (**5**). We

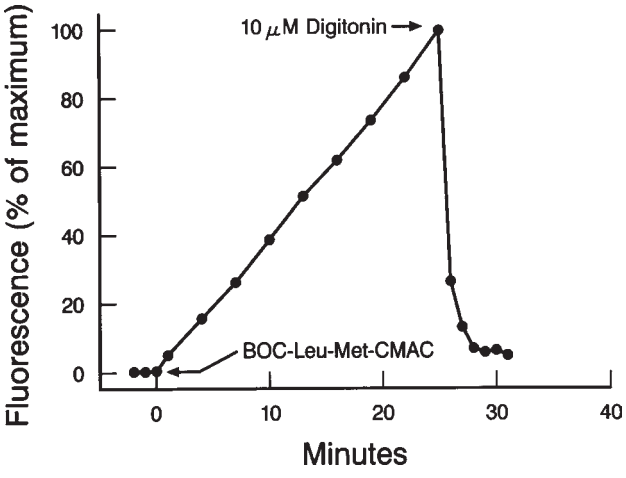

Fig. 2. Intracellular fluorescence increases in a linear manner after addition of Boc-Leu-Met-CMAC and is released from the cell by digitonin. Cultured hepatocytes in KRH at 37°C were mounted on the microscope stage. Following addition of Boc-Leu-Met-CMAC (10 μ*M*) intracellular fluorescence was measured using DVFM. After 25 min, digitonin (10 μ*M*) was added to the buffer to release cytosolic contents. Intracellular fluorescence was quantitated for an additional 5 min showing complete release of fluorescence products accompanying cytosol release. Data represent the mean ± standard error of five experiments. (From **ref. *5*** with permission.)

subsequently tested whether the probe could be used in the study of physiologic and pathophysiologic calpain activity.

3.4.1. Calcium Signaling and Boc-Leu-Met-CMAC Hydrolysis

We hypothesized that physiologic increases in $[Ca^{2+}]_i$ induced by calcium-mobilizing receptor agonists and IP_3-mediated calcium release might increase hepatocyte calpain activity. If this were true, increases in $[Ca^{2+}]_i$ induced by these agonists should increase the rate of Boc-Leu-Met-CMAC hydrolysis. Micromolar concentrations of calcium mobilizing agonists adenosine triphosphate (ATP), uridine triphosphate (UTP), vasopressin and phenylephrine (through purinergic [ATP, UTP], vasopressin and adrenergic receptors, respectively) reliably stimulate $[Ca^{2+}]_i$ increases in hepatocytes as measured using DVFM and the calcium fluorophore Fura-2-AM **(Fig. 3)** (***5,6,9,10***). We noted increased calpain activity with ATP stimulation (***5***). However, as ATP may induce nonspecific permeabilization of cell membranes that may affect probe retention, we decided to use the other calcium-mobilizing agents to study calpain activity (***11***). We studied the response of hepatocytes to the calcium mobilizing agents vasopressin, UTP, and phenylephrine. Glucagon and adenos-

ine, which stimulate cyclic AMP release but not calcium mobilization, were used as noncalcium mobilizing controls (***6***).

1. Isolate, culture, and mount hepatocytes on the DVFM stage with appropriate optical settings in KRH as outlined previously.
2. Assess calpain activity **(Subheading 3.2.2., step 2.)** before and after addition of vasopressin (final concentration 100 n*M*).
3. As a control, preincubate hepatocytes with the membrane permeant specific calpain inhibitor Cbz-Leu-Leu-Tyr-CHN_2 (100 μ*M*) for 30 min.
4. Add vasopressin as above, and assess calpain activity.
5. The experiments may be repeated using other calcium mobilizing agents (50 μ*M* phenylephrine, 100 μ*M* UTP) and non-calcium mobilizing agents (100 μ*M* adenosine, 1 m*M* glucagon) in the presence and absence of the calpain inhibitor Cbz-Leu-Leu-Tyr-CHN_2.

As seen in **Fig. 3**, a transient, approximately threefold increase above baseline in maximal rate of probe hydrolysis was noted under conditions that generated increased $[Ca^{2+}]_i$. Glucagon and adenosine had no effect on probe hydrolysis. No increase of rate of fluorescence generation was noted in response to calcium mobilization when hepatocytes were pretreated with the calpain inhibitor Cbz-Leu-Leu-Tyr-CHN_2, preventing an increase in calpain activity in response to these calcium mobilizing agonists (***12***). This observation suggested that the observed changes in the rate of fluorophore generation were related to calpain protease activity.

These data suggest that calpain activity is increased following exposure to calcium-mobilizing receptor agonists and may play a role in $[Ca^{2+}]_i$-mediated signaling processes in hepatocytes; and that Boc-Leu-Met-CMAC may be a useful tool to evaluate potential physiologic roles of calpains in mammalian cells.

3.4.2. Application of Boc-Leu-Met-CMAC Assay to Study Pathologic Calpain Activity in Anoxic Liver Cell Injury

1. Induction of anoxia in hepatocytes in suspension:
   a. Isolate hepatocytes from rat livers by means of collagen perfusion and suspend them immediately in KRH.
   b. Place the hepatocytes in an anaerobic chamber similar to standard culture chambers used in microbiology labs for anaerobic bacterial culture and rendered anoxic ($pO_2 < 1.5$ torr).

   Carry out experimental manipulations with the reagents of choice to determine the effect of anoxia on large numbers of hepatocytes in suspension.
2. Induction of anoxia in hepatocytes cultured on slides:
   a. Isolate hepatocytes from rat livers by means of collagen perfusion, culture them on collagen-coated coverslips and mount on a fluorescence microscope stage in a custom modified gas-impermeant Leiden chamber in KRH.
   b. Render the cells anoxic in the chamber ($pO_2 < 5$ torr) by perifusion with N_2-saturated incubation buffers, allowing assessment of individual hepatocytes under conditions of anoxia (*see* **Note 3**).

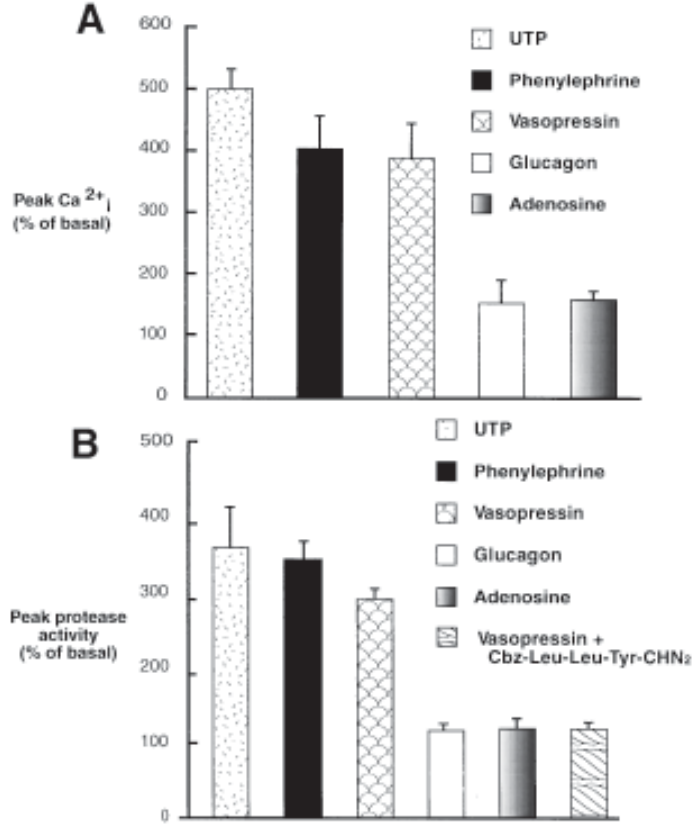

Fig. 3. Effect of hormone agonists on $[Ca^{2+}]_i$ and Boc-Leu-Met-CMAC fluorophore generation in hepatocytes. **(A)** Maximum $[Ca^{2+}]_i$ (% of basal $[Ca^{2+}]_i \pm$ standard error) was measured in hepatocytes loaded with fura-2-AM (10 μ*M* for 30 min) within 5 min of exposure to UTP (100 μ*M*), phenylephrine (20 μ*M*), and vasopressin (10 nM), along with glucagon (100 n*M*) and adenosine (100 μM). **(B)** Hepatocytes were exposed to Boc-Leu-Met-CMAC (20 μ*M*) and baseline protease activity was measured. Hepatocytes were then exposed to the agonists listed above and peak calpain activity determined over the subsequent 5–10 min (% basal activity ± standard error). Hepatocytes were then preincubated with the calpain inhibitor Cbz-Leu-Leu-Tyr-CHN_2 (100 μ*M*) prior to measurement of calpain activity before and after vasopressin exposure to confirm that the increased fluorophore generation was related to calpain protease activity (From **ref. *6***.)

3.4.3. Measurement of Calpain Activity in Anoxic Hepatocytes

1. Maintain hepatocyte suspensions in anoxic conditions for 30 min in the presence of 1 μ*M* propidium iodide to confirm that cell necrosis is minimal (*see* **Note 4**).
2. Assess calpain activity in the cell suspension after 30 min of anoxia using Succ-Leu-Leu-Val-Tyr-AMC **(Subheading 3.3.)**, in comparison with control aerobic

hepatocytes. This will determine if calpain activity increases before the onset of hepatocyte necrosis during anoxia.
3. Inspect hepatocytes cultured on slides after exposure to anoxia in the modified Leiden chamber for 30 min to rule out necrosis (loss of granularity of cytoplasm, significant cell membrane blebbing) (***8***).
4. After 30 min anoxia in the Leiden chamber, stop perifusion and inject Boc-Leu-Met-CMAC into the anaerobic chamber.
5. Assess calpain activity in individual, viable hepatocytes (**Subheading 3.2.2., step 1**) and compare to activity in hepatocytes perifused with oxygenated KRH.

3.5. Measurement of Calpain and Calpastatin Transcriptional Activity Using Fluorescence In Situ Hybridization

To assess whether the increase in calpain activity measured during anoxia was due to increased transcription of calpain mRNA or to decreased transcription of calpastatin mRNA, a technique was developed for measurement of calpain aand calpastatin transcriptional activity in isolated cultured hepatocytes using fluorescence *in situ* hybridization and a novel ratio imaging technique.

1. Label cDNA probes for m-calpain and calpastatin with the Prime-It Fluor fluorescent labeling kit (Stratagene, La Jolla, CA).
2. Denature the probes at 95°C for 10 min together with random primers from the kit, and cool slowly to permit annealing of the primers.
3. Elongate and fluorescently label the probes by incubation in the manufacturer's nucleotide buffer, containing 80 µ*M* tetramethylrhodamine-5-dUTP and 10 U of the exonuclease-deficient Klenow fragment of DNA polymerase I for 30 min at 37°C.
4. Stop reaction by addition of EDTA, and separate the labeled cDNA probes from tetramethylrhodamine-5-dUTP by addition of sodium acetate and ethanol precipitation.
5. Render isolated cultured hepatocytes anoxic by perifusion with N_2-saturated incubation buffers in the modified Leiden chamber, fix with fresh 4% paraformaldehyde in PBS for 20 min at room temperature, and wash with 1 mL of PBS.
6. Carry out fluorescence in situ hybridization measurement of calpain and calpastatin probe binding to hepatocyte RNA as described (***15***), using DVFM and a ratio imaging strategy.
7. Determine the ratio of the specific fluorescence of the hybridized probe to the fluorescence of total cellular DNA, after subtraction of background fluorescence:
   a. Quantitate rhodamine-labeled cDNA hybridized to its mRNA with a rhodamine filter set (excitation wavelength 546 nm, emission wavelength 590 nm).
   b. Measure total cellular DNA by incubating the cells with 1 pg/mL 4',6'-diamidino-2-phenylindole (DAPI) for 5 min, and quantitating DAPI fluorescence with a UV filter set (excitation and emission wavelengths of 360 and 440 nm, respectively).
   c. Determine background fluorescence (autofluorescence and non-specific binding of rhodamine-conjugated dUTP): (i) by pretreating the cells with 100 µg/mL RNase A at 37°C for 30 min before the hybridization procedure; (ii) by

performing hybridization with the nucleotide buffer containing tetramethylrhodamine-conjugated dUTP in the absence of cDNA. Both of these methods yielded virtually identical values for nonspecific fluorescence.

3.6. Measurement of $[Ca^{2+}]_i$ Using Fura-2-AM

1. Incubate isolated cultured hepatocytes with the calcium fluorophore Fura-2-AM (5 μ*M* for 30 minutes) and then wash the cells 3 times with KRH.
2. Mount Fura-2-AM loaded hepatocytes on the microscope stage in the modified Leiden chamber and render them anoxic over 60 min by N_2 perifusion.
3. Measure cytosolic calcium concentration $[Ca^{2+}]_i$ using DVFM during anoxia.
4. Compare peak $[Ca^{2+}]_i$ levels in anoxic cells to levels in hepatocytes perifused over a similar time period with aerobic KRH (***5***).

3.7. Results

Previous studies in our laboratory had demonstrated that during lethal anoxic cell injury, hepatocytes showed increased nonlysosomal, calcium-dependent protease activity (***8***), and inhibition studies suggested that calpains were involved (***16***). During anoxic hepatocyte injury, an increase in calpain activity could be caused by a variety of mechanisms, including:

- Increased calpain transcription and translation;
- Decreased calpastatin transcription and translation;
- Increased $[Ca^{2+}]_{i;}$
- Release of small acidic phospholipids which lower $[Ca^{2+}]_i$ requirements for calpain activation. These four possibilities were addressed experimentally.

1. Calpain activity increased early during anoxic hepatocyte injury, before loss of cell viability, and was not a manifestation of postnecrotic calpain release and activation in the high calcium, extracellular environment **(Fig. 4)**.
2. Despite the observed increase in calpain activity after 30 min of anoxia, no changes in calpain or calpastatin transcription were detected by fluorescence *in situ* hybridization (FISH) analysis and this observation was confirmed using standard Northern blot methods *(7)*. Pre-incubation of hepatocytes with the protein synthesis inhibitor cycloheximide (100 μ*M*) and the transcriptional inhibitor actinomycin D (5 μg/mL) before anoxia also failed to prevent the increase in calpain activity observed after 30 min of anoxia **(Table 1)** confirming that the observed increase in calpain activity during hepatocyte anoxia was not secondary to changes in transcription or translation of calpain or calpastatin mRNA *(7)*.
3. Significant changes in $[Ca^{2+}]_i$ were not observed in hepatocytes perifused with either anoxic or aerobic buffers *(7)*.
4. Much further work suggests that anoxia-induced calpain activation measured by our assays is secondary to increased phospholipase activity but the exact mechanisms remain unclear **(Table 1) (7)**. Taken together, the data presented demonstrate the utility of intracellular calpain measurement with Boc-Leu–Met-CMAC in the study of pathophysiology of calpains and support the hypothesis

that calpain activity is stimulated during anoxic hepatocyte injury by release of phospholipid degradation products.

4. Notes

1. Despite its utility as a measure of calpain protease activity in hepatocytes, Boc-Leu–Met-CMAC has characteristics that may limit its utility in some cell types, in tissue specimens, and under certain experimental conditions **(Table 2)**.
   Boc-Leu–Met-CMAC must reach the intracellular environment to allow measurement of calpain activity. If large amounts of extracellular GSH or sulfhydryl groups are present (as in tissue specimens), and especially if extracellular GST is present, Boc-Leu–Met-CMAC will bind them extracellularly before reaching the cell of interest and would be unavailable for concentration in the cell and calpain hydrolysis. Boc-Leu–Met-CMAC is unsuitable for measurement of extracellular calpain activity or calpain activity in cell fractions (e.g., cytosolic or membrane fractions). Intact cells with GSH and GST are required to concentrate Boc-Leu–Met-MAC-SG to a level close to the K_m for calpains (>1 m*M*). For extracellular assays, succ-Leu–Leu-Val-Tyr-AMC or other fluorogenic substrates with a lower K_m for calpain (1–10 μ*M*) are required.

   Boc-Leu–Met-CMAC requires adequate amounts of cellular GSH (>1–2 m*M*) to reach concentrations close to the K_m for calpains. In cells with low GSH content or under experimental conditions leading to GSH depletion (e.g., oxidative stress), the conjugate Boc-Leu–Met-MAC-SG will not reach a sufficient concentration, and will not be useful for accurate measurement of intracellular calpain activity. Preincubation with membrane-permeable GSH esters may be considered in such cell types but has not been validated with this probe. In addition, the conjugation of Boc-Leu–Met-CMAC to GSH must be rapid, so that its formation is not rate limiting in generation of intracellular fluorescence. Since different cells may have different levels of GST activity, each cell type should be assessed for GSH content and its ability to conjugate CMAC to GSH.
   As Boc-Leu–Met-CMAC binds to GSH in cells, it depletes cells of GSH reserves. No effect on viability in hepatocytes was noted in our studies, probably because hepatocytes are rich in GSH and other anti-oxidant systems ***(17)***. However, cells susceptible to injury from GSH depletion are clearly at risk of toxicity induced by Boc-Leu–Met-CMAC and this must be ruled out **(***see* **Note 2)**. Again, preincubation of cells with GSH esters may be considered to overcome this problem. Some cells (especially tumor cells) have increased organic anion and drug transporter activity ***(5,18)***. Boc-Leu–Met-CMAC, Boc-Leu–Met-MAC-SG or MAC-SG may be excreted from the cytosol in these cells. If transport out of the cell is significant, measurements of protease activity become too erratic to be useful. We confirmed stable MAC-SG retention in isolated hepatocytes over time periods of at least 20–30 min ***(5)***. This time period is adequate for assessment of calpain activity in most cells. Therefore, MAC-SG retention after CMAC loading should be assessed in all cells before using Boc-Leu–Met-CMAC for calpain assessment.

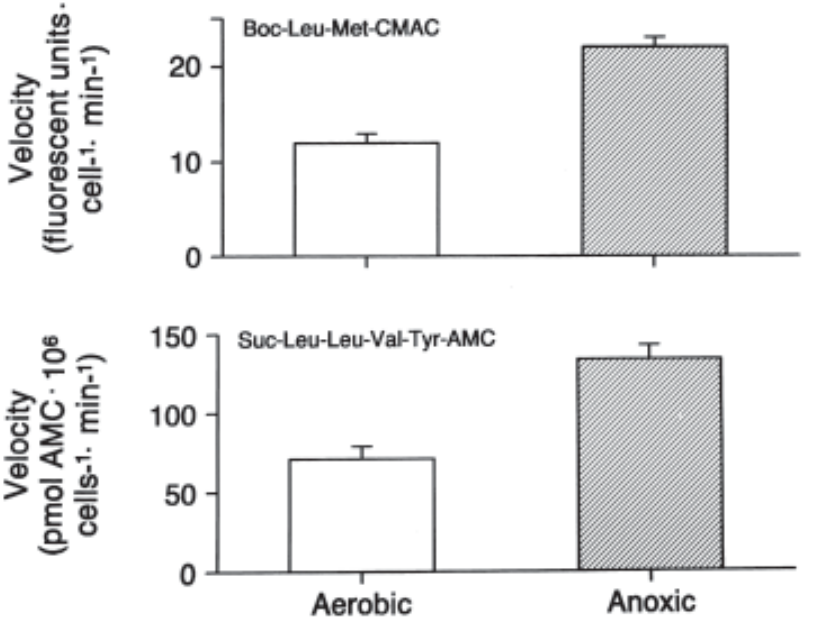

Fig. 4. Calpain catalytic activity is stimulated during anoxia. **(A)** Cultured hepatocytes were mounted on the microscope stage in a Leiden chamber. Cells were perifused with KRH at 37°C, and anoxia ($_{P}0_2$ <5 mmHg) was achieved by perifusion (1 mL/min) with N_2-saturated KRH. After 30 min of perifusion, Boc-Leu-Met-CMAC (20 μ*M*) was added and intracellular fluorescence was measured using DVFM. **(B)** Hepatocyte suspensions (10^5 cells/mL) in KRH were incubated under aerobic conditions or in an anaerobic chamber at 37°C. After 30 min of incubation, calpain activity was measured by adding Succ-Leu-Leu-Val-Tyr-AMC (20 μ*M*) and measuring the rate of free AMC generation over a period of 10 min fluorometrically (From reference *7*, with permission.)

Although the MAC-SG fluorophore did not undergo significant photobleaching in our initial studies, we did note some photobleaching in isolated hepatocytes with intense or frequent UV exposure (>3 exposures per minute) (*B.G. Rosser, unpublished observations*). This can be avoided by decreasing the frequency of image acquisition and using appropriate interference filters to lower excitation light intensity, along with sensitive cameras that do not require intense fluorescence for detection. Unfortunately, Boc-Leu–Met-CMAC does not allowing ratio imaging to control for changes of photobleaching, leak of substrate or leak of product from the cell.

Determination of the subcellular site of calpain activity, an important issue relating to the activation of calpains in living cells, is not possible with Boc-Leu–Met-CMAC. Since Boc-Leu–Met-CMAC is rapidly conjugated by GST to GSH

Table 1
Calpain Activity in Anoxic and Aerobic Hepatocytes During Pharmacologic Modification of Protein Synthesis, Transcription and Phospholipase Activity

	Velocity of Succ-Leu-Leu-Val-Tyr-AMC hydrolysis, p*M* AMC × 10^6 cells^{-1} min^{-1}			
Treatment	Aerobic	Anoxic	Difference, %	*P* value
No treatment	71 ± 8	133 ± 9 [a]	+87	<.05
Cycloheximide	81 ± 6	125 ± 9 [a]	+54	<.05
Actinomycin D	69 ± 7	120 ± 7 [a]	+74	<.05
Fluphenazine	67 ± 7	71 ± 11 [b]	+6	
Melittin	432 ± 25 [a]			NS

Cell suspensions (10^5/mL) were incubated in 3 mL of KRH buffer at 37°C in an anaerobic chamber or in presence of atmospheric oxygen. In selected experiments, 5 µg/mL of actinomycin D, 100 µ*M* cycloheximide, or 100 µ*M* fluphenazine was present throughout the incubation period. In addition, hepatocytes were incubated with 1 µg/mL melittin in the presence of atmospheric oxygen. After 30 min of incubation, the fluorogenic calpain substrate Succ-Leu-Leu-Val-Tyr-AMC (20 µ*M*) was added. AMC fluorescence was quantitated over the next 10 min, and this initial rate of peptidyl-AMC hydrolysis was interpreted as a measure of calpain activity.

[a] *P*<.05 compared to no treatment aerobic hepatocytes.
[b] *P*<.05 compared to no treatment anoxic hepatocytes.
Modified from **ref. *7***.

(both of which are predominantly found in the cytoplasm), most Boc-Leu–Met-MAC-SG available to calpain should be present in the cytosolic compartment. Supporting this assumption, selective permeabilization of the plasma membrane after loading with Boc-Leu–Met-CMAC failed to demonstrate any fluorescent product generation (probe hydrolysis) in residual intact mitochondria or nuclei *(5)*. Even if cytosolic Boc-Leu–Met-MAC-SG were cleaved in specific cellular compartments (e.g., at the plasma membrane or in the nucleus), the MAC-SG product is soluble and would rapidly diffuse throughout the cytoplasm generating the same diffuse pattern of fluorescence in the cell regardless of the initial site of calpain activity.

The specificity of Boc-Leu–Met-CMAC and other fluorogenic peptides needs to be documented in the cell system of interest by confirming suppression of MAC-SG generation by specific calpain inhibitors, and lack of suppression by noncalpain inhibitors. Boc-Leu–Met-CMAC does not differentiate between specific subtypes of calpain proteases (µ, m, or other tissue specific calpains). In addition, some overlap between inhibitor and peptide specificity is noted between calpains, the proteasome, and lysosomal cathepsin L ***(12)***.

2. If CMAC fluorescence in cells exposed to DEM is greater than 25% of baseline CMAC fluorescence (i.e., CMAC fluorescence in cells not depleted of GSH), it is unlikely that the Boc-Leu-Met-CMAC probe will be useful in the cell being studied because of a low cellular GSH content or inadequate GST activity. If in doubt, repeat

Table 2
Potential Limitations of Boc-Leu-Met-CMAC Assay

Extracellular binding with failure to reach intracellular environment
Unsuitable for extracellular calpain measurement
Insufficient conjugation to GSH
Insufficient cellular GSH
Insufficent GST activity
Oxidative stress injury to susceptible cells
Excretion of probe from some cell lines
Photobleaching with intense illumination
Inability to discriminate subcellular site of calpain activity
Inability to differentiate calpain subtype activity
Specificity tested in hepatocytes only

protocol using isolated hepatocytes to confirm that the cell line of interest has similar GSH content (and/or functional GST activity) to that of hepatocytes.

3. Both experimental models have been well described and validated by us (***8***,***13***,***14***).
4. Results suggested < 5% hepatocyte necrosis over this time interval despite the presence of anoxia (***7***,***8***).

Acknowledgments

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References

1. Saido, T. C., Sorimachi, H., and Suzuki, K. (1994) Calpain: New perspectives in molecular diversity and physiological-pathological involvement. *FASEB J.* **10,** 814–822.
2. Suzuki, K. and Ohno, S. (1990) Calcium activated neutral protease: Structure-function relationships and functional implications. *Cell Struct. Function* **15,** 1–6.
3. Mellgren, R. L. (1987) Calcium-dependent proteases: an enzyme system active at cellular membranes? *FASEB J.* **1,** 110–115.
4. Sasaki, T., Kikuchi, T., Yumoto, N., Yoshimura, N., and Murachi, T. (1984) Comparative specificity and kinetic studies on porcine calpain I and calpain II with naturally occurring peptides and synthetic fluorogenic substrates. *J. Biol. Chem.* **259,** 12,489–12,494.
5. Rosser, B. G., Powers, S. P., and Gores, G. J. (1993) Calpain activity increases in hepatocytes following addition of ATP. *J. Biol. Chem.* **268,** 23,593–23,600.
6. Rosser, B. G. and Gores, G. J. (1993) Calpain protease activation: A universal event in calcium- mediated signal transduction? *Hepatology* **18,** A437.

7. Arora, A. S., de Groen, P., Emori, Y., and Gores, G. J. (1996) A cascade of degradative hydrolase activity contributes to hepatocyte necrosis during anoxia. *Am. J. Physiol.* **270,** G238–G245.
8. Bronk, S. F. and Gores, G. J. (1993) pH dependent, non-lysosomal proteolysis contributes to lethal anoxic cell injury of rat hepatocytes. *Am. J. Physiol.* **264,** G744–G751.
9. Grynkiewicz, G., Poenie, M., and Tsien, R. (1985) A new generation of calcium indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **102,** 1030–1038.
10. Berridge, M. J. (1993) Inositol triphosphate and calcium signaling. *Nature* **361,** 315–325.
11. Zoeteweij, J. P., van de Water, B., deBont, H. J., Mulder, G. J., and Nagelkerke, J. F. (1993) Calcium-induced cytotoxicity in hepatocytes after exposure to extracellular ATP is dependent on inorganic phosphate. *J. Biol. Chem.* **268,** 3384–3388.
12. Anagli, J., Hagmann, J., and Shaw, E. (1991) Investigation of the role of calpain as a stimulus-response mediator in human platelets using new synthetic inhibitors. *Biochem. J.* **274,** 497–502.
13. Dickson, R. C., Bronk, S. F., and Gores, G. J. (1992) Glycine cytoprotection during lethal hepatocellular injury from adenosine triphosphate depletion. *Gastroenterology* **102,** 2098–2107.
14. Fujii, Y., Johnson, M. E., and Gores, G. J. (1994) Mitochondrial dysfunction during anoxia/reperfusion injury of sinusoidal endothelial cells. *Hepatology* **20,** 177–185.
15. Nouri-Aria, K. T., Arnold, J., Davison, F., Portman, B. C., Meager, A., Morris, A. G., Alexander, G. J., Eddleston, A. L. and Williams, R. (1991) Hepatic interferon-α gene transcripts and products in liver specimens from acute and chronic hepatitis B virus infection. *Hepatology* **13,** 1029–1034.
16. Nichols, J. C., Bronk, S. F., Mellgren, R. L., and Gores, G. J. (1994) Inhibition of non-lysosomal calcium-dependent proteolysis by glycine during anoxic injury of rat hepatocytes. *Gastroenterology* **106,** 168–176.
17. Rosser, B. G., and Gores, G. J. (1995) Liver cell necrosis: Cellular mechanisms and clinical implications. *Gastroenterology* **108,** 252–275.
18. Zimniak, P., and Awasthi, Y. C. (1993) ATP-dependent transport systems for organic anions. *Hepatology* **17,** 330–339.

30

Calpain Methods in Hepatic Ischemia–Reperfusion Injury

David Sindram and Pierre-Alain Clavien

1. Introduction

Ischemia–reperfusion (I/Rp) injury to the liver occurs after liver transplantation, shock or in surgical procedures in which vascular supply to the liver is temporarily abrogated. The extent of I/Rp-mediated injury is dependent on several factors including duration of ischemia, and in liver transplantation the injury depends also on the temperature of the liver and the type of preservation solution used ***(1)***. A variety of proteases has been found to be important in mediating I/Rp injury in several organs. Cytoplasmic calcium concentrations rise dramatically during both ischemia and reperfusion, mainly due to release from intracellular organelles ***(2–7)***. Increased cytoplasmic calcium concentrations activate several calcium-dependent systems including calpain-like proteases ***(8–11)***. Calpain-like proteolytic activity has recently been found to be involved in the pathogenesis of the ischemic brain ***(12)***, heart ***(13)***, kidney ***(14),*** and liver ***(15)***. Other factors may also contribute to increased calpain-like activities such as increased synthesis of calpains, inhibition by calpastatin, or increased phospholipase activity ***(16)***.

Increased calpain-like activities have been documented after reperfusion of transplanted human livers, and were found to correlate with the degree of graft injury after reperfusion ***(17)***. In a rat model of liver transplantation, we studied the role of calpains as a mediator of injury during each phase of I/Rp injury (cold storage, rewarming, and reperfusion) ***(15)***. Calpain-like protease activity, measured as described below, was found to increase during each phase of transplantation with the most significant increase occurring rapidly after reperfusion **(Fig. 1)**. The

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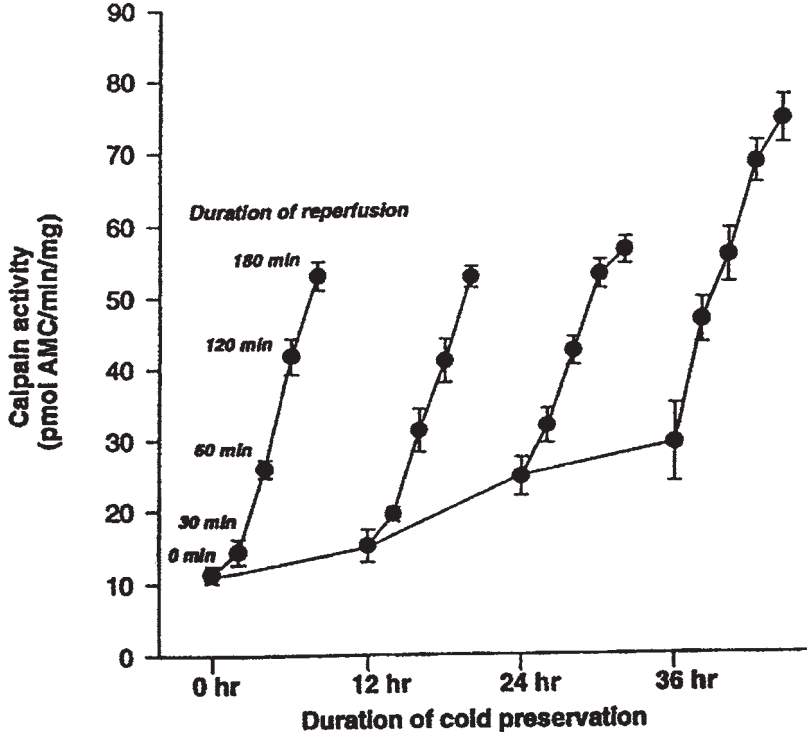

Fig. 1. Calpain activity was assessed in livers perfused in an isolated perfused rat liver (IPRL) system after different periods of cold ischemia. Significant increase in calpain activity was noted after 30 min of reperfusion in each cold ischemia group. At each duration of reperfusion calpain activity was significantly higher in livers preserved for 24 and 36 h compared with unpreserved or 12 h preserved livers ($n = 3$ for each cold ischemia time; $p < .01$, ANOVA). (This graph is reproduced with permission from the National Academy of Sciences of the U.S.A. ***[15]***).

use of a specific calpain inhibitor (Cbz-Val-Phe methyl ester) significantly reduced liver injury and improved animal survival following orthotopic liver transplantation.

Evidence was provided recently that apoptosis of the sinusoidal endothelial cell (SEC) occurring upon reperfusion of the liver graft represents a critical mechanism of injury affecting both graft and animal survival ***(18,19)***. As calpain-like activity is known to induce apoptosis under various conditions, we tested the hypothesis that increased calpain-like activity during each phase of transplantation is responsible for SEC apoptosis ***(20,21)***. Recently we have shown that calpain inhibition halved endothelial cell apoptosis following reperfusion of the liver graft ***(22)***. We describe below the method we have

developed to measure calpain-like activity in cytosol extracted from snap frozen tissue obtained from liver graft. No attempt was made in this assay to differentiate μ- from m-calpain activities.

2. Materials

1. Buffer 1 for tissue homogenization: 0.1 *M* Tris-HCl, pH 7.3, 0.145 *M* NaCl, 10 m*M* EDTA.
2. Buffer 2 for activation of cytosolic calpain in the homogenates: 0.1 *M* Tris-HCl, pH 7.3, 10 m*M* $CaCl_2$, 0.145 *M* NaCl (*see* **Note 1**).
3. Suc-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (SLLVY-AMC) stock substrate solution. SLLVY-AMC is supplied in 10-mg vials (Sigma, St. Louis, MO). Dissolve the contents of one vial first in 2.62 mL of DMSO, and add 2.62 mL of buffer 2 to give a stock solution of 2.5 m*M*.
4. SLLVY-AMC/Ca^{2+}: make a working solution for each experiment by diluting the stock substrate solution 50-fold in buffer 2 to give a final SLLVY-AMC concentration of 50 μ*M*.
5. SLLVY-AMC/EDTA: prepare an identical dilution of SLLVY-AMC using buffer 1 in place of buffer 2 for measuring calcium-independent proteolysis.
6. Calpain inhibitor Z-Leu-Leu-Tyr-CHN_2 (ZLLYCHN$_2$) (M_r 565.7, kind gift from Dr. John Agnali, Institute of Biochemistry, Basel, Switzerland): prepare a 10 m*M* solution of ZLLYCHN$_2$ in DMSO.
7. SLLVY-AMC/ZLLCYHN$_2$/Ca^{2+}: prepare a third SLLVY-AMC substrate solution with 400 n*M* ZLLYCHN$_2$: dilute the 10 m*M* ZLLYCHN$_2$ solution 250-fold in buffer 2, and add 20 μL of this to 2 mL of SLLVY-AMC/Ca^{2+}.
8. AMC stock solution: dissolve 175 mg of AMC in 100 mL of 50% (v/v) DMSO in H_2O. This solution is diluted a further 1000-fold in buffer 2 and serially diluted in 10-fold steps to generate a standard curve from 10 p*M* to 1 μ*M*.
9. 96-well white MaxiSorp FluoroNunc plate (Nunc A/S, Roskilde, Denmark).

3. Methods

3.1. Cytosol Extraction From Snap Frozen Tissue

1. Freeze tissue samples immediately by immersion in liquid nitrogen and store at –70°C.
2. Homogenize ~1 g of tissue in 2 mL of buffer 1 by polytron. The samples are kept on ice (*see* **Note 2**).
3. Centrifuge the samples at 12,000*g* for 30 min at 4°C.
4. Centrifuge the supernatants at 150,000*g* for 90 min at 4°C.
5. Transfer supernatant cytosol to Eppendorf tubes and freeze immediately in liquid nitrogen.
6. Assay protein concentrations with the Bio-Rad (Hercules, CA) protein assay reagent.

3.2. Calpain Assay

1. Add 200 μL of the serially diluted AMC standard to 8 wells of a 96-well microtiter plate.

2. Thaw tissue extract supernatant samples at room temperature, or at the temperature at which the liver was preserved (*see* **Note 3**).
3. Dilute cytosol samples by mixing 1 vol of cytosol with 1 vol of buffer 1.
4. Pipet 40 µL of each sample into each of three microtiter wells.
5. Add 160 µL of SLLVY-AMC/Ca^{2+} solution to one row of samples *(a)*.
6. Add 160 µL of SLLVY-AMC/EDTA solution to the next row of samples *(b)*.
7. Add 160 µL of SLLVY-AMC/$ZLLCHN_2$/Ca^{2+} solution to the last row of samples *(c)*.
8. Incubate at room temperature for 20 min (*see* **Note 4**).
9. Read AMC fluorescence in a fluorometer using 360 nm excitation and 460 nm emission filters (*see* **Note 5**). The samples are read quickly without stopping the reaction.
10. With the aid of the standard curve from the AMC dilution series, calculate the results in units of p*M* of substrate hydrolyzed per minute per milligram of cytosolic protein (*see* **Note 5**).
11. Calculate calpain proteolysis as a fraction of the total proteolysis (*see* **Note 6**) according to the formula (where *a* and *b* are the values resulting from **steps 5** and **6** above):

$$\text{calpain proteolysis (fraction of total)} = (a-b) / (a)$$

Notes

1. Conducting the assay at pH 7.3, at which the cathepsins are known to be inactive, was felt to be important ***(23,24)***.
2. When studying ischemia in cold-preserved liver, we felt that it was important to measure the calpain activity at the temperature at which the liver was stored. Warm ischemic samples can be read at room temperature.
3. We have shown that the reaction rate is linear for 20 min and is not limited by substrate. This was, however, not routinely determined for each assay. Four sequential measurements can be taken at 5-min intervals in order to establish the linearity and to calculate the initial velocity.
4. The range of measurements in the liver lies between 10 and 100 p*M*/min/mg. We have found a basal calpain activity of 10 p*M*/min/mg in normal rat liver controls.
5. The formula simply subtracts proteolysis which is not Ca^{2+}-dependent *(b)* from proteolysis which is Ca^{2+}-dependent *(a)*. However we find it important to check also for the occurrence of proteolysis (which may or may not be Ca^{2+}-dependent) but is not caused by calpain, and this information is supplied by the value in the SLLVY-AMC/$ZLLCHN_2$/Ca^{2+}-containing sample *(c)*.
6. These results make it possible to confirm that the measurements refer only to Ca^{2+}-dependent, calpain-catalyzed, proteolysis.

References

1. Clavien, P. A., Harvey, P. R., and Strasberg, S. M. (1992) Preservation and reperfusion injuries in liver allografts. An overview and synthesis of current studies. *Transplantation* **53,** 957–978.

2. Snowdowne, K. W., Freudenrich, C. C., and Borle, A. B. (1985) The effects of anoxia on cytosolic free calcium, calcium fluxes, and cellular ATP levels in cultured kidney cells. *J. Biol.Chem.* **260,** 11,619–11,626.
3. Nieminen, A. L., Gores, G. J., Wray, B. E., Tanaka, Y., Herman, B., and Lemasters, J. J. (1988) Calcium dependence of bleb formation and cell death in hepatocytes. *Cell Calcium* **9,** 237–246.
4. Nicotera, P., McConkey, D. J., Dypbukt, J. M., Jones, D. P., and Orrenius, S. (1989) Ca^{2+}-activated mechanisms in cell killing. *Drug Metab. Rev.* **20,** 193–201.
5. Geeraerts, M. D., Ronveaux-Dupal, M. F., Lemasters, J. J., and Herman, B. (1991) Cytosolic free Ca^{2+} and proteolysis in lethal oxidative injury in endothelial cells. *Am. J. Physiol*. **261,** C889–896.
6. Nauta, R. J., Tsimoyiannis, E., Uribe, M., Walsh, D. B., Miller, D., and Butterfield, A. (1991) The role of calcium ions and calcium channel entry blockers in experimental ischemia-reperfusion-induced liver injury. *Ann. Surg*. **213,** 137–142.
7. Thomas, C. E. and Reed, D. J. (1989) Current status of calcium in hepatocellular injury. *Hepatology* **10,** 375–84.
8. Clavien, P. A., Sanabria, J. R., Upadhaya, A., Harvey, P. R., and Strasberg, S. M. (1993) Evidence of the existence of a soluble mediator of cold preservation injury. *Transplantation* **56,** 44–53.
9. Takei, Y., Marzi, I., Kauffman, F. C., Currin, R. T., Lemasters, J. J., and Thurman, R. G. (1990) Increase in survival time of liver transplants by protease inhibitors and a calcium channel blocker, nisoldipine. *Transplantation* **50,** 14–20.
10. Nichols, J. C., Bronk, S. F., Mellgren, R. L., and Gores, G. J. (1994) Inhibition of non-lysosomal calcium-dependent proteolysis by glycine during anoxic injury of rat hepatocytes. *Gastroenterology* **106,** 168–176.
11. Ferguson, D. M., Gores, G. J., Bronk, S. F., and Krom, R. A. (1993) An increase in cytosolic protease activity during liver preservation: Inhibition by glutathione and glycine. *Transplantation* **55,** 627–633.
12. Lee, K. S., Frank, S., Vanderklish, P., Arai, A., and Lynch, G. (1991) Inhibition of proteolysis protects hippocampal neurons from ischemia. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 7233–7237.
13. Tolnai, S. and Korecky, B. (1986) Calcium-dependent proteolysis and its inhibition in the ischemic rat myocardium. *Can. J. Cardiol.* **2,** 42–47.
14. Edelstein, C. L., Wieder, E. D., Yaqoob, M. M., Gengaro, P. E., Burke, T. J., Nemenoff, R. A., and Schrier, R. W. (1995) The role of cysteine proteases in hypoxia-induced rat renal proximal tubular injury. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 7662–7666.
15. Kohli, V., Gao, W., Camargo, C. A., Jr., and Clavien, P. A. (1997) Calpain is a mediator of preservation-reperfusion injury in rat liver transplantation. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 9354–9359.
16. Croall, D. E. and DeMartino, G. N. (1991) Calcium-activated neutral protease (calpain) system: Structure, function, and regulation. *Physiol. Rev.* **71,** 813–847.

17. Aguilar, H. I., Steers, J. L., Wiesner, R. H., Krom, R. A., and Gores, G. J. (1997) Enhanced liver calpain protease activity is a risk factor for dysfunction of human liver allografts. *Transplantation* **63,** 612–614.
18. Clavien, P. A. (1998) Sinusoidal endothelial cell injury during hepatic preservation and reperfusion. *Hepatology* **28,** 281–285.
19. Gao, W., Bentley, R. C., Madden, J. F., and Clavien, P. A. (1998) Apoptosis of sinusoidal endothelial cells is a critical mechanism of preservation injury in rat liver transplantation. *Hepatology* **27,** 1652–1660.
20. Sarin, A., Clerici, M., Blatt, S. P., Hendrix, C. W., Shearer, G. M., and Henkart, P. A. (1994) Inhibition of activation-induced programmed cell death and restoration of defective immune responses of HIV+ donors by cysteine protease inhibitors. *J. Immunol*. **153,** 862–872.
21. Squier, M. K., Miller, A. C., Malkinson, A. M., and Cohen, J. J. (1994) Calpain activation in apoptosis. *J. Cell. Physiol*. **159,** 229–237.
22. Sindram, D., Kohli V., Madden, J. F., and Clavien, P. A. (1999) Calpain inhibition prevents sinusoidal endothelial cell apoptosis in the cold ischemic rat liver. *Transplantation,* **68,** 136–140.
23. Crawford, C., Mason, R. W., Wikstrom, P., and Shaw, E. (1988) The design of peptidyldiazomethane inhibitors to distinguish between the cysteine proteinases calpain II, cathepsin L and cathepsin B. *Biochem. J*. **253,** 751–758.
24. Barrett, A. J. and Kirschke, H. (1981) Cathepsin B, cathepsin H, and cathepsin L. *Methods Enzymol.* **80,** 535–561.

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Myocardial Ischemia–Reperfusion Injury and Proteolysis of Fodrin, Ankyrin, and Calpastatin

Ken-ichi Yoshida

1. Introduction

Erythrocyte spectrin and nonerythroid spectrin (fodrin, calspectin) are major constituents of the membrane cytoskeleton, forming a two-dimensional meshwork beneath the outer cell membrane ***(1,2)***. The three-dimensional organization of the membrane cytoskeleton has been extensively studied in the erythrocyte, providing a model shown in **Fig. 1** ***(1)***. Spectrin and fodrin are rod-shaped proteins and consist of α and β subunits (several isoforms exist) ***(1)***.

Ankyrin links several integral membrane proteins with the fodrin-based membrane cytoskeleton **(Fig. 1)** ***(2)***. The anion exchanger, Na^+-K^+-ATPase, a voltage-dependent Na^+ channel, a Na^+/Ca^{2+}-exchanger, a ryanodine receptor, and adhesion molecules have all been reported to interact with ankyrin **(Fig. 2)** ***(2).*** Fodrin and ankyrin are therefore thought to maintain cell shape and membrane integrity ***(1,2),*** and degradation of fodrin or ankyrin is assumed to disrupt membrane integrity, resulting in irreversible cell injury (infarction) and cell death. Proteolysis of fodrin and ankyrin had been reported previously in ischemic brain and kidney ***(3–5)***, but not in the ischemic heart before our studies ***(6–10)***. We have also shown that m-calpain is the predominant calpain isoform in the rat heart and that calpastatin is downregulated by calpain during postischemic reperfusion.

I summarize here the methods we use to study the effects of calpain-mediated proteolysis of fodrin and ankyrin in the sarcolemma on contractile dysfunction and myocardial infarction in the rat heart.

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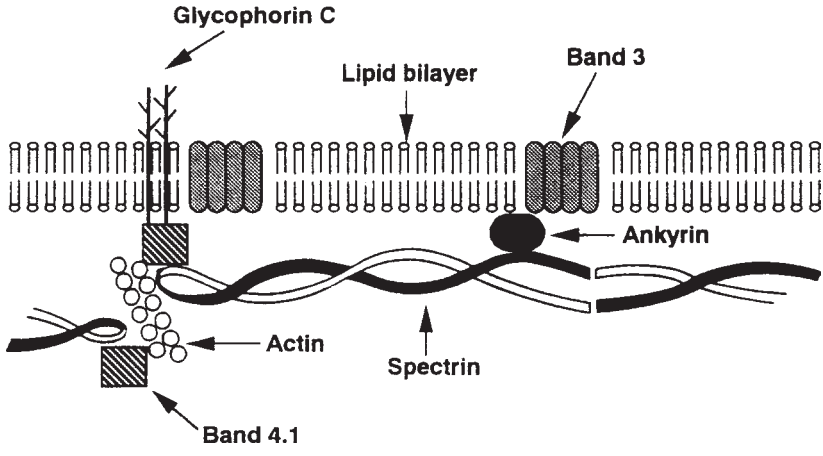

Fig. 1. Schematic representation of the organization of spectrin cytoskeleton of erythrocyte.

2. Materials

1. KH buffer: modified Krebs-Henseleit buffer: 124 m*M* NaCl, 1.2 m*M* KH_2PO_4, 4.7 m*M* KCl, 2.5 m*M* $CaCl_2$, 1.2 m*M* $MgCl_2$, 5.5 m*M* glucose, 2 m*M* sodium pyruvate, pH 7.4.
2. Calpain inhibitor I: *N*-acetyl-leucyl-leucyl-norleucinal (ALLnaL) (Nacalai Tesque, Kyoto, Japan), 50 m*M* in DMSO (*see* **Note 1**).
3. STE buffer: 10 m*M* Tris-HCl, pH 7.4, 1 m*M* EGTA, 0.32 *M* sucrose, 5 m*M* NaN_3, 10 m*M* 2-ME, 20 μ*M* leupeptin, 0.15 μ*M* pepstatin A, 0.2 m*M* PMSF.
4. Tris-buffered saline (TBS): 10 m*M* Tris-HCl, pH 7.4, 0.15 *M* NaCl, 0.05% Tween-20.
5. Antibodies to: brain α-fodrin ***(7,11)*** or (Biohit); brain β-fodrin ***(7)***; chicken erythrocyte $ankyrin_R$ (Transformation Laboratory); brain $ankyrin_B$ ***(12)***; 150-kDa fragment of α-fodrin ***(5)***.
6. Antibody binding detection system: enhanced chemiluminescence (ECL) Western blotting detection kit (Amersham).
7. Polygraph, RMP-6004, Nihon-Koden.

3. Methods

3.1. Perfusion: Ischemia and Reperfusion

1. Anesthetize adult male rats (180–250 g) with intraperitoneal sodium pentobarbital (1 mg/ kg body weight).
2. Rapidly excise the hearts and mount for Langendorff perfusion ***(6–10)***.
3. Perfuse with KH buffer at 37°C gassed with 95% O_2 – 5% CO_2 at a constant pressure of 80 cm of H_2O for 10–20 min.

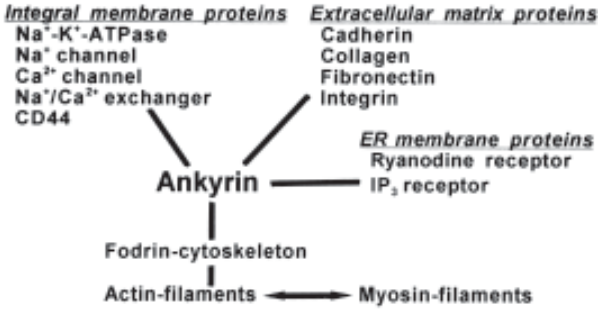

Fig. 2. Ankyrin-binding proteins.

4. Subject to global ischemia either simply by stopping perfusion, or for longer periods by incubating the hearts in plastic bags in KH buffer without oxygenation at 37°C for 5–60 min.
5. Reperfuse the hearts with KH buffer gassed with 95% O_2–5% CO_2 at a constant pressure of 80 cm H_2O for 10–60 min.
6. To study the effects of calpain inhibition, the perfusion buffer before ischemia and the reperfusion buffer contain 50 μ*M* ALLnaL.
7. To study the effects of reducing Ca^{2+}, the reperfusion buffer is prepared without Ca^{2+}.
8. After reperfusion, snap-freeze the hearts by immersion in liquid N_2, and store at –70°C until analyzed.
9. To assay function during ischemia/reperfusion, monitor the left ventricular developed pressure (LVDP) and its first derivative (*dP*/*dt*) through a latex balloon connected to a pressure transducer that is attached to a polygraph or to a computer with appropriate software *(7)*.

3.2. Subcellular Fractionation

All procedures are carried out on ice or at 4°C.

1. Homogenize frozen hearts in 2 mL/g wet weight of STE buffer with a Polytron homogenizer for 4 × 30 s.
2. To the homogenate, add a further 4 mL/g wet weight of STE buffer .
3. Centrifuge the homogenate at 600*g* for 10 min. The pellet is designated as the nucleus-myofibril fraction.
4. Centrifuge the supernatant fraction at 10,000*g* for 20 min. The pellet is designated as the sarcolemma.
5. Centrifuge the supernatant fraction at 100,000*g* for 60 min. The pellet from this step is designated as sarcolemma with intercalated discs and T tubules, and the supernatant is designated as the cytosolic fraction, on the basis of marker activities *(7)*.

6. In the study of ankyrin and calpastatin ***(8,10)***, the first centrifugation was performed at 1000*g* for 10 min, providing a pellet referred to as nucleus-myofibril (P1) fraction; centrifugation at 10,000*g* was omitted, and after centrifugation at 100,000*g* for 60 min the 100,000*g* pellet is referred to as the membrane (P2) fraction.

3.3. Gel Electrophoresis and Immunoblotting

1. Run protein samples (~ 2 μg for P_1 fraction and 10 μg for P_2 fraction) on 6.5% polyacrylamide SDS gels by standard methods ***(13)***, followed by electroblotting ***(14)***.
2. Block the blots in 5% (w/v) skim milk in TBS for ~ 1h.
3. Incubate the blots with the desired antibodies diluted 1000–2000-fold in 1% bovine serum albumin (BSA) in TBS for 1 h at room temperature, followed by washing three times for 5-10 min in TBS.
4. Incubate with second antibodies, wash as before, and detect binding by means of ECL detection.

3.4. Immunohistochemistry

3.4.1. Fodrin

1. For the study of fodrin ***(5,7)***, fix hearts after ischemia–reperfusion in 2% paraformaldehyde in 0.1 *M* sodium phosphate, pH 7.4 overnight at 4°C.
2. Embed tissues in paraffin, and cut sections onto glass slides.
3. Deparaffinize, and incubate the sections with anti-α–fodrin 240-kDa antibody or anti-150-kDa antibody, diluted 1000–2000 in 1% BSA in TBS.

3.4.2. Ankyrin

1. For the study of ankyrin ***(8)***, immerse the hearts in OCT and freeze rapidly in liquid N_2.
2. Cut cryosections onto glass slides and fix either with 4% paraformaldehyde-lysine-phosphate (PLP), or with 70% acetone-30% methanol at –20°C for 10 min.
3. Permeabilize with 0.1% Triton X-100 in phosphate-buffered saline for 10 min.
4. Treat the sections with cold 0.3% H_2O_2 in methanol for 30 min.
5. Block with 2% normal horse serum in PBS for 1 h.
6. Biotin-block with the commercial kit (Dako).
7. Incubate with antiankyrin$_R$ or antiankyrin$_B$ antibodies, diluted 1000-fold in 20 m*M* Tris-HCl, pH 7.8, 0.3 *M* NaCl, 0.05% Tween-20.
8. Stain with the avidin-biotin-peroxidase complex method (Vectastain ABC kit) (Vector Laboratories) ***(15)***.

Some of the results obtained with these procedures are briefly summarized here ***(7–10)***.

Ischemia for up to 60 min did not induce fodrin proteolysis, but reperfusion (30 min) following only 10 min of ischemia induced a substantial proteolysis of α-fodrin (240-kDa), generating the 150-kDa fragment both in the 10,000*g* pellet (sarcolemma) **(Fig. 3)** and in the 100,000*g* pellet (T tubules). β-Fodrin is

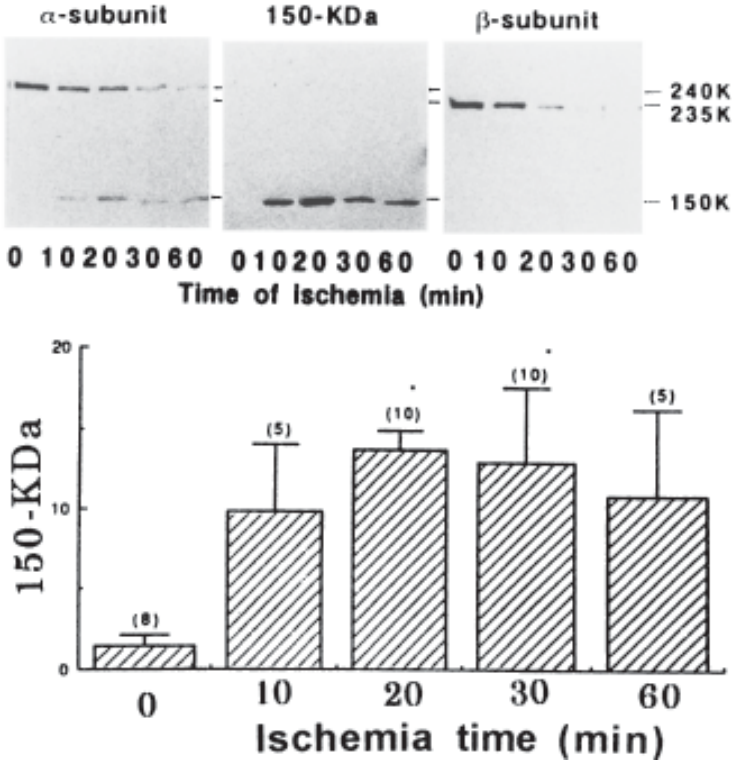

Fig. 3. Western blotting showing the proteolysis of α-and β-fodrins after 30 min of reperfusion following 10–60 min of ischemia. The antibody to the 150-kDa fragment of α-fodrin specifically recognizes the proteolytic product of α-fodrin. Modified from **ref. 7**. The lower panel shows the quantification of the 150-kDa fragment.

similarly degraded in the 10,000*g* pellet after ischemia–reperfusion **(Fig. 3)**. The results of subcellular fractionation were consistent with the immunohistochemistry ***(7)***, suggesting that the anti-150-kDa antibody detects preferential calpain cleavage of fodrin in T tubules, sarcolemma, and intercalated discs.

The presence of ALLnaL improved contractile function during reperfusion to 80% of the preischemic level **(Fig. 4**, upper panel). Also the extent of α-fodrin proteolysis and LVDP recovery after ischemia were inversely correlated **(Fig. 4**, lower panel).

ALLnaL greatly inhibits the infarct size in the model of coronary artery occlusion followed by recirculation, through suppresion of fodrin proteolysis *(unpublished observations)*.

Both forms of ankyrin are found in the nucleus–myofibril fraction, but about 5% of ankyrin$_R$ is found in the membrane fraction ***(8)***. Proteolysis of ankyrin$_R$

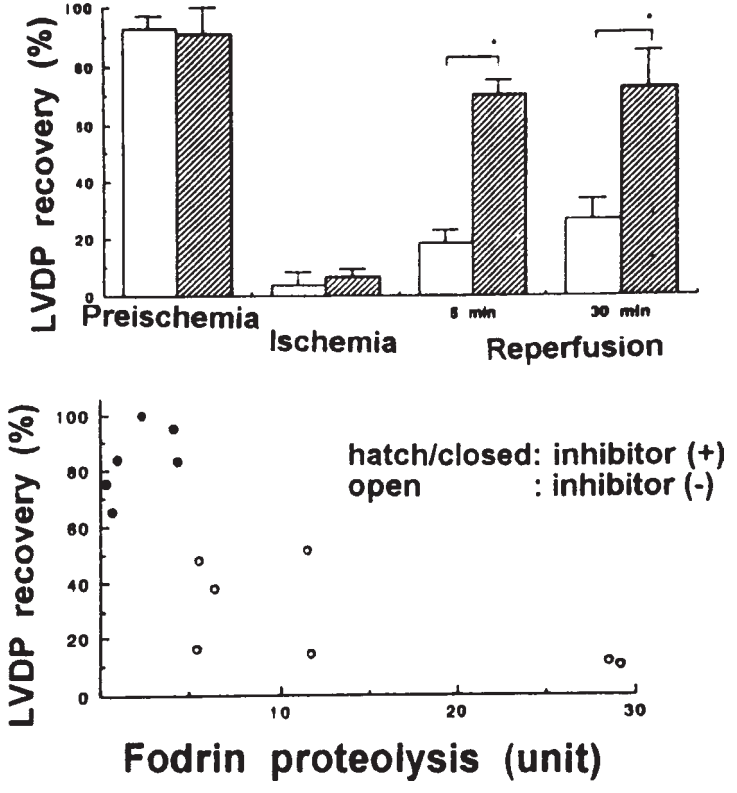

Fig. 4. The effects of calpain inhibitor I on fodrin proteolysis and LVDP during ischemia and reperfusion *(7)*.

was most marked in the membrane fraction following postischemic reperfusion **(Fig. 5)**.

3.5. Calpain Assay

Calpain activity is determined by caseinolysis after chromatographic separation, or by means of casein zymography, while distribution and autolytic activation are determined by immunoblotting *(6,10)*. For studies on calpastatin activity, the homogenates are prepared with STE buffer without calpain inhibitors.

1. Separate calpain and calpastatin by chromatography of the 100,000*g* supernatant on a column of diethylaminoethyl (DEAE)-cellulose (2 mL packed volume) preequilibrated in 20 m*M* Tris-HCl, pH 7.4, 50 m*M* NaCl, 1 m*M* EGTA, 5m*M* NaN_3, 10 m*M* 2-ME.
2. Wash the column with 2 × 3 mL of this buffer, and elute μ-calpain and calpastatin with 2 × 5 mL of this buffer containing 0.18 *M* NaCl.
3. Elute m-calpain with 2 × 2 mL of this buffer containing 0.4 *M* NaCl.

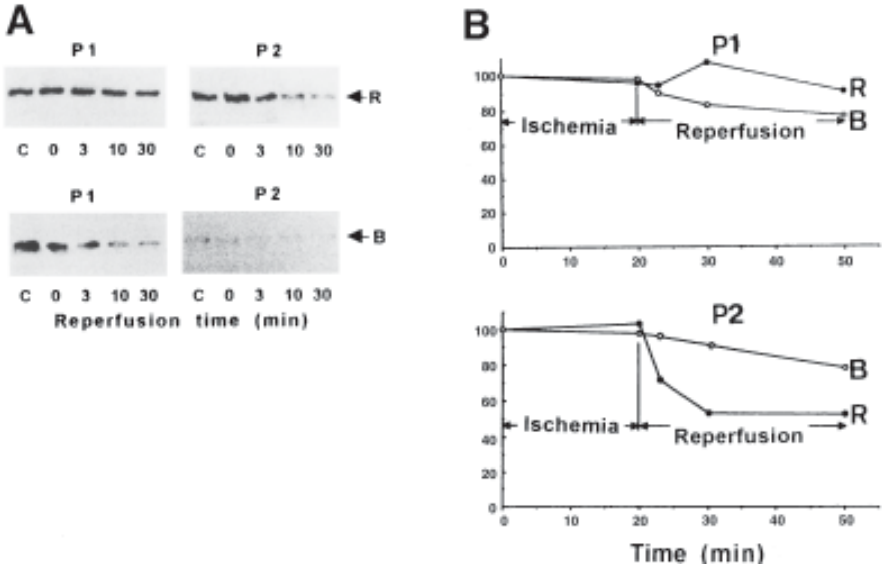

Fig. 5. Western blotting showing the proteolysis of ankyrin$_R$ and ankyrin$_B$ isoforms in the nucleus- myofibril (P2) and membrane (P1) fractions during reperfusion for 30 min following 20 min of ischemia. Ankyrin$_R$ is proteolyzed specifically in the membrane fraction during the reperfusion. Modified from **ref. *7***.

4. Measure m-calpain activity as the release of TCA-soluble peptides from azocasein in an assay mixture of 1.5 mL containing: 20 m*M* Tris-HCl, pH 7.4, 2 mg/mL azocasein, 20 m*M* 2-ME, 1% Triton X-100, 0.75 mL of sample, 2 m*M* $CaCl_2$, incubating at 25°C for 60 min ***(6,10)***.
5. To determine calpastatin activity, inactivate endogenous calpain by boiling the sample for 5 min, and centrifuge for 10 min at 10,000*g* to remove denatured protein.
6. Assay samples of the supernatant in the presence of standard μ-or m-calpain. The calpastatin activity (inhibition rate, %) is defined as ([calpain activity without sample] minus [calpain activity with the sample]) / (calpain activity without sample).

No μ-calpain activity could be detected in rat heart extracts, and no change in m-calpain activity could be detected following ischemia–reperfusion ***(10)***. Since μ-calpain mRNA can be detected by Northern blotting in rat heart, further attempts were made to detect μ-calpain activity by means of casein zymography. No band was detected in the position expected for m-calpain. The m-calpain band did not change in intensity following ischemia for 20 min and reperfusion for 30 min, suggesting that no irreversible calpain activation had occurred ***(10)***. Both 0.18 *M* NaCl and 0.4 *M* NaCl eluates from the DEAE-cellulose column contained a protease band of lower mobility than μ-calpain, which was inhibited by AALnaL, suggesting that this may be a novel form of calpain ***(10)***.

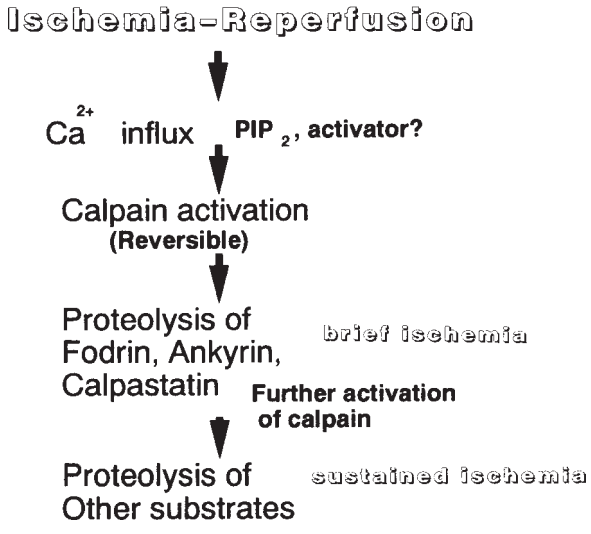

Fig. 6. Flow chart of calpain activation and proteolysis of fodrin, ankyrin, and calpastatin, leading to myocardial injury during ischemia–reperfusion.

Without proceeding here to a full analysis of our work and its physiological relevance ***(17,18)***, the accumulated evidence suggests that the process of myocardial ischemia-reperfusion injury through calpain activation proceeds as shown in **Fig. 6**.

Notes

1. There is no calpain-specific inhibitor that can penetrate the sarcolemma without toxicity. ALLnaL is the only inhibitor that can be used without apparent toxicity, and it is known that this compound inhibits both calpain and the proteasome ***(7–10)***.

References

1. Bennet, V. and Lambert, S. (1991) The spectrin skeleton: From red cells to brain. *J. Clin. Invest*. **87,** 1438–1489.
2. Bennett, V. (1992) Ankyrins. Adapters between diverse plasma membrane proteins and the cytoplasm. *J. Biol. Chem*. **267,** 8703–8706.
3. Doctor, R. B., Bennett, V., and Mandel, L. J. (1993) Degradation of spectrin and ankyrin in the ischemic rat kidney. *Am. J. Physiol*. **264,** C1003–C1013.
4. Seubert, P., Lee, K., and Lynch, G. (1989) Ischemia triggers NMDA receptor-linked cytoskeletal proteolysis in hippocampus. *Brain Res*. **492,** 366–370.
5. Saido, T. C., Yokota, M., Nagao, S., Yamamura, I., Tani, E., Tsuchiya, T., Suzuki, K., and Kawashima, S. (1993) Spatial resolution of fodrin proteolysis in postischemic brain. *J. Biol. Chem.* **268,** 25,239–25,243.

6. Yoshida, K., Yamasaki, Y., and Kawashima, S. (1993) Calpain activity alters in rat myocardial subfractions after ischemia or reperfusion. *Biochim. Biophys. Acta* **1182,** 215–220.
7. Yoshida, K., Inui, M., Harada, K., Saido, T. C., Sorimachi, Y., Ishihara, T., Kawashima, S., and Sobue, K. (1995) Reperfusion of rat heart after brief ischemia induces proteolysis of calspectin (nonerythroid spectrin or fodrin) by calpain. *Circ. Res*. **77,** 603–610.
8. Yoshida, K. and Harada, K. (1997) Proteolysis of erythrocyte-type and brain-type ankyrins in rat heart after postischemic reperfusion. *J. Biochem*. **122,** 279–285.
9. Yoshida, K., Sorimachi, Y., Fujiwara, M., and Hironaka, K. (1995) Calpain is implicated in rat myocardial injury after ischemia or reperfusion. *Jpn. Circ. J*. **59,** 40–48.
10. Sorimachi, Y., Harada, K., Saido, T. C., Ono, T., Kawashima, S., and Yoshida, K. (1997) Downregulation of calpastatin in rat heart after brief ischemia and reperfusion. *J. Biochem*. **122,** 743–748.
11. Sobue, K., Fujio, Y., and Kanda, K. (1988) Tumor promoter induces reorganization of actin filaments and calspectin (fodrin or nonerythroid spectrin) in 3T3 cells. *Proc. Natl. Acad. Sci. U.S.A*. **85,** 482–486.
12. Kunimoto, M., Otto, E., and Bennett, V. (1991) A new 440-kD isoform is the major ankyrin in neonatal rat brain. *J. Cell Biol*. **115,** 1319–1331.
13. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* **227,** 680–685.
14. Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A*. **76,** 4350–4354.
15. Hsu, S. M., Raine, L., and Fanger, H. (1981) The use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabelled antibody (PAP) procedures. *J. Histochem*. **29,** 577–580.
16. Raser, K. J., Posner, A., and Wang, K. K. W. (1995) Casein zymography: A method to study μ- calpain, m-calpain, and their inhibitory agents. *Arch. Biochem. Biophys*. **319,** 211–216.
17. Harada, K., Fukuda, S., Kunimoto, M., and Yoshida, K. (1997) Distribution of ankyrin isoforms and their proteolysis after ischemia and reperfusion in rat brain. *J. Neurochem*. **69,** 371–376.
18. Fukuda, S., Harada, K., Kunimatsu, M., Sakabe, T., and Yoshida, K. (1998) Postischemic reperfusion induces a-fodrin proteolysis by m-calpain in synaptosome and nucleus in rat brain. *J. Neurochem*. **70,** 2426–2532.

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Calpains in the Lens and Cataractogenesis

Thomas R. Shearer, Hong Ma, Marjorie Shih, Chiho Fukiage, and Mitsuyoshi Azuma

1. Introduction

Many types of cataracts in the lens of the eye show elevated concentrations of calcium, which could activate calpains, and thus this chapter presents the techniques specialized for measuring calpain activity in lens. Ubiquitous calpain has been assayed in lenses from man, cow, pig, rat, sheep, mouse, guinea pig, and rabbit ***(1)***. Further, a lens-specific calpain, termed Lp82, was recently discovered in young rat lens ***(2)***. Cloning and sequencing of the cDNA for Lp82 calpain showed that Lp82 is actually a splice variant of muscle-type calpain p94 missing several exons and containing a new exon 1. Examples of calpain-induced proteolysis are found in rodent cataracts induced by selenite, buthionine sulfoximine, calcium ionophore A23187, hydrogen peroxide, diamide, xylose, galactose, and streptozotocin; and in several animal models, Nakano mice, UPL hereditary rat cataract, Shumiya cataract rat (SCR), and transgenic mice expressing human immunodeficiency virus (HIV) protease ***(1)***. Calpain-induced proteolysis of lens crystallins is also a common feature of maturing rodent lenses ***(3)***.

2. Materials

2.1. Purification of Lens Calpains

1. Homogenization buffer I: 20 m*M* imidazole-HCl, pH 6.8, 50 µ*M* EGTA, 0.02% NaN_3, 2 mM DTE.
2. Buffer A: 20 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, 1 m*M* EGTA, 10 m*M* 2-ME (or 2 m*M* DTE).
3. Gel filtration elution buffer: 20 m*M* imidazole-HCl, pH 7.0, 1 m*M* EGTA, 1 m*M* EDTA, 10 m*M* 2-ME, 100 m*M* Na_2SO_4.

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2.2. Reverse Transcriptase-Polymerase Chain Reaction for Lens Calpains

1. Polymerase chain reaction (PCR) buffer: 10 m*M* Tris-HCl, pH 9.0 at 25°C, 50 m*M* KCl, 0.01% Triton X-100, 2.5 m*M* $MgCl_2$, 0.2 m*M* each dNTP, 0.2 m*M* primers as listed in Table 1.
2. Reverse transcriptase (RT)-PCR primers for rat calpains **(Table 1)**.

2.3. Casein Zymography

1. 10% polyacrylamide gels, 1 mm thick, are copolymerized with 0.05% casein in 375 m*M* Tris-HCl, pH 7.5. The stacking gels contain 4% acrylamide in 125 m*M* Tris-HCl, pH 6.8.
2. Running buffer: 25 m*M* Tris, 192 m*M* glycine, pH 8.3, 1 m*M* EGTA, 1 m*M* DTT.
3. Calcium incubation buffer: 20 m*M* Tris-HCl, pH 7.4, 10 m*M* DTT, 20 m*M* $CaCl_2$.

2.4. Selenite Injection Solution

Measure the average body weight of the pups in each litter of rats to be injected. Prepare selenite injection solution by dissolving anhydrous sodium selenite in distilled water using the following formula, which is designed to provide a dose of 5 µg of sodium selenite per gram body weight in a single subcutaneous injection of 50 µL **(***see* **Note 1)**:

$$\text{mg } Na_2SeO_3 \text{ / 10 mL} = (\text{body weight/pup}) \times 1.038$$

3. Methods

3.1. Purification of Lens Calpains

3.1.1. Lens-Specific Calpain Lp82

1. Homogenize ~50 lenses from 12-day old rats in 2 mL of buffer I by hand at room temperature until well disrupted **(***see* **Note 2)**.
2. Centrifuge the suspension at 13,000*g* for 15 min and recover the supernatant.
3. Fractionate the soluble proteins by high-performance-liquid chromatography (HPLC) using a 7.5 mm × 7.5 mm DEAE 5-PW column (TOSOH, Tokyo, Japan) with a linear 0.0–0.5 *M* NaCl gradient in a total volume of 70 mL of buffer A at a flow rate of 1 mL/min ***(6)***.
4. Identify the Lp82 peak by means of enzyme-linked immunosorbent assay (ELISA):
   a. mix 25 µL of each column fraction with 25 µL of 0.1 *M* $NaHCO_3$, pH 9.3, and absorb overnight onto 96-well flat-bottom plates (Corning, Corning, NY);
   b. wash the wells, and incubate with Lp82 specific-antibody available from this laboratory;
   c. detect bound primary antibody by incubation with alkaline phosphatase-conjugated secondary antibody, followed by incubation with *p*-nitrophenol phosphate and reading of the plates at 405 nm.

   Lp82 elutes as a sharp peak at 185 m*M* NaCl well before the m-calpain peak at 317 m*M* NaCl **(Fig. 1A) (***see* **Note 3)**.

Table 1
RT-PCR Primers for Rat Lens Calpains

Primer name	Position within cDNA (Nucleotides)	T_m (°C)	Primer sequence, 5' - 3'
m-Calpain sense (wild-type)	m-Calpain (1324–1353)	60	GGGCAGACCAACATC-CACCTCAGCAAAAAC
m-Calpain antisense (wild-type)	m-Calpain (1727–1699)	60	GTCTCGATGCTGAAG-CCATCTGACTTGAT
m-Calpain deletion, standard antisense	m-Calpain (1727–1699 ....1598–1579)	60	GTCTCGATGCTGAAG-CCATCTGACTTGATC-CATCTCCAATGTCCT-CCTC
m-Calpain deletion, standard sense	T7-RNA polymerase binding region + m-Calpain (1324–1353)	60	AATTTAATACGACTC-ACTATAGGGAGGGCA-GACCAACATCCACCT-CAGCAAAAAC
Lp82 sense (wild-type)	Lp82 (272–302)	58	GAAAGCCAAGATGAA-GGCCATCACTTGGAAG
Lp82 antisense (wild-type)	Lp82 (829–797)	58	GAAAGCCAAGATGAA-GGCCATCACTTGGAAG
Lp 82 deletion, standard antisense	Lp82 (822–797 ....662–642)	65	GAAAGCCAAGATGAA-GGCCATCACTTGGAAG
Lp82 deletion, standard sense	SP6 RNA polymerase binding region + Lp82 272–302)	65	ATTTAGGTGACACTAT-AGAATACGAAAGCCA-AGATGAAGGCCATCA-CTTGGAAG

5. Further purify the Lp82 calpain by chromatography on a 7.5 × 75 mm phenyl-PW HPLC column (TOSOH) using a linear descending gradient from 0.5 to 0 *M* $(NH_4)_2SO_4$ in a total volume of 15 mL of buffer A, at a flow rate of 1 mL/min.

3.1.2.Purification of Lens m-Calpain by Three Sequential Chromatography Steps

See **ref. *7.***

1. Homogenize 120 lenses from rats less than 6 mo of age in 12 mL of buffer A on ice.

2. Centrifuge the mixture at 10,000*g* for 30 min, and recover the supernatant.
3. Apply this supernatant to a 2.5 × 10 cm column of DEAE Bio-Gel A (Bio-Rad Laboratories), wash, and elute with a linear gradient from 0 to 300 m*M* NaCl in buffer A at 50 mL/h.
4. Assay fractions for m-calpain by direct enzyme assay or ELISA, and concentrate the peak by ultrafiltration (YM5 filter, Amicon).
5. Further purify the m-calpain on a 7.5 × 300 mm TSK 3000 HPLC column using gel filtration elution buffer. Pool active fractions and dilute 1:1 with buffer A containing 2 *M* $(NH_4)_2SO_4$.
6. Apply the m-calpain solution in 1 *M* $(NH_4)_2SO_4$ to a 7.5 × 75 mm TSK phenyl 5-PW HPLC column equilibrated with buffer A containing 1 *M* $(NH_4)_2SO_4$, and elute with a linear descending gradient of 1 M to 0 $(NH_4)_2SO_4$ in a total volume of 15 mL of buffer A.

3.2. Measurement of mRNA for Lens Calpains

3.2.1. RT-PCR for Lp82 and m-Calpain

1. Extract total RNA from lens in TRIzol reagent (GIBCO BRL) (1 mL/100 mg tissue).
2. Treat isolated RNA with DNase I to remove genomic DNA and quantify the recovered RNA at 260 nm.
3. Perform reverse transcription (RT) and PCR using standard conditions *(8)*. Use 1 µL RT mixture (equal to 200 ng of total RNA) for gene-specific RT-PCR. Perform the PCR in a final volume of 50 µL with the primers and annealing temperatures shown in **Table 1**.
4. Size-separate 15 µL of cDNA products by electrophoresis on a 1–1.5% agarose gel and stain with ethidium bromide (10 µg/mL).

3.3. Quantification of Lp82 and m-Calpain mRNAs by Competitive RT-PCR

1. Prepare internal competitor RNAs by first producing a cDNA for a portion of Lp82 with a 134 bp deletion from the wild-type sequence, and a cDNA for m-calpain with a 100 bp deletion from the wild-type sequence *(8)* (*see* **Note 4**). For example, the m-calpain cDNA standard is produced by using the antisense m-calpain deletion primer **(Table 1)** for reverse transcription of m-calpain mRNA. PCR is then performed with the sense m-calpain deletion standard primer, which contains the T7 RNA polymerase binding region. The resulting PCR product is a cDNA containing a 100 bp deletion and the T7 RNA polymerase binding region. This is then transcribed with T7 RNA polymerase to produce the internal competitor RNA for use in subsequent competitive RT-PCR. The Lp82 deletion standard is similarly produced using the antisense and sense Lp82 standard primers (the latter containing the Sp6 RNA polymerase binding site) in **Table 1**.
2. Perform PCR with the wild-type primers in the presence of serial dilutions of internal competitor RNA standards.

3. Run PCR products derived both from wild-type mRNA and from internal RNA standards on 1.5% ethidium bromide-stained gels, which are scanned and digitized.
4. Use NIH Image software version 1.57 to invert the image, and to determine the uncalibrated optical density of the PCR cDNA bands corresponding to specific calpain RNAs and to their competitor RNAs.
5. Perform equivalence point analysis and linear regression to calculate copies of mRNA per 1 µg total RNA ***(9)***.

3.4. Casein Zymography for Lp82 and m-Calpain in Lens

1. Prerun casein gels with zymography running buffer for 15 min at 4°C ***(10,11)*** (*See* Chapter 13).
2. Load 80 µg of lens protein per lane and run the gel at 125 V for 120 min at 4°C.
3. After electrophoresis, incubate gels with slow shaking overnight at room temperature in calcium incubation buffer .
4. Stain gels with Coomassie brilliant blue. Bands of caseinolytic activity appear white on a blue background. Lens-specific calpain runs near the top of the gel, µ-calpain runs a little faster, and m-calpain is toward the middle **(Fig. 1B)**.

3.5. Activation of Calpains in Lens In Vivo by Production of Selenite Cataract

1. Inject 0.05 mL of sodium selenite solution subcutaneously behind the neck of each 12-day-old, suckling rat ***(5)*** **(***see* **Note 5)**.
2. Use uninjected, litter mate animals as controls **(***see* **Note 6)**.
3. Observe severe, bilateral nuclear cataract within 4–6 days **(***see* **Note 7)**.

3.6. Activation of Calpains in Cultured Lenses

1. Dissect lenses carefully from 4-week-old Sprague-Dawley rats by dissecting the globe at the region near the optic nerve ***(14)***.
2. Culture lenses at 37°C under 5% CO_2 in 6 mL of Eagle's minimum essential medium (MEM, Gibco BRL, Life Technologies) with 10% fetal bovine serum (Gibco BRL).
3. Use the first 24 h of culture to identify and discard damaged lenses.
4. Increase lens Ca^{2+} concentration and activate calpain by culturing for the next 24 h only in medium containing 10 µ*M* calcium ionophore A23187 (Calbiochem, La Jolla, CA).
5. Perform control incubations with 100 µ*M* E64 in addition to ionophore ***(14)*** **(***see* **Note 8)**.
6. After 24 h, replace the culture medium with fresh medium containing no ionophore.
7. Photograph lenses daily under a dissecting microscope for 5 d. Control lenses remain clear except for a very thin subcapsular opacity. Expect the following changes in clarity of the ionophore-treated lenses: day 1, equatorial opacity; day 2 or 3 cortical opacity; day 5 nuclear opacity.

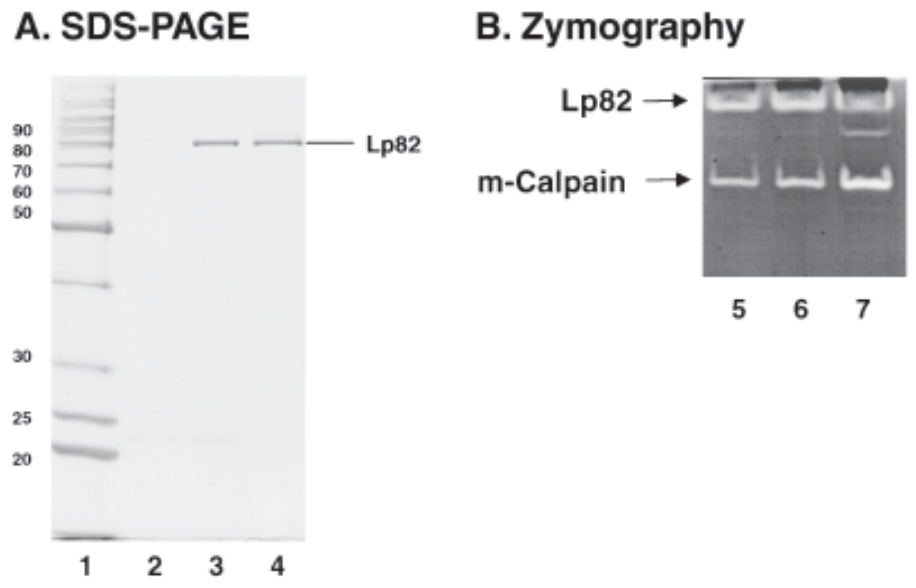

Fig. 1. **(A)** SDS-PAGE of purifed Lp82 from rat lens. The lanes contain material eluted from the phenyl-PW column: *lane 1*, 100kDa protein ladder; *lane 2*, phenyl fraction 9; *lane 3*, phenyl fraction 10; *lane 4*, phenyl fraction 11. The faint band above Lp82 in *lane 4* is another isoform, Lp85, containing a 28 amino acid insert ***(6)***. **(B)** Casein zymography of soluble proteins from whole lenses from 2-wk-old rats: *lane 5*, 10 µg; *lane 6*, 30 µg; *lane 7*, 90 µg.

3.7. Activation of Calpains in Lens Homogenates: In Vitro Precipitation of Lens Crystallins by Calpain

1. Homogenize lenses from 12-day old rats in buffer I (10 µL/lens).
2. Centrifuge the homogenate at 8000*g* for 15 min at room temperature (*see* **Note 9**).
3. Determine protein concentration by the BCA assay (Pierce Chemical Co., Rockford, IL) using bovine serum albumin as a standard and adjust to 30–50 mg protein/mL in buffer I with 120 m*M* KCl.
4. Add 80 µL of this solution to each well of smaller-volume, flat bottom, microtiter plates (96–wells, Costar, Cambridge, MA) ***(16)***.
5. Prepare control wells containing in addition 100 µ*M* E64.
6. To activate natural, endogenous lens calpains and cause light scattering, add 1 m*M* free Ca^{2+} (final concentration).
7. To test exogenous calpains, use 80 µL of 10 mg protein/mL in buffer I + KCl, but add 3 U of purified m-calpain from porcine heart per mg protein (*see* **Note 9**) and 1 mM free Ca^{2+}.
8. Cover the microtiter plates with pressure-sensitive plate sealers (Costar, 3095) and incubate at 37°C in an incubator containing an open tray of water to maintain humidity.

9. After 24 h, add 125 µ*M* E64 to all calpain-activated, experimental wells to inhibit calpain as well as to inhibit bacterial growth ***(16)***.
10. Measure light scattering daily in each well using an ELISA microtiter plate reader set at 405 nm. Before each measurement, adjust the volume of each well to 80 µL with 0.02% azide in 5 m*M* imidazole-HCl, pH 6.8, (using a micropipetor set to 80 µL), and resuspend insoluble proteins by repeated mixing in the pipetor tip. Add fresh E64 every third day (*see* **Note 11**).
11. Express light scattering values as change in optical density units after subtracting the initial light scattering of lens soluble proteins at time 0 (*see* **Note 10**).

4. Notes

1. Selenium safety: Sodium selenite is moisture-sensitive, and injection solutions should be prepared fresh from the powder for each experiment. A significant portion of the cataractogenic dose of selenite is retained in the carcass due to incorporation of selenium into tissue proteins ***(4)***. Selenium is also excreted in urine, feces, and expired air. Much of the selenium injection solution is not used since injection volumes are only 0.05 mL/pup. Precautions are therefore needed for animal handlers and for disposal of selenium injection solutions, animal carcasses, and wastes. The sodium selenite solutions cannot be dumped in the drainage. Remove by commercial or university chemical hygiene officers for disposal by an EPA-approved disposal facility. House animals in a separate animal room with adequate air turnover. Animal handlers should wear gloves, laboratory coats and surgical masks. Double-bag and incinerate all contaminated bedding material and carcasses ***(5)***.
2. Lp82 decreases rapidly in lens with age; do not use rats older than 3 wk of age. Lp82 is more concentrated in rat lens nucleus than in cortex or epithelium and nucleus may be substituted for whole lens. Lp82 has also been identified in 1-wk-old rabbit lenses ***(6)***.
3. The single DEAE-5PW step partially purifies the Lp82 enzyme to a useful level (**Fig. 1A**). DEAE-purified Lp82 concentrated from forty 13-d-old rat pups produces approximately 5 µg fluorescein isothiocyanate (FITC)-labeled casein fragments/min/mL. This preparation contains 20% Lp85 isoform.
4. The wild-type Lp82 PCR product is 525 bp, and the wild-type m-calpain PCR product is 403 bp in length.
5. For unknown reasons, production of selenite cataract sometimes becomes variable. A lower percentage of animals develop cataract, and the nuclear cataract is only pinpoint. The only consistent remedy has been to order animals from a vendor where other investigators are currently having success. Animals from B&K Universal, (Fremont, CA) currently work well in our laboratory. Repeated injections of smaller doses of selenite ***(12)*** or oral administration ***(13)*** are also cataractogenic, but the single subcutaneous injection is most convenient. Do not use older animals since selenite is cataractogenic only when injected into young rats up to the completion of the critical maturation period of the lens (~16 d of age).

6. The controls are simply uninjected litter mate animals. Control injections of sodium sulfite do not cause cataracts ***(13)*** or changes in mRNA for m-calpain, and are therefore not used.
7. Precursor stages: posterior subcapsular cataract (day 1), swollen fibers (days 2–3), and perinuclear refractile ring (day 3) can be observed with slit lamp biomicroscopy. This is a model for cataract formation in the center (nucleus) of the lens. An outer (cortical) cataract also forms after 15–30 d, but the cortical cataract clears after several months ***(15)***, while the nuclear cataract is permanent.
8. The technique for solubilizing E-64 peptide is to add a small amount of water or buffer (~1 mL) to the weighed powder and vigorously vortex until dissolved, then bring to volume.
9. Centrifuge at room temperature so as not to remove a cold-precipitable factor necessary for calpain-induced light scattering of crystallins.
10. 120 mM KCl is the physiological level of KCl in lens and markedly slows the rate of calpain-induced in vitro precipitation of crystallins. However, the pattern of crystallins eventually precipitated more closely resembles the *in vivo* pattern observed in a rodent model of cataract ***(12)***.
11. Daily addition of freshly prepared 2 m*M* DTE to the incubation wells prevents in vitro light scattering, but not calpain-induced proteolysis. Thus, oxidation of proteolyzed crystallins may enhance calpain-induced light scattering in this model.

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References

1. Shearer, T. R., Ma, H., Shih, M., Fukiage, C., and Azuma, M. (1999) Calpains in lens of the eye, in *Calpain: Pharmacology and Toxicology of a Calpain-Dependent Cellular Protease*, Wang, K. K. and Yuen, P.-W. ed. (1999) Taylor and Francis, Washington, DC., Chapter 17, 331-347.
2. Ma, H., Fukiage, C., Azuma, M., and Shearer, T. R. (1998) Cloning and expression of mRNA for calpain Lp82 from rat lens: Splice variant of p94. *Invest. Ophthal. Vis. Sci*. **39,** 454–461.
3. David, L. L., Azuma, M., and Shearer, T. R. (1994) Cataract and the acceleration of calpain-induced beta-crystallin insolubilization occurring during normal maturation of rat lens. *Invest. Ophthal. Vis. Sci*. **35,** 785–793.
4. Shearer, T. R. and Hadjimarkos, D. M. (1973) Comparative distribution of ^{75}Se in the hard and soft tissues of mother rats and their pups. *J. Nutr*. **103,** 553–559.
5. Shearer, T. R., Ma, H., Fukiage, C., and Azuma, M. (1997) Selenite nuclear cataract: Review of the model. *Mol. Vis.* **3,** 8.
6. Ma, H., Shih, M., Hata, I., Fukiage, C., Azuma, M., and Shearer, T. R. (1998) Protein for Lp82 calpain is expressed and enzymatically active in young rat lens. *Exp. Eye Res.* **67,** 221–229.
7. David, L. L., and Shearer, T. R. (1986) Purification of calpain II from rat lens and determination of endogenous substrates. *Exp. Eye Res*. **42,** 227–238.

8. Ma, H., Shih, M., Throneberg, D. B., David, L. L., and Shearer T. R. (1997) Changes in calpain II mRNA in young rat lens during maturation and cataract formation. *Exp. Eye Res*. **64,** 437–445.
9. Reidy, M., Timm, E., Jr., and Stewart, C. (1995) Quantitative RT-PCR for measuring gene expression. *Biotechniques* **18,** 70–76.
10. Fukiage, C., Azuma, M., Nakamura, Y., Tamada, Y., and Shearer, T. R. (1997) Calpain-induced light scattering by crystallins from three rodent species. *Exp. Eye Res.* **65,** 757–770.
11. Raser, K. J., Posner, A., and Wang, K. K. (1995) Casein zymography: A method to study mu-calpain, m-calpain, and their inhibitory agents. *Arch. Biochem. Biophys.* **319,** 211–216.
12. Huang, L. L., Zhang, C. Y., Hess, J. L., and Bunce, G. E. (1992) Biochemical changes and cataract formation in lenses from rats receiving multiple, low doses of sodium selenite. *Exp. Eye Res*. **55,** 671–678.
13. Shearer, T. R., Anderson, R. S., and Britton, J. L. (1983) Influence of selenite and fourteen trace elements on cataractogenesis in the rat. *Invest. Ophthalmol. Vis. Sci.* **24,** 417–423.
14. Azuma, M., David, L. L., and Shearer, T. R. (1992) Superior prevention of calcium ionophore cataract by E64d. *Biochim. Biophys. Acta* **1180,** 215–220.
15. Shearer, T. R., David, L. L., Anderson, R. S., and Azuma, M. (1992) Review of selenite cataract. *Curr. Eye Res*. **11,** 357–369.
16. Shearer, T. R., Shih, M., Azuma, M., and David, L. L. (1995) Precipitation of crystallins from young rat lens by endogenous calpain. *Exp. Eye Res*. **61,** 141–150.

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Proteolysis of Cortactin by Calpain in Platelets and In Vitro

Cai Huang and Xi Zhan

1. Introduction

Circulating platelets undergo dramatic morphological changes in response to platelet agonists, resulting in attachment, spreading, secretion, and aggregation. These rapid dynamic physiological activities require a series of rapid biochemical reactions, including tyrosine phosphorylation of various intracellular proteins and remodeling of the cytoskeleton. Calpain, a Ca^{2+}-dependent thiol protease, plays an important role in remodeling the platelet cytoskeleton by selective hydrolysis of platelet proteins in a manner dependent on platelet activation *(1)*. One of the substrates of calpain was found to be cortactin *(2)*, a filamentous actin (F-actin) crosslinker *(3)*, and a prominent substrate of Src protein tyrosine kinase *(4–6)*. Cortactin is enriched in platelets and in many motile adherent cells such as metastatic breast tumor cells *(7,8)*. Tyrosine phosphorylation of cortactin is also implicated in endothelial cell migration *(9)*. Because cortactin is highly tyrosine-phosphorylated during platelet activation, study of cortactin proteolysis may reveal the mechanism by which tyrosine phosphorylation and calpain-mediated hydrolysis regulate the reorganization of the platelet cytoskeleton.

In this chapter, we describe procedures to analyze the calpain-mediated proteolysis of cortactin both in platelets and in vitro. Proteolysis of cortactin occurs after tyrosine phosphorylation in a manner dependent on platelet aggregation and activation of GpIIbIIIa ($\alpha_2\beta_3$), a major platelet surface glycoprotein *(2)* (*see* **Note 1**). The cleavage of platelet cortactin is best analyzed by immunoblotting platelet lysates prepared using either Triton-containing buffer or sodium dodecyl sulfate (SDS) gel sample buffer.

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Cortactin can also be expressed in bacteria and purified in a functional form, and the purified protein can be efficiently tyrosine-phosphorylated by Src. Digestion of cortactin and the effects of phosphorylation on calpain-mediated digestion of cortactin can therefore be studied with purified components in vitro.

2. Materials

Unless indicated, all chemical reagents are from Sigma-Aldrich Co (St. Louis, MO).

1. Purified μ-calpain derived from pig erythrocytes (ICN, Costa Mesa, CA).
2. 10 m*M* calpeptin in DMSO (Biomol, Plymouth Meeting, PA).
3. Type I tendon collagen (Chrono-Log, Havertown, PA).
4. 1 mg/mL prostaglandin E1 in ethanol.
5. Bovine thrombin (2000 U/mg protein, ICN, Costa Mesa, CA).
6. DEAE-Sepharose FF and glutathione-Sepharose (Pharmacia, Piscataway, NJ).
7. CPD anticoagulant: dissolve 2.63 g sodium citrate, 2.54 g dextrose, 2.99 g citric acid, and 2.1 g NaH_2PO_4 in 1 L sterile water (pH does not need to be adjusted).
8. Monoclonal antibody mAb 4F11 against cortactin (Upstate Biotechnology, Lake Placid, NY).
9. Platelet washing buffer: 4.26 m*M* NaH_2PO_4, 7.46 m*M* Na_2HPO_4, pH 6.5, 5.5 m*M* dextrose, 128 m*M* NaCl, 4.77 m*M* sodium citrate, 2.35 m*M* citric acid, 3.5 mg/mL of bovine serum albumin (BSA). We usually prepare a 10 × stock solution without BSA and store it at –20°C. BSA is added to freshly prepared 1 × solution before use.
10. Modified Tyrode-HEPES buffer: 10 m*M* HEPES, pH 7.35, 136.7 m*M* NaCl, 5 m*M* glucose, 2.6 m*M* KCl, 13.8 m*M* $NaHCO_3$, 1.0 m*M* $MgCl_2$, 0.36 m*M* NaH_2PO_4, 3.5 mg/mL BSA. Prepare a BSA-free 10 × stock buffer and store at –20°C.
11. 1 *M* IPTG.
12. 100 m*M* ampicillin. Sterilize by filtering through a 0.2-μm syringe filter (Gelman Sciences, Ann Arbor, MI).
13. Triton lysis buffer (4×): 200 m*M* Tris-HCl, pH 7.2, 4% (v/v) Triton X-100, 20 m*M* EGTA, 40 μg/mL leupeptin, 40 μg/mL aprotinin, 4 m*M* PMSF, 4 m*M* benzamidine, 4 m*M* Na_3VO_4. The protease inhibitors (leupeptin, aprotinin, and PMSF) are added immediately before use.
14. Fusion protein extraction buffer: 50 m*M* Tris-HCl, pH 8.2, 134 m*M* NaCl, 50 m*M* NaF, 1% (v/v) Triton X-100, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 1 m*M* PMSF.
15. Fusion protein elution buffer: 50 m*M* Tris-HCl, pH 8.2, 10 m*M* glutathione.
16. Thrombin cleavage buffer: 100 m*M* Tris-HCl, pH 8.6, 100 m*M* NaCl, 2 m*M* $CaCl_2$.
17. DEAE column buffer: 20 m*M* Tris-HCl, pH 7.6, 20 m*M* KCl, 1 m*M* $MgCl_2$, 1 m*M* DTT, 1 m*M* EGTA.
18. Calpain digestion buffer (2 ×): 100 m*M* Tris-HCl, pH 7.4, 268 m*M* KCl, 2 m*M* $MgCl_2$, 150 μ*M* EGTA, 150 μ*M* $CaCl_2$. The free Ca^{2+} concentration in this solution is close to 3 μ*M*. This solution is stable at room temperature for at least 6 mo.
19. Preactivate 1 μ*M* recombinant human Src (Glaxo and Wellcome, NC) by incubation in a buffer containing 40 μ*M* ATP, 0.8 m*M* $MgCl_2$, and 1 mg/mL BSA on ice for 30 min. Make aliquots of the activated Src and store at –20°C.

20. Kinase reaction buffer (4 ×): 200 m*M* Tris-HCl, pH 7.4, 20 m*M* $MgCl_2$, 40 m*M* ATP. Adjust the pH to 7.4 with NaOH. The solution is stable at –20°C for 1 mo.
21. SDS gel sample buffer (2 ×): 4% (w/v) SDS, 10% (v/v) 2-ME, 20% (v/v) glycerol, 125 m*M* Tris-HCl, pH 6.8, 20 m*M* EGTA, 0.004% bromphenol blue. For analyzing cortactin proteolysis in platelets, add 20 μ*M* calpeptin before use.
22. Chrono-Log aggregometer (Havertown, PA).

3. Methods

3.1. Analysis of Cortactin Degradation in Human Platelets

The monoclonal antibody 4F11 specifically recognizes human cortactin in a standard immunoblotting analysis. Thus, the degradation of cortactin occurring in activated platelets can be detected by a direct immunoblot on the total platelet proteins solubilized in SDS gel sample buffer. Precautions must be taken to prevent calpain action, which may occur after the experiment itself, but during preparation and heating of the platelet lysate. The use of SDS gel sample buffer, containing in addition EGTA, minimizes this proteolysis. If a Triton X-100 containing lysis buffer is used to solubilize the platelets, both cortactin and calpain are retained in the Triton X-100 insoluble fraction, where cortactin can then be degraded ***(10,11)***. In this case, calpain inhibitors such as calpeptin ***(12)*** must be added to the lysis buffer to inhibit calpain during lysis.

3.1.1. Preparation of Human Platelet Suspensions (See **Note 2)**

Unless otherwise indicated all procedures are performed at room temperature.

1. Collect human blood (500 mL) from healthy aspirin-free volunteers into 70 mL of CPD anticoagulant.
2. Centrifuge the fresh blood at 200*g* for 16 min at room temperature and transfer the top layer (platelet-rich plasma) to a 6 × 50 mL plastic centrifuge tubes. To prevent platelet activation during their preparation, citric acid and prostaglandin E_1 are added to platelet-rich plasma to final concentrations of 4 m*M* and 1 μg/mL, respectively.
3. Centrifuge the platelet-rich plasma at 700*g* for 10 min. Cautiously resuspend the platelet pellet in 15 mL washing buffer by gentle pipetting. After platelets are fully dispersed, add washing buffer to 50 mL.
4. Centrifuge the platelet suspension at 700*g* for 10 min to pellet platelets. Cautiously resuspend the pellet in 10 ml of modified Tyrode-HEPES buffer, and adjust the concentration of platelets to 1×10^9 platelets/mL. Keep the platelet suspension at room temperature for 30 min before use.

3.1.2. Analysis of Cortactin in SDS-Lysed Platelets

1. Preincubate 0.4 mL of platelet suspension at 1×10^9 cells/mL at 37°C for 2 min in an aggregometer cuvette containing a magnetic stir bar. For controls, add calpeptin at a concentration of 20 μ*M*.

2. While stirring, add $CaCl_2$ to platelet suspension to a final concentration of 1 m*M* and incubate for 2 min.
3. Add 1 µL of 1 mg/mL collagen to the platelet suspension while stirring. The final concentration of collagen is 2.5 µg/mL. Platelets start to aggregate within 30 s, as recorded by the aggregometer.
4. At 0 to 10 min after collagen stimulation, add to the platelets an equal volume of 2 × SDS gel sample buffer supplemented with 20 µ*M* calpeptin (*see* **Note 3**).
5. Immediately transfer the lysate mixture to a 1.5 mL microcentrifuge tube and boil for 10 min.
6. Analyze an equal amount of protein (100 µg) from each sample on a 7.5% SDS-PAGE, and analyze the gel by immunoblotting. We use mAb 4F11 to quantitate cortactin in the immunoblot analysis.

3.1.3. Analysis of Cortactin in Platelets Lysed with Triton Buffer

The initial steps are the same as the **steps 1–3** in **Subheading 3.1.2.**, except that 0.6-mL aliquots of the platelet suspensions are used for aggregation experiments.

1. At different time points after addition of collagen, add 200 µL of 4 × Triton lysis buffer to the activated platelets, transfer the lysate mixture into a 1.5 mL microcentrifuge tube and immediately place the tube on ice.
2. Centrifuge the sample at 15,000*g* at 4°C for 10 min in a microcentrifuge. Transfer the supernatant to a new tube and label the tube as soluble fraction. The pellet, labeled as Triton-insoluble fraction, is solubilized with 100 µL of 2 × SDS gel sample buffer.
3. To analyze cortactin in the soluble fraction, the soluble fraction is incubated with 1 µg of mAb 4F11 and 20 µL protein A Sepharose (50% v/v) at 4°C in a rotator for 1 h.
4. Recover the immunoprecipitate by centrifugation and wash once with 1 × Triton-lysis buffer; solubilize the pellet in 100 µL of 2 × SDS gel sample buffer.
5. Analyze both soluble and insoluble fractions in a 7.5% (w/v) SDS-PAGE and immunoblot the gel with mAb 4F11.

3.2. Analysis of Calpain-Mediated Proteolysis of Cortactin In Vitro

3.2.1. Expression and Purification of Recombinant Cortactin

We prepare recombinant cortactin using a glutathione-*S*-transferase (GST) fusion expression system in *E. coli*, as previously described ***(3,13)***.

1. Inoculate 5 mL of Luria broth (LB) containing 100 µg/mL ampicillin with a single bacterial colony transformed with pGEX-2T/cortactin plasmid. Incubate the bacterial culture at 37°C for 12 h.
2. Transfer 2 mL of the bacterial culture into 2 L of LB medium containing 100 µg/mL ampicillin, and incubate the culture at 37°C in a shaker at 220 rpm. When the culture density has reached $OD_{600\ nm}$ = 0.4, add IPTG to 1 m*M* and continue to incubate for 2 h.

3. Harvest the cells by centrifugation at 3,000*g* for 20 min, and resuspend the pellet with ice-cold fusion protein extraction buffer. The final total volume of the suspension is 35 mL. All further steps are performed at 4°C.
4. Sonicate the bacterial suspension on ice using a microtip at output limit for 50 x 2 s impulses.
5. Centrifuge the lysate at 2000*g* for 15 min. The resulting supernatant is transferred into a 50-mL tube containing 3 mL of glutathione-Sepharose and incubated on a rotator for 90 min.
6. Wash the glutathione-Sepharose beads three times with 50 mL of fusion protein *extraction* buffer lacking protease inhibitors by brief centrifugation (1000*g* for 5 min) and resuspension of the pellet.
7. Finally resuspend the beads in 10 mL of fusion protein *elution* buffer and incubate on ice for 2 min.
8. Centrifuge the suspension at 1000g for 5 min and transfer the supernatant that contains the GST-cortactin fusion protein into a new 50-mL tube. Repeat the elution procedure three times and pool all the eluates.
9. Concentrate the pooled supernatants to approximately 15 mL by centrifugation using a Centriprep-30 device (Amicon).
10. Add the concentrated cortactin solution to an equal volume of thrombin cleavage buffer containing 1 to 2.5 units of thrombin. Incubate the mixture for 3–4 h at room temperature with constant rotation.
11. Take an aliquot into SDS gel sample buffer at this point in order to examine later by SDS-PAGE the extent of GST-cortactin cleavage. Complete cleavage should generate two bands: one at 80 kDa (cortactin), and the other at 27 kDa (GST).
12. Dilute the thrombin digestion reaction mixture with DEAE column buffer to 100 ml. Apply the solution to a DEAE-Sepharose FF column (1.6 × 10 cm) and elute the column with a linear gradient from 20 to 600 m*M* KCl in a total volume of 200 mL.
13. Analyze the eluted fractions both by $A_{280\ nm}$ reading and SDS-PAGE, and pool the fractions containing the desired recombinant cortactin.
14. Concentrate the protein solution to a final protein concentration of 1 mg/mL (~2 mL) with a Centriprep-30 device (*see* **Note 4**).

3.2.2. Phosphorylation of Cortactin In Vitro

Incubate purified cortactin (4 μg) with Src (250 n*M*) in 20 μL of kinase reaction buffer containing 5 m*M* ATP at room temperature for 1 h. To monitor tyrosine phosphorylation of cortactin, add a trace amount of [^{32}P]γ-ATP (1 μCi) to the kinase buffer.

3.2.3. Digestion of Cortactin with μ-Calpain In Vitro

1. Incubate 4 μg of purified recombinant cortactin (or phosphorylated cortactin) with μ-calpain in 40 μl of calpain digestion buffer at room temperature. The concentration of μ-calpain is in the range of 1 to 5 μg/mL, in the presence of ~3 μ*M* Ca^{2+}. For nonphosphorylated cortactin, a complete digestion requires 20–90 min; for phosphorylated cortactin, the digestion requires only 2–10 min.

2. Terminate the calpain digestion by adding an equal volume of 2 × SDS gel sample buffer. Analyze the digested samples by a gradient SDS–PAGE (4–20%, w/v) followed either by Coomassie staining or by immunoblotting with mAb 4F11.

4. Notes

1. The proteolysis of cortactin is highly dependent on the degree of platelet aggregation, and it is essential to use blood samples that are drug-free. The concentration of platelet suspensions should be ~1×10^9 platelets/mL. A lower platelet concentration such as 4×10^8/mL can result in a decrease in the proteolysis of cortactin, but concentrations higher than 1×10^9 platelets/mL can cause a spontaneous degradation of cortactin.
2. The quality of platelets is critical for the analysis of cortactin proteolysis. Because platelets adhere to glass, no glassware should be used in platelet isolation.
3. Platelet calpain can become activated during the lysis of platelets ***(14)***. High Ca^{2+} (1 m*M*) is necessary for the platelet aggregation reaction, and the presence of residual Ca^{2+} as low as 1 μ*M* in cell lysates is sufficient to activate calpain. This will cause rapid degradation of cortactin, which does not, however, reflect proteolysis of cortactin occuring within the activated platelets. The presence of 20 m*M* EGTA and calpain inhibitors such as calpeptin is therefore essential in either the Triton lysis buffer or the SDS gel sample buffer.
4. It is necessary to use pure cortactin free of GST in the in vitro proteolysis experiments. We have found that the following tips may help in the purification.
   a. The pH of the fusion protein extraction buffer should be 8.2 instead of 8.0, which has been recommended by most other protocols since a lower pH may reduce the recovery of GST fusion proteins.
   b. Sonication of cell lysates is a critical step for the success in GST fusion protein preparation. While insufficient sonication causes a poor release of GST-proteins and therefore reduces the yield, prolonged sonication can degrade proteins. We found that sonication at 2-s impulse for 45 to 60 times gives a satisfactory result.
   c. In the final steps of the preparation of GST-cortactin and GST-free cortactin, the buffers used to wash glutathione beads or DEAE beads should not include protease inhibitors because these inhibitors can also inhibit thrombin and calpain.
   d. The GST-cortactin eluted from glutathione-Sepharose is often contaminated by small molecular weight proteins, and DEAE Sepharose chromatography of cortactin following thrombin-catalyzed cleavage of GST significantly improves the purity of recombinant cortactin. If the final cortactin preparation still contains GST, as shown by SDS-PAGE, it can be removed by passing the protein solution through a minicolumn (1 mL) of glutathione-Sepharose.
5. Purified cortactin is highly susceptible to calpain. Therefore, the concentrations of Ca^{2+} and calpain used for in vitro proteolysis should be carefully adjusted. In most applications, we use Ca^{2+} at μ*M* levels and μ-calpain at concentrations from 1–2 μg/mL.

References

1. Fox, J. E., Goll, D. E., Reynolds, C. C., and Phillips, D. R. (1985) Identification of two proteins (actin-binding protein and P235) that are hydrolyzed by endogenous Ca^{2+}-dependent protease during platelet aggregation. *J. Biol. Chem.* **260,** 1060–1066.
2. Huang, C., Tandon, N. N., Greco, N. J. Ni, Y., and Zhan, X. (1997) Proteolysis of platelet cortactin by calpain. *J. Biol. Chem.* **272,** 19,248–19,252.
3. Huang, C.,. Ni, Y., Gao, Y., Wang, T., Haudenschild, C. C., and Zhan, X. (1997) Downregulation of the F-actin cross-linking activity of cortactin by Src.-mediated tyrosine phosphorylation *J. Biol. Chem.* **272,** 13,911–13,915.
4. Wu, H., Reynolds, A. B. Kanner, S. B., Vines, R. R., and Parsons, J. T. (1991) Identification and characterization of a novel cytoskeleton-associated pp60src substrate. *Mol. Cell Biol.* **11,** 5113–5124.
5. Zhan, X., Plourde, C., Hu, X., Friesel, R., and Maciag, T. (1994) Association of fibroblast growth factor receptor-1 with c-Src correlates with association between c-Src and cortactin. *J. Biol. Chem.* **269,** 20,221–20,224.
6. Wong, S., Reynolds, A. B., and Papkoff, J. (1992) Platelet activation leads to increased c-src kinase activity and association of c-src with an 85-kDa tyrosine phosphoprotein. *Oncogene* **7,** 2407–2415.
7. Zhan, X., Haudenschild, C. C., Ni, Y., Smith, E., and Huang, C. (1997) Up-regulation of cortactin expression during the maturation of megakaryocytes. *Blood* **89,** 457–464.
8. Schuuring, E. D., Verhoeven, E., Litvinov, S., and Michalides, R. J. A. M. (1993) The product of the EMS1 gene, amplified and overexpressed in human carcinomas, is homologous to a v-src substrate and is located in cell-substratum contact sites. *Mol. Cell Biol.* **13,** 2891–2898.
9. Huang, C., Liu, J., Haudenschild, C. C., and Zhan, X. (1998) The role of tyrosine phosphorylation of cortactin in the locomotion of endothelial cells. *J. Biol. Chem.* **273,** 25,770–25,776.
10. Ozawa, K., Kashiwada, K., Takahashi, M., and Sobue, K. (1995) Translocation of cortactin (p80/85) to the actin-based cytoskeleton during thrombin receptor-mediated platelet activation. *Exp. Cell Res.* **221,** 197–204.
11. Fox, J. E., Lipfert,L., Clark, E. A., Reynolds, C. C., Austin, C. D., and Brugge, J. S. (1993) On the role of the platelet membrane skeleton in mediating signal transduction: Association of GP IIb-IIIa, pp60c-src, pp62c-yes, and the p21ras GTPase-activating protein with the membrane skeleton. *J. Biol. Chem.* **268,** 25,973–25,984.
12. Tsujinaka, T., Kajiwara, K., Kambayashi, J., Sakon, M., Higuchi, N., Tanaka, T., and Mori, T. (1988) Synthesis of a new cell penetrating calpain inhibitor (calpeptin). *Biochem. Biophys. Res. Commun.* **153,** 1201–1208.
13. Smith, D. B. and Johnson, K. S. (1988) Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione *S*-transferase. *Gene* **67,** 31–40.
14. Elce, J.S., Sigmund, L., and Fox, M.J. (1989) Calpain I activation is not correlated with aggregation in human platelets. *Biochem. J.* **261,** 1039–1042.

34

Proteolysis of p53 Protein by Ubiquitous Calpains

Marc Piechaczyk

1. Introduction

p53 is a key regulator of the cell cycle and apoptosis which is essential for maintenance of genome integrity ***(1–4)***. It can function as a transcription factor, but other roles in DNA repair, homologous recombination and the regulation of its own mRNA translation have been proposed. p53 has also been reported to display intrinsic proteolytic activity ***(5,6)***. Importantly, p53 can adopt different tertiary structures. These can be monitored with a panel of monoclonal antibodies that react with the various conformations associated with different functions of the protein ***(7,8)***.

p53 is a short-lived protein that is frequently mutated in tumor cells, and the mutations are often responsible for altered functions which correlate with partial destabilization of its tertiary structure. In some cases these mutations are also responsible for a reduced rate of turnover ***(1,3,4,9–11)***. The ubiquitin-proteasome pathway accounts for most of wild-type p53 degradation in vivo ***(10,12,13)***, but several lines of data suggest that the ubiquitous calpains may also contribute to p53 turnover, at least under certain specific conditions ***(14–16)***. Zhang et al. ***(14)*** have reported that entry of calpain into the nucleus of human fibroblasts in late G1 phase correlated with decreased abundance of p53 and that the fall in p53 was inhibited by a specific calpain inhibitor. Similarly, Kubbutat and Vousden ***(16)*** showed that calpain inhibition in MCF7 cells increased the half-life of p53. In our present state of knowledge, however, it is not possible to exclude the possibility that calpains regulate p53 steady-state levels in vivo by indirect mechanisms, rather than by direct proteolysis of p53.

This chapter deals with (i) the use of calpains as topological probes for investigating the tertiary structure of mutant p53 in vitro, and (ii) techniques

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for investigating the role of calpains in p53 breakdown in vivo. These methods have also been used for studying oncogenic transcription factors such as c-Fos and c-Jun. The latter are also calpain substrates in vitro and their activity and/or abundance is regulated to a significant extent by calpains in vivo (***17–22*** and *our unpublished results*), even though the ubiquitin–proteasome system is again responsible for most of the breakdown of both proteins in vivo ***(23–25)***.

2. Materials

1. In vitro expression plasmids for wild-type and mutant p53, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and dihydrofolate reductase (DHFR) ***(15)***. The cDNAs in these plasmids are under the control of the T3 or T7 phage promoter.
2. TNT transcription-translation kit (Promega).
3. [^{35}S]methionine, 800–1000 Ci/m*M* (Amersham).
4. Daudi (human B lymphoma) and Jurkat (human T lymphoma) cells (American Type Cell Collection) (*see* **Note 1**).
5. Bovine m-calpain (Sigma).
6. Protease inhibitors: leupeptin used at a final concentration of 5 µg/mL; aprotinin used at 200 µg/mL and soybean trypsin inhibitor used at 0.85 µg/mL (Sigma).
7. Calpastatin peptide synthesized in house ***(26)*** and used at a final concentration of 0.5 µg/mL.
8. PAb1620 and PAb240 monoclonal antibodies were prepared from hybridoma cells in the laboratory but they are commercially available from various sources (e.g., MedGene Sciences SA). They are used for immunoprecipitation as described ***(27)***.
9. SAOS and H358a cells (American Type Cell Collection). These cells express no detectable p53 protein.
10. Plasmids SV2Oli, pcDNAI-neo, SVp53, pRE-CAT, and PM194 ***(15)***.
11. Lipofectin and Lipofectamine (Gibco/BRL). Transfections are conducted using Lipofectin or Lipofectamine as specified by the supplier.
12. MCF7 human breast cancer cells (American Type Cell Collection). These cells express low levels of wild-type p53.
13. X77 anti-p53 monoclonal antibody ***(28)*** (*see* **Note 2**).
14. *ts20* cells ***(12,29)*** (*see* **Note 3**).
15. E-64d, calpain inhibitor I and calpain inhibitor II (Sigma) are used at a final concentration of 50 µ*M*.
16. Calcium ionophore A23187 (Sigma) used at a final concentration of 10 µg/mL.
17. Cell culture medium: Dulbecco modified Eagle medium (Gibco/BRL) supplemented with 10% fetal calf serum.

3. Methods

3.1. Preparation of S100 Extracts

1. Wash 5×10^8 cells twice in PBS (150 m*M* NaCl, 10 m*M* sodium phosphate, pH 7) by resuspension and centrifugation at 1000*g* for 10 min.

2. Resuspend the cells in 2 mL of hypotonic lysis buffer (20 m*M* HEPES, pH 7.5, 1.5 m*M* magnesium acetate, 10 m*M* potassium acetate) and stand the suspension on ice for 5 min.
3. Homogenize with a Dounce homogenizer and check that lysis is complete by microscopic examination.
4. Remove nuclei by centrifugation at 2000*g* for 5 min at 4°C.
5. Centrifuge the supernatants at 100,000*g* for 1 h at 4°C.
6. Aliquot the resulting S100 cytoplasmic extracts (5–12 mg of protein/mL) and store at –80°C until used ***(15,17,19,20)***.

3.2. Production of Target Substrates by In Vitro Transcription/Translation

p53, GAPDH and DHFR are synthesized from the appropriate plasmids in vitro using the TNT transcription–translation system from Promega according to the supplier's specifications.

1. Use 1 µg of each expression plasmid in the presence of 20 µCi of [^{35}S]methionine in a final volume of 30 µL.
2. Estimate the efficiency of translation by removing 1 µL aliquots for electrophoresis followed by overnight autoradiography of dried gels.
3. Translation products are stored frozen until used **(***see* **Note 4)**.

3.3. Degradation of p53 by Calpain In Vitro

Both murine and human p53 can be cleaved by µ-and m-calpain in vitro ***(15,16)***. The assays can be carried out either with recombinant p53 proteins purified from *E. coli* or from the baculovirus system, or as described here with proteins produced by in vitro translation in rabbit reticulocyte lysate, which is itself devoid of any detectable calpain activity. As the source of calpain, either an S100 extract from any given cell line or purified µ-or m-calpain may be used.

1. Add 1 µL of the translation mix to 33 µL of pure or diluted S100 extract and adjust the volume to 36 µL using either PBS or PBS containing a selected protease inhibitor.
2. Prewarm the reaction mixtures to 37°C for 1 min and start reaction by adding 4 µL of 10 m*M* $CaCl_2$ (final concentration of 1 m*M* Ca^{2+}) **(***see* **Note 5** and **6)**.
3. When degradation experiments are conducted in the presence of purified calpain, the latter is added to the reticulocyte lysate at a final concentration of 50 µg of calpain/ml.
4. Remove aliquots of 4 µL at various times up to 1 h and stop reaction by mixing them with SDS gel sample buffer ***(28)*** containing 1% (w/v) SDS.
5. Analyze the products by electrophoresis on 15% polyacrylamide gels ***(28)*** and transfer them to nitrocellulose membrane for autoradiography ***(15,25)*** **(Fig. 1)** **(***see* **Note 7)**.
6. Carry out control incubations with calpain-resistant proteins such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and dihydrofolate reductase (DHFR) to rule out noncalpain proteolysis. Assessment of specificity is also achieved by omission of Ca^{2+} and by adding a variety of protease inhibitors in the presence of

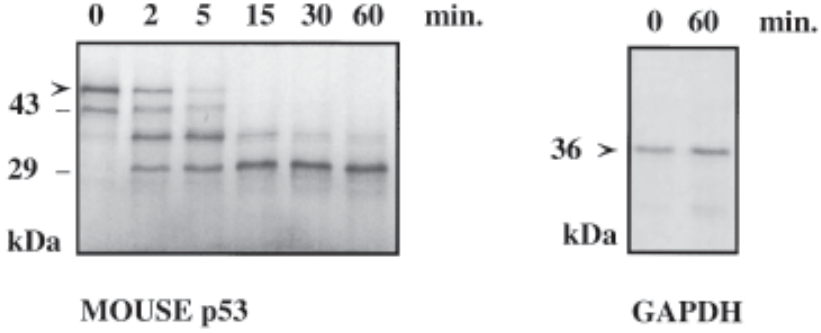

Fig. 1 In vitro cleavage of wild-type mouse p53 by calpains**.** Wild-type mouse p53 (arrow) and hamster GAPDH were produced in reticulocyte lysate and incubated for the indicated periods of time in a Jurkat cell S100 cytoplasmic extract in the presence of 1 mM $CaCl_2$. A shorter translation product with an apparent molecular weight of 43 kDa is visible at time zero. The proteolytic product pattern of the mouse p53 is very simple as only two peptides with apparent molecular weights of 41 and 33 kDa are observed. Immunoanalysis of these peptides indicates that calpains cleave on both sides of the central core domain of p53 and that N and C termini are sequentially removed, with the N terminus being cleaved first ***(15)***. In contrast to p53, GAPDH is resistant to cleavage by calpains.

Ca^{2+}, such as soybean trypsin inhibitor and aprotinin, which do not inhibit calpains, PMSF, which partially inhibits calpains, and leupeptin or a 27-mer calpastatin-derived peptide, which fully inhibit calpain activity ***(15,17,19,20)*** **(***see* **Note 8)**.

3.4. Modulation of p53 Turnover by Calpains In Vivo

Direct assessment of the in vivo effects of calpains on turnover of nuclear proteins such as p53 or other transcription factors, is difficult for several reasons **(Note 9)**. It is approached here in three ways, involving (in **Subheading 3.4.**, **step 1**) an assay of the effects of calpastatin on the transcriptional regulatory activity of p53, by means of choline acetyl transferase (CAT) reporter assays; in **Subheading 3.4.**, **step 2** an assay of the effects of calpastatin on the steady state level of p53 protein, measured immunochemically; and finally (in **Subheading 3.4.**, **step 3**) studies of the steady-state level of p53 in temperature-sensitive cells lacking the ubiquitin-proteasome pathway. These assays do not discriminate between the effects of μ-and m-calpains.

3.4.1. Effect of Calpastatin on p53 Transcriptional Activity

1. Seed H358a and SAOS cells (which lack p53) in Dulbecco modified Eagle medium (Gibco/BRL) supplemented with 10% fetal calf serum at a density of 2×10^5 cells per well in six-well culture plates (Nunc) and place in the incubator at 37°C.

2. Transfect the cells 18 h later in the presence of Lipofectin, using 500 ng of pRE-CAT, 10 ng of SVp53 and, when present, various amounts of PM194 ranging from 250 ng to 5 µg per well. (*see* **Notes 10** and **11**).
3. Leave the cells for 36 h at 37°C.
4. Perform CAT assays on cell lysates according to standard procedures ***(29)*** using a Packard Instant Imager Instrument for analysis of thin layer chromatography plates.
5. In the presence of calpastatin produced from the calpastatin expression vector, transcriptional activity of p53 is enhanced because inhibition of calpain allows an increase in the amount of p53 in transfected cells **(Fig. 2)**.

3.4.2. Effect of Calpastatin on the Steady-State Level of p53

The aim is to determine whether inhibition of calpain results in higher steady-state levels of p53. Cells expressing low, but detectable, levels of wild-type p53 are transfected with a calpastatin expression vector and p53 abundance is monitored by immunoblotting.

1. Seed MCF7 cells at a density of 10^6 cells per 60 mm-diameter culture dish.
2. Transfect the cells using Lipofectamine or Lipofectin, and 0–10 µg of PM194 plasmid per well.
3. Adjust the total amount of plasmid to10 µg per transfection using pcDNAI-neo (*see* **Note 11**).
4. Culture the cells for 48 h posttransfection and prepare cell lysates.
5. Measure p53 abundance by analysis of 50 µg samples of protein by gel electrophoresis and immunoblotting, using the X77 monoclonal antibody and enhanced chemiluminescence (Dupont), as described elsewhere ***(15,25)*** **(Fig. 3)** (*see* **Note 12**).

3.4.3. Calcium Ionophore-Induced Degradation of p53

The idea is to determine whether calpain activation leads to p53 breakdown in *ts20* cells, in which the ubiquitin-proteasome pathway is inactivated (*see* **Note 3**).

1. Culture *ts20* cells at 32°C until almost confluent.
2. Transfer the cells to an incubator at 39°C.
3. After 8 h at 39°C, add calcium ionophore A23187 to a final concentration of 10 µg/mL.
4. Culture the cells in the presence of calcium ionophore for 4 h.
5. Prepare cell extracts and measure p53 abundance by immunoblotting.
6. In parallel control experiments, add E64d, calpain inhibitor I, or calpain inhibitor II, at final concentrations of 50 µ*M*, 10 min before the addition of calcium ionophore **(Fig. 4)** (*see* **Note 13**).

4. Notes

1. All mammalian cells tested in the laboratory contain enough calpain activity for carrying out the experiments described.

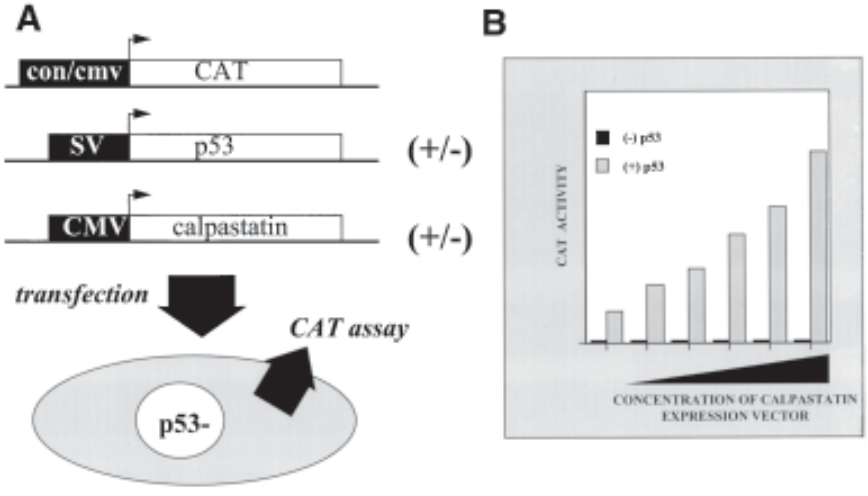

Fig. 2 Effect of calpains on p53 transcriptional activity. (**A**) *Principle of the assay*. p53-null cells are transfected with various combinations of plasmids. These include a reporter plasmid in which the CAT gene is under the transcriptional control of a p53-responsive element (CON); an expression plasmid for p53; an expression plasmid for calpastatin. When present, the p53 expression plasmid and the reporter plasmid are used in constant amounts. By contrast, the calpastatin expression vector is used at various concentrations to inhibit endogenous calpains to different degrees. CAT assays are performed 36-h posttransfection. (**B**) *Interpretation of the experimental data.* In the absence of p53 expression vector (black boxes), no p53 activity is detectable whatever the amount of calpastatin expression vector. In the presence of p53 expression vector and in the absence of calpastatin expression vector, a basal level of p53 transcriptional activity (gray boxes) is observed. In the presence of increasing concentrations of the calpastatin expression vector, calpain activity is inhibited to a greater extent and p53-dependent transcription activity increases in transfected cells in a dose-dependent manner.

2. In addition to p53, the X77 anti-p53 monoclonal antibody detects an uncharacterized protein of 30 kDa in human and mouse cell extracts. The latter has been shown to be invariant in many of our experiments, and is routinely used as a control for normalization of immunoblotting assays.
3. *ts20* cells are thermosensitive for the ubiquitin pathway (see legend to **Fig. 4**). The ubiquitin pathway is active at the permissive temperature, 32°C, but is inactive at the restrictive temperature, 39°C. At the latter temperature, p53 accumulates steadily over a period of 24 h. If calpains are activated by addition of A23187 during this time, cytoplasmic p53 is vulnerable to calpain attack and less protein enters the nucleus (where it is stable because of the inactivation of the ubiquitin pathway).

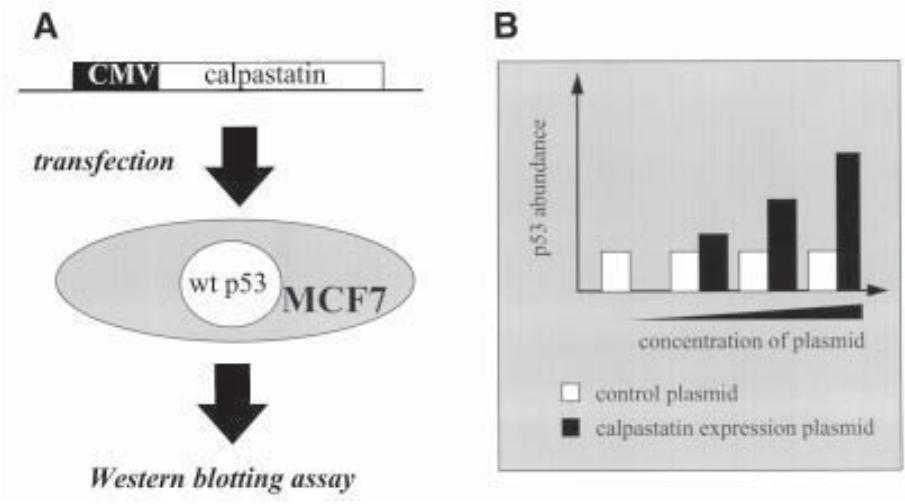

Fig. 3 p53 accumulation upon inhibition of calpain activity. (**A**) *Principle of the assay*. Cells expressing a basal level of wild-type p53 are transfected with a calpastatin expression vector to inhibit the endogenous calpain activity. p53 abundance is monitored by immunoblot analysis of transfected cells. To demonstrate the specificity of the effect of calpastatin expression on p53 steady-state level, control transfection experiments are conducted in parallel with the parental cloning vector lacking calpastatin sequence. (**B**) *Interpretation of the experimental data.* Whatever the concentration of control plasmid, no change in p53 abundance is observed. In the presence of increasing amounts of the calpastatin expression vector, endogenous calpain activity is progressively inhibited and p53 accumulates to higher levels in a dose-dependent manner.

4. When required, the conformation of in vitro translated p53 proteins is assessed by immunoprecipitation using the PAb240 and PAB1620 panel of monoclonal antibodies.
5. Concentrations as low as 75–100 μ*M* Ca^{2+} can be used for activating μ-calpain alone. Degradation experiments are usually conducted at 37°C, but may be conducted at lower temperatures to reduce the rate of degradation.
6. Significant variations in calpain activity are observed from one S100 extract to another, including those from the same cell type. Susceptibilities to calpains of different proteins are always compared in parallel experiments using the same S100 cytoplasmic extract batch. Calpain activity is not constant over time in reaction mixtures and is more or less rapidly exhausted depending on the cell type.
7. Calpain cleavage of p53 is influenced by the conformation of the monomer of p53, but is not affected by its quaternary structure ***(15)***. Like most calpain substrates, p53 is not fully degraded, but stable proteolytic products accumulate in reaction mixtures ***(15,16)***. The proteolysis pattern derived from mouse p53, as shown in **Fig. 1**, is simpler than that from human p53.

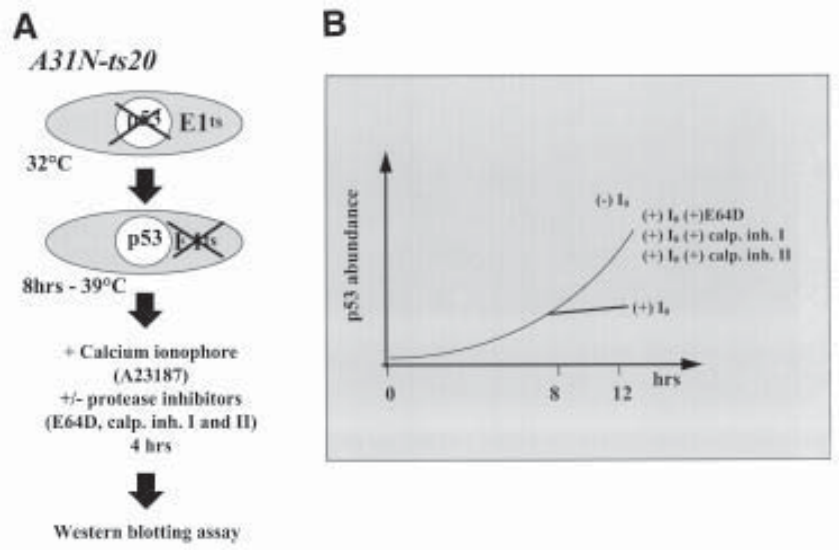

Fig. 4 Calcium ionophore-induced degradation of p53. (**A**) *Principle of the assay. ts20* cells are thermosensitive for the E1 enzyme of the ubiquitin pathway. At the permissive temperature, 32°C, p53 is not detectable in these cells because it is degraded *via* the ubiquitin/proteasome pathway. By contrast, p53 is no longer degraded upon shifting the cells to the restrictive temperature, 39°C, and it accumulates in the nucleus where it is stable and protected from calpains. Calcium ionophore A23187 is added to cells to activate calpains at a time when p53 synthesis is in progress. Newly synthesized p53 is accessible to activated calpains and accumulation of p53 ceases. To show that the effect is actually due to calpains and not to other effects of A23187, the same experiments are carried out in the presence of various cell-permeant calpain inhibitors. (**B**) *Interpretation of the experimental data*. In the absence of calcium ionophore [(-) I_0], p53 steadily accumulates in heat-treated *ts20* cells over a period as long as 24 h, as monitored by immunoblotting. If calcium ionophore is added 8 h after the temperature shift [(+) I_0], p53 accumulation is stopped for at least 4 h. p53 accumulates normally in cells treated with both calcium ionophore and calpain inhibitors [(+) I_0 (+) E64d, (+) I_0 (+) calpain inhibitor I, (+) I_0 (+) calpain inhibitor II], showing that the effect depends on calpain activation.

8. When testing a new protein for sensitivity to calpain in this system, further experiments are required to prove that the protein is an actual substrate of calpains and not a substrate of a different protease in the cell extract, which is itself activated by calpain ***(15,17,19,20)***. To this end, we routinely use the following two-step degradation assay: (i) the protein is first incubated for 30 min in the S100 extract in the presence of calcium, that is, a time sufficient for activation of a second possible protease and for degradation of the protein; (ii) fresh protein is then added to the reaction, and incubation is continued for another 30 minutes

under conditions that are permissive for calpain action (i.e., in the presence of calcium) or nonpermissive (i.e., in the presence of calpastatin peptide or of EGTA at a concentration of 5 m*M*). If proteolysis of the freshly added protein is not inhibited in the latter case, a protease other than calpain must be suspected.

9. Several considerations should be borne in mind. The calpains are assumed to be largely, if not exclusively, cytoplasmic although a small fraction has been reported to be associated with the nucleus ***(15)***, and the regulation of calpain activity in vivo is unclear. The ubiquitin–proteasome pathway can contribute to a greater or lesser extent to the degradation of the studied proteins and may mask the calpain contribution. Exposure to calpain may vary for proteins constitutively transported to the nucleus, for proteins shuttling between the nucleus and the cytoplasm, and for proteins whose nuclear import is regulated so that they may remain cytoplasmic for longer.
10. p53 null cells are cotransfected with combinations of plasmids which include: (i) a wild-type p53 expression vector (SVp53) in limiting amounts (10 ng) so that an increase in p53-dependent transcriptional activity will be seen upon p53 stabilization; (ii) a reporter plasmid (pRE-CAT) in which transcription of the bacterial CAT gene is under the control of a p53-responsive element (CON); and (iii) an expression plasmid for calpastatin (PM194).
11. Expression of p53 and the calpastatin gene are under the transcriptional control of the SV40 late promoter and the cytomegalovirus promoter, respectively. As a control, SVp53 is replaced in transfections with 10 ng of the SV2oli plasmid which carries an SV40 late promoter sequence. Similarly, reduced amounts of PM194 are compensated for by addition of increased amounts of pcDNAI-neo (the vector used for cloning the calpastatin cDNA to form PM194).
12. The increase in abundance is modest, in the range of 2- to 3-fold, because the ubiquitin–proteasome system predominates in p53 turnover.
13. It is important to run controls to show that the effect is due to calpain. Parallel experiments are conducted in the presence of various cell-permeant calpain inhibitors that prevent p53 degradation. E64d, calpain inhibitor I and calpain inhibitor II are used. None of them is strictly specific for calpains. However, all three inhibit calpains, and it follows that a common effect is likely to involve calpain inhibition.

Acknowledgments

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References

1. Haffner, R. and Oren, M. (1995) Biochemical properties and biological effects of p53. *Curr. Opin. Genet. Dev.* **5,** 84–90.
2. Bates, S. and Vousden, K. H. (1996) p53 in signalling checkpoint arrest and apoptosis. *Curr. Opin. Genet. Dev.* **6,** 12–18.

3. Hansen, R. and Oren, M. (1997) p53, from inductive signal to cellular effect. *Curr. Opin. Genet. Dev.* **7,** 46–51.
4. Ko, J. K. and Prives, C. (1996) p53: Puzzle and paradigm. *Genes Dev.* **10,** 1054–1072.
5. Okorokov, A. L., Ponchel, F., and Milner, J. (1997) Induced N-and C-terminal cleavage of p53: A core fragment of p53, generated by interaction with damaged DNA, promotes cleavage of the N-terminus of full-length p53, whereas ssDNA induces C-terminal cleavage of p53. *EMBO J.* **16,** 6008–6017.
6. Okorokov, A. L. and Milner, J. (1998) Proteolytic cleavage of p53: A model for the activation of p53 in response to DNA damage. *Oncol. Res.* **9,** 267–273.
7. Milner, J. (1994) Forms and functions of p53. *Cancer Biol.* **5,** 211–219.
8. Milner, J. (1995) Flexibility: the key to p53 function? *Trends Biochem. Sci.* **20,** 49–51.
9. Donehower, L. A. and Bradley, A. (1993) The tumor suppressor gene p53. *Biochem. Biophys. Acta* **1155,** 181–205.
10. Michael, H. G., Kubbutat, H. G., and Vousden, K. H. (1998) Keeping an old friend under control: regulation of p53 stability. *Mol. Med. Today* **4,** 250–256.
11. Greenblatt, M. S., Bennett, W. P., Hollstein, M., and Harris, C. C. (1994) Mutations in the p53 tumor suppressor gene: Clues to cancer etiology and molecular pathogenesis. *Cancer Res.* **54,** 4855–4878.
12. Chowdary, D. R., Dermody, J. J., Jha, K. K., and Ozer, H. L. (1994) Accumulation of p53 in a mutant cell line defective in the ubiquitin pathway. *Mol. Cell. Biol.* **14,** 1997–2003.
13. Maki, C. G., Huibregtse, J., and Howley, P. (1996) In vivo ubiquitination and proteasome-mediated degradation of p53. *Cancer Res.* **56,** 2649–2654.
14. Zhang, W., Lu, Q., Xie, Z. J., and Mellgren, R. L. (1997) Inhibition of the growth of WI-38 fibroblasts by benzyloxycarbonyl-Leu-Leu-Tyr diazomethyl ketone: Evidence that cleavage of p53 by a calpain-like protease is necessary for G1 to S-phase transition. *Oncogene* **14,** 255–263.
15. Pariat, M., Carillo, S., Molinari, M., Salvat, C., Debüssche, L., Bracco, L., Milner, J., and Piechaczyk, M. (1997) Proteolysis by calpains: A possible contribution to degradation of p53. *Mol. Cell Biol.* **17,** 2806–2815.
16. Kubbutat, M. H. G., and Vousden, K. H. (1997) Proteolytic cleavage of human p53 by calpain: A potential regulator of protein stability. *Mol. Cell Biol.* **17,** 460–468.
17. Steff, A.-M., Carillo, S., Pariat, M., and Piechaczyk, M. (1997) v-FOS-FBR and v-JUN-ASV17, but not v-FOS-FBJ, are resistant to calpains. *Biochem. J.* **323,** 685–692.
18. Pariat, M., Bébien, M., Brockly, F., Carillo, S., Jariel-Encontre, I., Altieri, E., and Piechaczyk, M. *(1999)* The sensitivity of c-fos and c-jun proteins to calpains depends on conformational determinants and not on the formation of the dimer. (Submitted.)
19. Carillo, S., Pariat, M., Steff, A.-M., Jariel-Encontre, I., Poulat, F., Berta, P., and Piechaczyk, M. (1996) PEST motifs are not required for rapid calpain-mediated proteolysis of *c-fos* protein. *Biochem. J.* **313,** 245–251.
20. Carillo, S., Pariat, M., Steff, A.-M., Etienne-Julan, M., Lorca, T., and Piechaczyk, M. (1994) Differential stability of FOS and JUN family members to calpains. *Oncogene* **9,** 1679–1689.

21. Hirai, S., Kawasaki, H., Yaniv, M., and Suzuki, K. (1991) Degradation of transcription factors, c-Jun and c-Fos, by calpain. *FEBS Lett.* **287,** 57–61.
22. Watt, F., and Molloy, P. L. (1993) Specific cleavage of transcription factors by the thiol protease, m-calpain. *Nucleic. Acids Res.* **21,** 5092–5100.
23. Stancovski, I., Gonen, H., Orian, A., Schwartz, A. L., and Ciechanover, A. (1995) Degradation of the proto-oncogene product c-fos by the ubiquitin proteolytic system in vivo and in vitro; Identification and characterization of the conjugating enzymes. *Mol. Cell Biol.* **15,** 7106–7116.
24. Treier, M., Staszewsk, L. M., and Bohman, D. (1994) Ubiquitin-dependent c-jun degradation in vivo is mediated by the δ domain. *Cell* **78,** 787–798.
25. Salvat, C., Jariel-Encontre, I., Acquaviva, C., Omura, S., and Piechaczyk, M. (1998) Differential directing of c-Fos and c-Jun proteins to the proteasome in serum-stimulated mouse embryo fibroblasts. *Oncogene* **17,** 327–337.
26. Maki, M., Bagci, H., Hamaguchi, K., Ueda, M., Murachi, T., and Hatanaka, M. (1989) Inhibition of calpain by a synthetic oligopeptide corresponding to an exon of the human calpastatin gene. *J. Biol. Chem.* **254,** 18,866–18,869.
27. Medcalf, E. A., Takahashi, T., Chiba, I., Minna, J., and Milner, J. (1992) Temperature-sensitive mutants of p53 associated with human carcinoma of the lung. *Oncogene* **7,** 71–76.
28. Laemmli, E. K. (1971) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.
29. Chiu, R., Boyle, W., Meek, J., Smeal, T., Hunter, T., and Karin, M. (1988) The c-fos protein interacts with c-jun/AP-1 to stimulate transcription of AP-1 responsive genes. *Cell* **54,** 541–552.
30. Legros, Y., Lacabanne, V., D'agay, M. F., Larsen, C. J., Pla, M., and Soussi, T. (1993) Isolation of human p53 specific monoclonal antibodies and their use in immunohistochemical studies of tumor cells. *Bull. Fr. Cancer* **80,** 102–110.
31. Salvat, C., Acquaviva, C., Scheffner, M., Robbins, I., Piechaczyk, M., and Jariel-Encontre, I. *(1999)* Molecular characterization of the E1 ubiquitin-activating enzyme thermosensitive mutant *ts20*: Differential requirement upon E1 for ubiquitination of the various protein substrates in vivo. (Submitted.)

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Strategies for Regulating Calpain Activities in Living Cells

Neil E. Forsberg and Jing Huang

1. Introduction

The goals of our research program have been to understand the mechanism(s) responsible for the turnover of myofibrillar proteins of skeletal muscle. This understanding would be useful in development of strategies to treat muscle wasting diseases and, possibly, to augment efficiency of muscle growth in domestic animals. The challenge in this area is that several proteolytic systems have been implicated in this process. For example, Goll et al. ***(1,2)*** have summarized in considerable detail a logical rationale for the participation of the calpains in this process. However, other researchers ***(3–5)*** have provided compelling evidence that the proteasome, the large, multifunctional ATP-dependent protease, plays a key role in this process as well. Earlier studies ***(6)*** have also documented the participation of the lysosome in this process.

The simplest strategy for elucidating the roles of individual proteases in living cells is to apply inhibitors to tissue culture systems. For the study of calpains, several candidate inhibitors are available, including calpain inhibitors I and II, E-64 and its derivatives, leupeptin, and calpeptin. While each of these effectively inhibits calpain, none is truly specific and none allows differentiation of the individual functions of the various calpain isoforms. Lactacystin is a proteasome inhibitor ***(7)***; however, it has not been studied in detail and could conceivably exert nonspecific actions in cultured cells. Because of these limitations, we designed, and describe here, two approaches which may be used to study the specific actions of calpain in living cells: overexpression of a dominant negative (DN) mutant form of m-calpain, and overexpression of the inhibitory domain of calpastatin. To prepare DN-m-calpain, we mutagen-

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ized the active site cysteine (Cys) residue of rat m-calpain to an alanine (Ala) and used the LacSwitch expression system to express it in cultured muscle cells. To prepare the calpastatin inhibitory domain (CID) we expressed a peptide fragment corresponding to the consensus calpain inhibitory domain of calpastatin ***(8)***. The DN-calpain was expected to compete with endogenous m-calpain for substrate, or to sequester 30-kDa subunit, and thereby specifically to inhibit calpain-dependent proteolysis. The CID fragment was expected to bind to and inhibit both μ-and m-calpain.

2. Materials

1. L8 muscle cells (ATCC, Manassas, VA).
2. Muta-Gene T7 Enzyme Refill Pack and Bradford Assay Reagent, (BioRad, Hercules, CA).
3. A plasmid (pT7-7fN-m80k), which included the cDNA for the coding region of rat m-calpain large subunit, was a gift of Dr. John Elce (Queen's University, Canada) ***(9)***.
4. T4 kinase, Hgal, Taq polymerase, RQ1 DNase and T4 ligase (Promega, Madison, WI).
5. LacSwitch expression kit and LacI antibody (Stratagene, LaJolla, CA) **(Note 1)**.
6. Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin, trypsin, and hygromycin B (GIBCO, Gaithersburg, MD).
7. Fetal bovine serum (FBS) and horse serum (HS) (HyClone, Logan, UT).
8. Enhanced Chemiluminescence Detection System (ECL) (Amersham, Arlington Heights, IL).
9. Radioactive tyrosine (New England Nuclear, (Boston, MA).
10. Optitran blotting membranes (Schleicher & Schuell, Keene, NH).
11. Spectrin (fodrin) antibody (Chemicon, Temecula, CA).
12. Mutagenic (antisense) Cys105Ala primer for rat m-calpain, containing a diagnostic *Hga*I site, and 5'-phosphorylated by means of T4 polynucleotide kinase:

    5'-P-AGCCAGAAGCCA**GGC**GTCCCCAAGGGCTCC-3'

13. Other materials including general reagents, A23187 (Ca^{2+}-ionophore), IPTG, DMSO, phenol and HEPES are available from Sigma Chemical Co. (St. Louis, MO).
14. Myoblast growth medium: DMEM with addition of: 1 g/L D-glucose, 584 mg/L L-glutamine, 110 mg/L sodium pyruvate, 4 mg/L pyridoxine hydrochloride, 3.7 g/L sodium bicarbonate, 100 U/mL penicillin-streptomycin and 10% (v/v) FBS.
15. 2 × HeBS solution: 50 m*M* HEPES acid, 282 m*M* NaCl, 0.8 m*M* Na_2HPO_4, pH 7.05.

3. Methods

3.1. Preparation of DN-m-Calpain cDNA

1. Mutagenize the cDNA for the rat m-calpain 80-kDa large subunit in the plasmid pT7-fN by the Kunkel method, using Muta-Gene T7 Enzyme Refill Pack Version 2.

2. Confirm the desired mutation by means of *Hga*I digestion and sequencing.
3. Digest pOP13CAT with *Not*I and purify the larger plasmid backbone fragment by gel elution to remove endogenous CAT cDNA.
4. Transfer the mutated 80-kDa cDNA by means of *Not*I/*Sal*I digestion and *Not*I linkers into the *Not*I site of pOP13CAT. The insertion can occur in either orientation.
5. Analyze by means of appropriate digestions and designate the plasmid harbouring mutagenized m-calpain cDNA in the correct orientation in pOP13 as pOP13DN. Prepare this plasmid on a large-scale and purify using CsCl centrifugation ***(10)***.

3.2. Preparation of Calpastatin Inhibitory Domain

Croall and McGrody ***(8)*** described a consensus calpain inhibitory domain 24-residue peptide (CID) which occurs four times in calpastatin: EKLGERDDTIPPEYRELLEKKTGV. An 88-base oligodeoxynucleotide encoding this fragment (lower case sequence) was synthesized in the Oregon State University Central Services Laboratory. A *Not*I site and a translation start codon (ATG, underlined) and a Kozak sequence were included at the 5'-end. A translation stop codon (TGA, underlined) and *Not*I site were included at the 3'-end. The CID nucleotide sequence was:

T*GCGGCCGC*C-ATG gag aag ctg ggc gag agg gac gac acc atc ccc ccc gag tac agg gag ctg ctg gag aag aag acc ggc gtg TGA *GCGGCCGC*A

This sequence was amplified by means of PCR using forward primer 5'-TGCGGCCGCC ATG GAG AAG CT-3' and reverse primer 5'-TGCGGCCGC TCA CAC GCC GGT-3'. The PCR product was digested with *Not*I and subcloned into the *Not*I site of pOP13CAT. The correct orientation was verified by sequencing and the plasmid designated as pOP13CID.

3.3. Cell Culture, Transfection, and Preparation of Cell Lines

Transfect cells either with pOP13DN (for dominant negative m-calpain expression) and p3'SS (for LacI expression); or with pOP13CID (for expression of the calpastatin fragment) and p3'SS. Control transfections with pOP13CAT and p3'SS are also carried out.

1. Seed L8 myoblasts onto plastic tissue culture dishes at low density in myoblast growth medium. Maintain cultures at 37°C at 5% CO_2.
2. One day before calcium-phosphate transfection, split one 10 cm plate of 80% confluent myoblasts onto four 10-cm plates. On the next day feed each of the four plates with fresh medium 4 h before transfection.
3. Purify plasmids by CsCl banding ***(10)*** and precipitate them with ethanol; wash the DNA pellets in 70% ethanol.
4. For each transfection, suspend 10 µg of each plasmid DNA together in 450 µL of sterile water and add 50 µL of sterile 2.5 *M* $CaCl_2$.
5. Add the DNA/$CaCl_2$ suspension dropwise into 500 µL of 2 × HEBS solution, and vortex the mixture for 5 s.

6. Allow the suspension of DNA precipitate to sit for 20 min at room temperature and then distribute evenly over a 10-cm plate of cells.
7. Duplicate plates of each transfection should be completed.
8. Following 24 h of exposure to DNA, replace the medium with fresh growth medium medium containing hygromycin and G418 (400 µg/mL) to select colonies which harbor both plasmids.
9. Recover resistant colonies using cloning rings (*see* **Note 2)** and grow up these cells. Several criteria may be used to select appropriate cell lines for subsequent experiments (*see* **Notes 3** and **4)**.

Cells which express DN-m-calpain are designated as L8/DN cells. Cells that express CID are designated as L8/CID cells. Cells transfected with original pOP13CAT and p3'SS are designated as L8/PC (plasmid control) cells.

3.4. Validation of Cell Lines Using RT-PCR Analysis

1. Assay expression of the DN and CID constructs in L8 cells following treatment with IPTG (5 m*M*) by means RT-PCR. As an internal control, LacI expression is also assessed.
2. Isolate total RNA by standard procedures, and carry out the RT reaction with RQ1 DNAase and oligo dT 18-mer.
3. For m-calpain the forward PCR primer is 5'-GATGAAACTGGCCAAAGA-3' and the reverse primer is 5'-AGCTCCTCTGGGACCTCATAGATG-3'.
4. For LacI, the forward PCR primer is 5'-TGTCGATGGTAGAAGGAAG-3' and the reverse primer is 5'-GTGGTTTTTCTTTTCACCAG-3'.
5. For CID, the forward PCR primer is 5'-CATGGAGAAGCTGGGCGA-3' and the reverse primer is TCACACGCCGGTCTTCTT-3'.
6. The PCR reactions consist of 2 µL of template, 1 µL (10 p*M*) of forward primer, 1 µL (10 p*M*) of reverse primer, with final concentrations of 1.5 m*M* $MgCl_2$, 0.2 m*M* *d*NTP, 1 × Taq DNA polymerase buffer, 0.5 µL Taq DNA polymerase. The cycles used are 30 cycles at 95°C for 1 min, 55°C for 1.5 min, and 74°C for 1 min.
7. The effects of IPTG on muscle cell m-calpain and LacI mRNA expression in L8/DN cells are shown in **Fig. 1**, and the effects of IPTG on CID and LacI mRNA expression in L8/CID cells are shown in **Fig. 2**. IPTG increased both m-calpain and CID mRNA but did not affect LacI mRNA.

3.5. Validation of Cell Lines by Western Blotting for m-Calpain

1. Culture L8/PC, L8/CID and L8/DN cells to 90% confluence and add IPTG (5 m*M*) for varying times (0 and 8 h, 1, 2, 3, 4, and 5 d).
2. Recover cell protein, run SDS polyacrylamide gels, and transfer the proteins onto Optitran membranes.
3. Probe the blots with m-calpain and LacI antibodies. m-Calpain antibody was prepared in our laboratory as a rabbit m-calpain N-terminal antipeptidic antibody and shown to be monospecific for rat m-calpain. LacI and m-calpain antibodies were diluted 1:1,000 and 1:2,000, respectively.

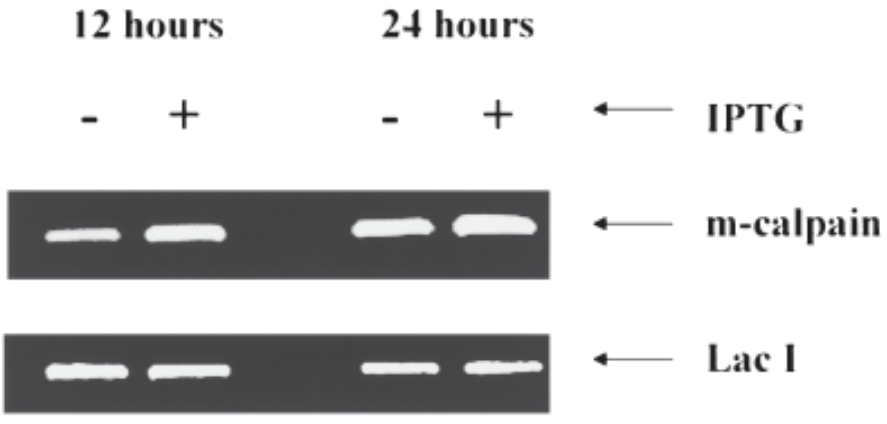

Fig. 1. Effects of IPTG on expression of mRNA for m-calpain and LacI in L8/DN cells, as shown by RT-PCR analysis. m-Calpain mRNA levels increased but LacI mRNAlevels remained constant over 24 h.

4. IPTG did not affect m-calpain concentrations markedly in L8/PC or L8/CID cells, but increased total m-calpain (endogenous + DN-m-calpain) markedly in L8/DN cells after 24 h **(Fig. 3)**. IPTG exerted this effect for 5 d in L8/DN cells.

3.6. Measurement of Total Protein Degradation

1. Culture myotubes in DMEM supplemented with 2% HS and label for 24 h with [^{3}H]tyrosine (2 µCi/mL). During this time, half the plates should be exposed to IPTG (5 m*M*).
2. After 24 h, replace the medium with DMEM containing 2% HS, 2 m*M* tyrosine (chase) and continue IPTG treatment (either 0 or 5 m*M*).
3. At this time 1.5 mL of medium should be taken and designated as radioactivity present at time 0.
4. Take samples of the media at various times later and determine their radioactivity.
5. From these observations, the effects of expressing either DN-m-calpain or CID on release of radioactive tyrosine into culture medium (i.e., total protein degradation) may be calculated. DN-m-calpain expression reduced degradation by 30% following 12 h of exposure and CID expression reduced degradation by 63% following 6 h of exposure **(Note 5)** ***(11)***.

3.7. Assessment of Effects of Ca^{2+}-Ionophore on Fodrin Concentration

1. A23187 was added to cell cultures to stimulate Ca^{2+} uptake and fodrin degradation. When A23187 (10–20 µ*M*) was added to cultures of L8/PC cells for 0–2 h, fodrin degradation was initiated **(Fig. 4)** ***(11)***.
2. Expression of CID and DN in L8/CID and L8/DN cells reduced A23187-dependent generation of fodrin cleavage product (150/145 kDa ***[11]***) (*see* **Note 6)**.

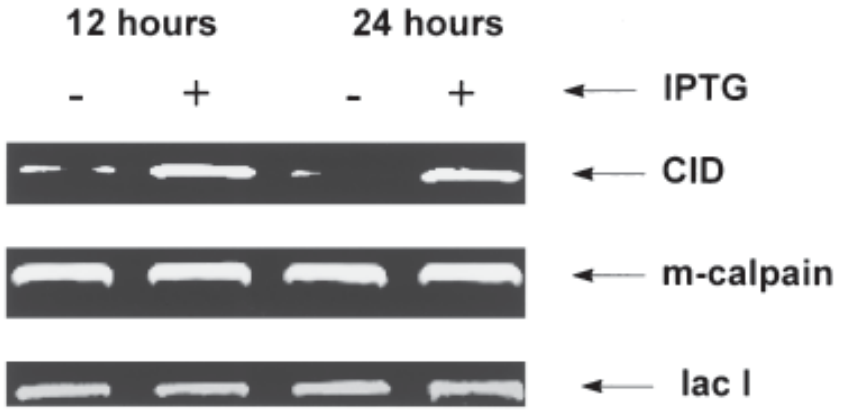

Fig. 2. Effects of IPTG on expression of mRNA for CID, m-calpain, and LacI in L8/CID cells as shown by RT-PCR analysis. CID mRNA levels increased in the cells in response to IPTG induction, while m-calpain and LacI mRNA levels remained constant.

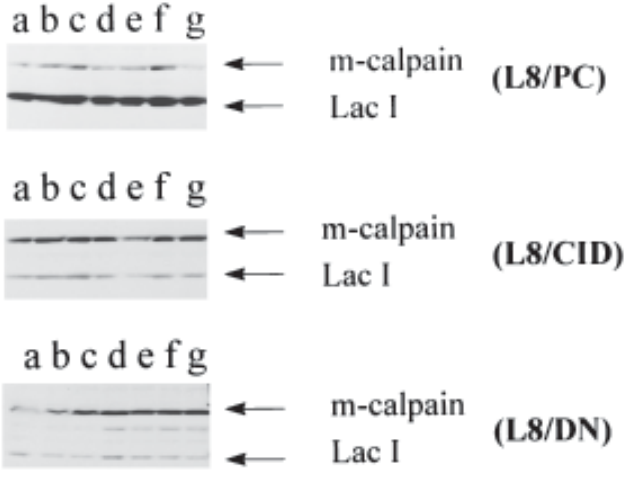

Fig. 3. Effects of IPTG induction on m-calpain and LacI protein levels measured by immunoblotting, in extracts of L8/PC, L8/CID, and L8/DN cells. The amount of m-calpain large subunit (wild-type plus DN-m-calpain) increased only in L8/DN cells, while the levels of other proteins, as controls, did not change. *Lanes a–g* correspond to exposure of cells to IPTG for 0 h, 8 h, and 1–5 d, respectively.

Notes

1. The Stratagene system includes two plasmids: the expression plasmid pOP13CAT, which includes the gene for hygromycin resistance, and the p3'SS plasmid which causes constitutive expression of the LacI repressor and contains the gene for gentamicin (G418) resistance.

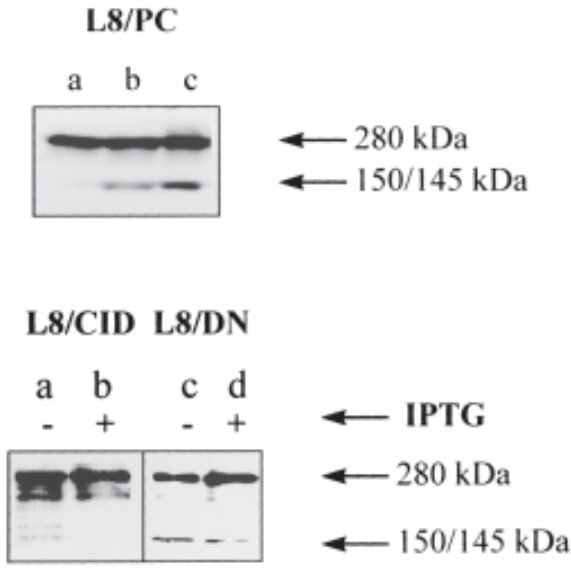

Fig. 4. Degradation of fodrin as shown by immunoblotting. *Lanes a–c* in the upper panel represent zero A23187, 10 μ*M* A23187 for 1 h, and 20 μ*M* A23187 for 2 h, respectively. The extent of formation of the 145/150 kDa fodrin degradation product was reduced in cells expressing CID or DN-m-calpain, in comparison with the control L8/PC cells.

2. Antibiotic-resistant colonies are selected by placing a small sterile cloning ring around a colony and adding a small amount of trypsin. The cells are recovered and transferred onto a 12-well plate, and gradually expanded in DMEM supplemented with 10% FBS and antibiotics.
3. It is generally necessary to screen large numbers of cells for antibiotic resistance to find one that performs properly. The first criterion for a cell line is its ability to differentiate. Individual clones are expanded, grown to confluence as myoblasts and then switched to differentiation medium (DMEM supplemented with 2% HS). These conditions will usually cause a confluent layer of myoblasts to fully differentiate within 4–5 d. In addition, we have verified constitutive expression of LacI by means of a Western blot. For DN-m-calpain expression, we assessed the total m-calpain (both endogenous and mutant m-calpain) content of cells following IPTG induction by Western blotting. For CID expression, we analyzed CID mRNA in induced cells by means of RT–PCR.
4. Preparation of cell lines which performed as expected was the most time-consuming aspect of this work. For each cell line desired, 20–30 individual clones were screened using the assays discussed in **Note 3**.
5. Expression of DN-m-calpain and CID occurs gradually over a period of 24 h, so that the inhibition of degradation occurs gradually following addition of IPTG to the culture medium. Hence, the percentage reduction in degradation caused by expression of DN-m-calpain or CID is a cumulative effect arising from changing inhibition over time.

6. By over-expressing DN-m-calpain or CID we believe we can identify the specific roles that calpain plays in turnover of individual proteins. It is not completely clear whether DN-m-calpain acts by binding to specific m-calpain substrates, or by sequestering calpain small subunit into an inactive enzyme. By contrast, CID allows us to assess the combined roles of μ- and m-calpains, since calpastatin inhibits both of these calpains. A similar strategy could be used for any other protein and, using pulse-chase approaches, effects of varying calpain activity on stability (half-life) of individual proteins could be assessed.

References

1. Goll, D. E., Kleese, W. C., and Szpacenko, A. (1989) Skeletal muscle proteases and protein turnover. in *Animal Growth Regulation*. Plenum, New York, NY. pp. 141–182.
2. Goll, D. E., Thompson, V. F., and Taylor, R. G. (1992) Role of the calpain system in muscle growth. *Biochimie* **74,** 225–237.
3. Fagan, J. M., Waxman, L., and Goldberg, A. L. (1989) Skeletal muscle and liver contain a soluble ATP and ubiquitin-dependent proteolytic system. *Biochem. J.* **243,** 335–343.
4. Furuno, K. and Goldberg, A. L. (1986) The activation of protein degradation in muscle by calcium or muscle injury does not involve a lysosomal mechanism. *Biochem. J.* **237,** 859–864.
5. Solomon, V. and Goldberg, A. L. (1996) Importance of the ATP-ubiquitin-proteasome pathway in the degradation of soluble and myofibrillar protein in rabbit muscle extracts. *J. Biol. Chem.* **271,** 26,690–26,697.
6. Gerard, K. W. and Schneider, D. L. (1979) Evidence for degradation of myofibrillar proteins by lysosomes: Myofibrillar proteins derivatized by intramuscular injection of N-ethylmaleimide are sequestered in lysosomes. *J. Biol. Chem.* **254,** 11,798–11,805.
7. Fenteany, G., Standaert, R. F., Lane, W. S., Choi, S., Corey, E. J., and Schreiber, S. L. (1995) Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. *Science* **268,** 726–731.
8. Croall, D. E. and McGrody, K. S. (1994) Domain structure of calpain: Mapping the binding site for calpastatin. *Biochemistry* **33,** 13,223–13,230.
9. DeLuca, C. I., Davies, P. L., Samis, J. A., and Elce, J. S. (1993) Molecular cloning and bacterial expression of cDNA for rat calpain II 80 kDa subunit. *Biochim. Biophys. Acta* **1216,** 81–93.
10. Ausubel, F. M., Brent, R., Kingston, R., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1990). *Current Protocols in Molecular Biology*. Wiley, New York.
11. Huang, J. and Forsberg, N. E. (1998) Role of calpain in skeletal muscle protein degradation. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 12,100–12,105.

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Assays of Apoptosis

Margaret K.T. Squier and J. John Cohen

1. Introduction

The phenomenon of apoptosis has gone from obscurity in 1972, when it was named ***(1)***, to the limelight of over 6000 publications in 1998 alone. Originally described as a peculiar morphology of cell death, seen when that death was "physiological" or programmed, apoptosis remains most clearly determined by morphology. Although there has been a great deal of interest in the biochemical and genetic regulation of the process, there is still no molecular definition of apoptosis for which a counterexample cannot be found. Thus in this chapter we have focused on morphology. The techniques we describe have the further virtue of being relatively inexpensive with respect to both reagents and equipment. We assume here that the investigator wants to determine whether the cells of interest have died by apoptosis or necrosis; if this question is not important, then any of the common dye-exclusion methods can be used to distinguish live from dead cells.

In a cell undergoing apoptosis, morphological changes take place that can be used to distinguish the process from necrosis. Necrosis is characterized by a loss of plasma membrane integrity, with other changes secondary to that. For example, a classic model of necrosis is the lysis of antibody-coated cells by the action of complement. In these cells, membrane permeability barriers are lost, and the cell is "dead" as shown by the uptake of trypan blue, eosin Y, propidium iodide, ethidium bromide, or other vital dyes. The morphology of the nucleus remains intact until lysosomal enzymes are released, after which nuclear disintegration begins and DNA is degraded. By contrast, apoptotic cells degrade their chromatin early in the process, and damaged DNA and nuclear collapse are seen while the plasma membrane is still intact. Thus an assay that

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can examine membrane integrity and nuclear condensation at the same time is highly desirable. Other canonical changes in apoptotic cells include: cellular shrinkage; fragmentation of DNA; "flipping" of phosphatidylserine from the inner leaflet of the plasma membrane to the outer; a peculiar "boiling" action of the plasma membrane, called zeiosis; the breaking up of the cell into apoptotic bodies; and its ingestion by a nearby phagocytic cell. All these changes happen while the plasma membrane is still intact and excluding vital dyes, and all can be measured depending on the design and needs of the experiment. An assay yet to be discovered is one that can monitor an absolute, universal protein marker of apoptosis: despite many candidates, no such protein has been definitively identified. For example, apoptosis can be delayed or prevented in many systems by the overexpression of Bcl-2, but this has been reported to inhibit necrosis as well ***(2)***.

Looking for a signaling system that would lead to death, we were attracted to the idea that such a system should involve proteases because their effects, like those of the Grim Reaper, are irreversible. Since a rise in intracellular calcium concentration often accompanies apoptosis, we focused on calpain. Inhibition of calpain with inhibitors of increasing specificity ***(3,4)*** blocks apoptosis in some but not all models of apoptosis: of the more familiar examples, apoptosis mediated by Fas (CD95) crosslinking is calpain-independent. By contrast, one of the oldest apoptosis models involves the death of thymus cells induced by glucocorticoids, and this death is blocked by calpain inhibition ***(5)***. Furthermore, calpain can be demonstrated to become activated before morphological apoptosis begins, and all aspects of apoptosis studied seem to be blocked by calpain inhibitors. Thus calpain appears to play its role upstream, as a signaling protease (it may also play a downstream, destructive role). Calpain has been implicated in a number of cell death systems, many of them of extreme biological importance, and most of these seem to involve apoptosis. Experimental use of calpain inhibitors in human disease of the central nervous system is now getting under way ***(6)***.

One issue that bears on the study of calpain in apoptosis is the molecular complexity of the death process. Other proteases also are involved in apoptosis, and of these the best characterized are the caspases. Like calpain they are cysteine proteases, but of an entirely separate family. In Fas-mediated cell death, where calpain is not involved, caspases are essential components of the signaling cascade. Whether calpain and the other proteases represent parallel pathways or have complex interactions with each other remains to be seen. Multiple calcium-dependent enzymes may also function in cell death. Another complication regarding calpain is its numerous functions: calpain is an enzyme of many faces, and doubtless does more things than we currently know. Interference with its roles in normal cellular housekeeping may damage cells,

and nonspecific damage, which might compromise a cell's function or promote tumorigenesis if left unchecked, often will lead a cell (for the benefit of the organism) to trigger its suicide process. Perhaps for this reason, we have observed that calpain inhibitors by themselves cause apoptosis in many cell types, particularly metabolically active or dividing transformed cells.

Described here are simple methods for detection and quantitation of apoptosis. They allow an investigator who does not focus primarily on cell death to assess quickly whether apoptosis is playing a role in a process of interest. We recommend beginning with a simple morphological determination **(Subheading 3.1.)**, which is rapid, highly informative, makes no *a priori* assumptions, uses an inexpensive blue-light fluorescence microscopy setup, and costs only pennies. More complex or advanced methods have been described in detail elsewhere *(7)*. The optimal combination of techniques to demonstrate apoptosis, in our extensive encounters with journal reviewers, would be morphology, plus an assay for DNA damage **(Subheadings 3.2.** and **3.3.)**, or annexin V binding (*see* **Note 15)**, demonstrated in cells with an intact plasma membrane permeability barrier. Many issues discussed elsewhere in this volume, including those of the specificity of calpain inhibitors and activity assays, and ways of resolving the function of calpain in other cellular processes, are also of particular relevance to apoptosis investigators.

2. Materials

2.1. Determination of Apoptosis by Light Microscopy

1. Acridine orange stock: 1 mg/mL acridine orange in 0.9% saline.
2. Ethidium bromide stock: ethidium bromide, 1 mg/mL in 0.9% saline.
3. Acridine orange / ethidium bromide (AO/EB) working solution: combine 100 µL of acridine orange stock solution, 100 µL of ethidium bromide stock solution, and 800 µL of 0.9% saline. The working solution and dye stocks are stable for >1 yr when stored in the dark (wrap the tubes in aluminum foil) at 4°C (*see* **Note 1)**.
4. Hoechst 33342 dye solution: 1 m*M* (0.62 mg/mL) Hoechst 33342 in water. Store as above.
5. Trypsin EDTA solution: use standard commercially available tissue culture formulation.
6. Microscope equipped with a 40 to 60X dry objective, using epiillumination and filters appropriate for observing fluorescein. An inexpensive quartz halogen bulb provides ideal light. Filters should provide for illumination at 488 nm, emission at 520 nm.

2.2. Quantitation of DNA Fragmentation by Diphenylamine or DNA Radiolabel Micromethods

1. Tris-Triton-EDTA (TTE): 10 m*M* Tris-HCl, pH 7.4, 0.2 % Triton X-100, 1 m*M* EDTA. Store at 4°C.

2. Tris-EDTA (TE): 10 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA. Store at 4°C.
3. TCA: 25% (w/v) in water; (corrosive; skin and eye protection must be worn). Store at room temperature.
4. Acetaldehyde solution: add 16 mg acetaldehyde (~20 µL) to 1 mL of water. Store at 4°C for up to 1 yr.
5. Diphenylamine (DPA) reagent: make this reagent fresh for each experiment, and no more than 1 h before use:
   a. dissolve 150 mg of diphenylamine in 10 mL of glacial acetic acid;
   b. add 150 µL of concentrated sulfuric acid and mix;
   c. add 50 µL of acetaldehyde solution and mix (*see* **Note 2**).
6. 2% (w/v) SDS in water.

2.3. Agarose Gel Electrophoresis to Observe Internucleosomal DNA Fragmentation

1. 5 *M* NaCl.
2. 10 × DNA loading buffer: 20% (w/v) Ficoll 400, 0.1 *M* EDTA, pH 8.0, 1% (w/v) SDS, 0.25 % (w/v) bromophenol blue.
3. TBE gel running buffer (10 × stock): 108 g Tris base (0.89 *M*), 55 g boric acid (0.89 *M*), 40 ml 0.5 *M* EDTA, pH 8.0 (10 m*M*), water added to make 1 L.

3. Methods

3.1. Determination of Apoptosis by Light Microscopy (*See* **Note 3.**)

1. Harvest of cells in suspension:
   a. if the cell concentration is ≥ 3×10^6/mL, combine 25 µL of cell suspension with 2 µL of AO/EB working solution and mix gently;
   b. if cells are at lower concentration: harvest cells by centrifugation at 200*g* for 10 min. Remove all but a small amount of supernatant, and resuspend cells in the residual fluid. Combine 25 µL of this suspension with 2 µL of AO/EB working solution and mix gently. If a series of samples is to be examined, store the cells on ice until nearly ready to read them, then add the dye mixture.
2. Harvest of adherent cells:
   a. remove the culture medium to a centrifuge tube;
   b. detach the adherent cells by covering them with trypsin/EDTA solution and incubating for several min at 37°C;
   c. when the cells have detached, transfer them to the tube containing the initial culture medium;
   d. wash the culture wells or plates twice with normal saline and add the washes to the same tube;
   e. centrifuge the tube at 200*g* for 10 min, remove all but a small amount of supernatant, resuspend in the residual fluid, and take 25 µL to combine with 2 µL of AO/EB working solution.

   Centrifugation of the mixture of medium, trypsinized cells, and washes, all

combined in one tube, will pellet apoptotic cells (which will primarily be floating in the medium in cell culture) along with normal cells (which will primarily be adherent).

3. Preparation of slides: place 10 µL of the cell/dye mixtures on a microscope slide and cover with a 10 × 10 mm No. 1 cover slip **(***see* **Note 4)**.
4. Examine with a 40 to 60X dry objective, using epiillumination and filters appropriate for observing fluorescein.
5. Scoring live vs dead cells: both acridine orange and ethidium bromide intercalate with DNA, and preferentially stain cell nuclei. Acridine orange is cell-permeant, allowing for visualization of nuclear structure in living cells. Nuclei stained with acridine orange appear yellow-green, and fluorescence of various colors may also be seen in the cytoplasm of normal cells (e.g., from RNA or mitochondrial DNA). Ethidium bromide, like trypan blue or eosin, does not penetrate intact cell membranes; however when a cell dies and its plasma membrane ruptures, ethidium bromide reaches the nucleus and, overwhelming the acridine effect, stains the nucleus orange-red.
6. Scoring apoptotic vs non-apoptotic cells by nuclear morphology: normal cell nuclei have "structure" or a lacy appearance: there are variations in intensity reflecting the distribution of euchromatin and heterochromatin. Apoptotic nuclei, by contrast, have very condensed chromatin. This can take the form of crescents around the periphery of the nucleus, or the entire nucleus can present as one or more featureless, bright, spherical beads **(***see* **Note 5)**. In advanced apoptosis, the cell will have lost DNA and the overall brightness may be less than that of a normal cell.
7. Differential counts: score 100 cells (minimum) into one of four categories:
   a. live nonapoptotic (green nuclei, normal distribution of chromatin);
   b. live apoptotic (green nuclei, condensed chromatin);
   c. dead nonapoptotic (orange nuclei, normal distribution of chromatin);
   d. dead apoptotic (orange nuclei, condensed chromatin) **(***see* **Notes 6–9)**.

3.2. Quantitation of DNA Fragmentation by Diphenylamine or Radiolabel Micromethods (See Note 10.*)*

1. Transfer cells ($1–5 \times 10^6$ per data point) to a 1.5 mL microcentrifuge tube labeled "B" (for "bottom").
2. Pellet cells by centrifugation at 200*g* for 10 min. Aspirate and discard supernatant **(Note 11)**.
3. Add 0.5 mL of TTE to the cell pellet and vortex. Let stand for at least 10 min to allow for cell and nuclear lysis.
4. Separate DNA fragments from intact chromatin by centrifugation at 13,000*g* for 10 min.
5. Carefully remove most of the supernatant to a separate microcentrifuge tube labeled "T" (for "top").
6. Add 0.5 mL of TE to the cell pellets in the "B" tubes.
7. Add 0.5 mL of 25% TCA to all (B and T) tubes and vortex. Place at 4°C overnight

to precipitate DNA.
8. Pellet precipitated DNA in all tubes by centrifugation at 13,000*g* for 10 min. Aspirate and discard supernatants.
9. Add 80 µL of 5% TCA and hydrolyze the DNA by heating the tubes to 90°C for 15 min in a heating block. Include a blank tube containing only 80 µL of 5% TCA.
10. Prepare diphenylamine (DPA) reagent (*see* **Note 12**). Add 160 µL of DPA reagent to each tube, including the blank, and vortex.
11. Allow color to develop overnight at room temperature.
12. Transfer 200 µL of the colored solution (ignore any dark particles) to wells of a 96-well plate and read OD at 600 nm, setting the blank to zero (*see* **Notes 13** and **14**).
13. Calculate percent DNA fragmentation as (T) × 100 / (T+B) (*see* **Notes 15** and **16**).
14. If cell lines are used and cell numbers are not sufficient to allow DNA quantitation by diphenylamine, a protocol for radiolabeled DNA may be substituted, as follows.
15. Radiolabel healthy, growing cells either with [^{3}H]thymidine (2 µCi/ml, overnight) or [^{125}I]UdR (1 µCi/ml, for 1 h).
16. Recover the cells, wash four times with tissue culture medium and check labeling by counting, both cell numbers and radioactivity, using a small aliquot of cells (*see* **Note 17**).
17. Carry out the desired experimental treatment of the cells, using 5–10 × 10^3 cells per data point.
18. After each experiment, transfer cells to a microcentrifuge tube and pellet along with 0.5 mL of unlabeled cells (the "cold" cells will help to bring the intact radiolabeled DNA down into the "B" fraction) (*see* **Note 18**).
19. Lyse cells with TTE as above, centrifuge at 13,000*g* to separate fragments from intact DNA, and determine the radioactivity in these "T" and "B" fractions (no TCA precipitation step is needed). Determination of [^{3}H]thymidine incorporation will involve liquid scintillation with an appropriate water-accepting fluor; incorporation of ^{125}IUdR-labeled DNA can be determined directly in a gamma counter. To solubilize "B" pellets for transfer to scintillation vials, add 0.5 mL of 2% SDS and allow to sit overnight.

3.3. Agarose Gel Electrophoresis to Observe Internucleosomal DNA Fragmentation

1. Prepare T and B tubes from cells as in **Subheading 3.2., steps 1–6 (Note 19)**.
2. Add 100 µL of ice-cold 5 *M* NaCl to each tube and vortex thoroughly.
3. Add 650 µL of ice-cold isopropanol to each; and vortex again.
4. Store at least overnight at –20°C.
5. Centrifuge at 13,000*g* for 10 min at 4°C. Discard supernatant.
6. Add 0.5 mL of ice-cold 70% ethanol to each tube, and centrifuge again as in **step 5**.
7. Stand tubes in inverted position over laboratory tissues to allow residual supernatant to drain away. After a few minutes, return tubes to upright position and allow to dry, open, for several hours.
8. Add 20-50 µL of TE; incubate for 1 (for T) to 3 (for B) d at 37°C to dissolve pellet (*see* **Note 20**).

9. Add 0.1 vol of 10 × DNA loading buffer, and heat for 10 min at 65°C.
10. Apply 10–20 µL to a full size 1% agarose gel in 1 × TBE (minigels do not resolve very well for this purpose), and carry out the electrophoresis.
11. Stain for several minutes with ethidium bromide solution and photograph on a UV light box (**Note 21**).

4. Notes

1. Note biohazard: both acridine orange and ethidium bromide are mutagens by the Ames test; skin and eye protection should be worn.
2. It is critical to follow these directions, in the precise order. Note biohazard: this reagent contains corrosives; skin and eye protection must be worn.
3. The gold standard for detection of apoptosis is the same as it was almost 30 yr ago: nuclear morphology ***(1)***. This can be seen, and quantitated, by assays such as the AO/EB method. For final confirmation on any new cell type, light microscopy should not stand alone: the definitive identification of apoptosis is by electron microscopy.
4. If counting multiple slides, prepare only one or two at a time, since drying of the slide will distort cellular architecture. A hemocytometer may be used instead of a slide, but nuclear morphology is more distinct on a slide because of the moderate compression provided by the No. 1 coverslip. A cover slip thicker than No.1 may provide too much compression, lysing the cells.
5. Positive and negative controls for nuclear morphology: in any experiment, untreated cells should be observed, to familiarize the eye with normal cellular architecture. To do the same for apoptotic nuclei, it is helpful to observe cells tested with an agent known to induce apoptosis in that cell type. If none has been characterized, serum starvation (growing cells in serum-free medium overnight) may be sufficient.
6. A color photograph of cells stained by this technique, with all four categories demonstrated, can be seen in Dwyer-Nield et al. ***(8)***.
7. Troubleshooting:
   a. excessive debris is observed. Common causes include cell lysis, by necrosis or at a late stage in apoptosis in vitro; damage to cells during harvest may also produce debris. Trying earlier time points may help. Careful handling of cells, including avoiding cytospinning, scraping, or vigorous pipetting, will also reduce debris.
   b. poor visualization of nuclei. Try additional dye. Also, if any agent in the experimental system is an intercalator, this may interfere with the AO/EB.
   c. bleaching. Check that filters are intact (not burned out), providing only blue light to the sample. Unfiltered UV light will produce relatively rapid bleaching with these dyes.
8. The power of the AO/EB combination lies in the ability, with one filter set, to examine both nuclear morphology and viability simultaneously on any given cell. This prevents common errors in data interpretation: for example, treatment of cells with distilled water might be seen as preventing apoptosis if nuclear

morphology was the only variable assessed (since a lysed cell cannot commit suicide), whereas it would be interpreted as causing apoptosis if cell death was the only variable assessed. An excellent source of information about fluorescent dyes and their uses may be found at the Molecular Probes, Inc. website: http://www.probes.com.

9. Alternative dyes and stains can be used to separately visualize nuclear morphology (e.g., Hoechst 33342, 4', 6'-diamidino-2-phenylindole [DAPI]) or viability (e.g., trypan blue, eosin). As shown in **Fig. 1**, Hoechst 33342 staining is particularly useful for visualizing nuclei in tissue sections, as nearly no other structures take up the dye. Hoechst dye stock should be 1 m*M* in water, and mixed with cells (or placed over a tissue section) at 10 μ*M* final concentration. Hoechst dyes require ultraviolet illumination, and filters that pass excitation light at 360 nm, and emitted light at 460 nm.
10. As discussed in detail elsewhere ***(7)***, other assays for chromatin damage include labeling methods for nicked DNA (e.g.,TUNEL), nucleosome enzyme-linked immunosorbent assays (ELISAs), and flow cytometric assays for size/complexity of stained nuclei. Each assay has its pluses and minuses. The assays in **Subheadings 3.2.** and **3.3.** detect oligonucleosomal DNA fragments, a common product of the nuclear destruction in apoptosis. In some cell types, chromatin damage has a different pattern: larger fragments (50–300 kbp), best detected on pulsed-field gels, or single-stranded nicks, which can be identified by TUNEL. Other pitfalls to watch for in various chromatin damage assays are (i) qualitative, not quantitative, results (e.g., agarose gels, where intact DNA cannot be completely solubilized and quantified, or flow cytometry for size of stained nuclei, where cells that separate into multiple DNA-containing apoptotic bodies are counted multiple times, inflating a sub-G_0 peak); (ii) inability to discriminate DNA damage in apoptosis from that occurring spontaneously at late stages of necrosis (e.g., TUNEL).
11. If apoptotic cells have lysed during the course of the experiment, some fragments may be present within this supernatant. If the supernatant may contain significant amounts of DNA, one may elect to keep it in a separate tube, labeled "S." It should be treated in a manner similar to the T and B fractions, beginning with TCA precipitation (12.5% TCA, overnight at 4°C, then centrifugation, as for the T and B tubes). In this case, the final calculation for percent DNA fragmentation is (S+T) divided by (S+T+B) times 100. Because constituents of tissue culture medium may interfere with the final OD reading, a separate blank tube containing medium should also be included, beginning at the TCA precipitation step.
12. The diphenylamine protocol is a micromethod ***(9)*** adapted from the original protocol of Burton ***(10)***.
13. The wavelength used should be within the range of 560 to 620 nm. An ELISA reader is easiest, but a spectrophotometer may be substituted.
14. Troubleshooting: lack of color development. Common causes include an inadequate cell number per point (at least 1 million cells per point, preferably 3 million to 5 million, must be used). Another is the diphenylamine reagent: it must be prepared exactly according to the protocol, and immediately before use.

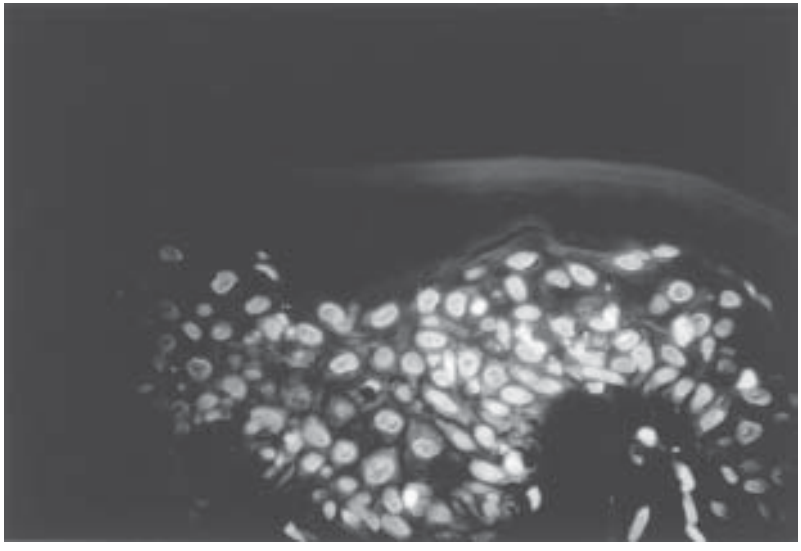

Fig. 1. Section of normal human skin stained with Hoechst 33342 1 d after exposure to two minimal erythema units of ultraviolet radiation. Note the bright-staining, collapsed morphology of the nuclei in the characteristic apoptotic "sunburn cells," scattered throughout the field. (Photograph courtesy of Dr. David A. Norris.)

The opposite problem, color development beyond the range of the ELISA reader, may occur if too many cells are used.

15. When comparing results by fragmentation with morphology, the percent DNA fragmentation will usually be lower than the percent apoptosis. This probably arises from incomplete separation of fragments from intact DNA: a small amount of fragmented DNA can typically be found in the "B" fraction.
16. If no fragmented DNA is detected but morphology strongly indicates apoptosis, the chromatin damage may be in the form of single-stranded nicks or large (50–300 kbp) fragments. Techniques that detect these types of chromatin damage might be informative (e.g., TUNEL or pulsed field gels, as discussed in **Note 10**). Another option would be to assay for membrane events in apoptosis: the most common technique uses labeled annexin V, which binds to phosphatidylserine on cell surfaces. This lipid is normally confined to the inner leaflet of the plasma membrane, but is "flipped" to the outside on an apoptotic cell ***(11)***. This is a sensitive and elegant assay for live apoptotic cells, but cannot distinguish dead apoptotic cells from dead necrotic cells, since the disrupted membrane gives annexin V access to the inner leaflet, where phosphatidylserine is plentiful. For this reason it is also not useful in tissue sections, unless annexin V is preadministered in vivo ***(12)***.
17. 2–3 dpm per cell would be considered optimal labeling. Suboptimal labeling might be caused by poor cell growth or contamination of the culture, especially

by mycoplasma.

18. Supernatants may be saved and counted (these may be contaminated with residual free radiolabel; a TCA precipitation step may be useful on the supernatant—12.5% TCA overnight, then centrifuge at 13,000*g* for 10 min and count pellet).
19. For this protocol, between 500,000 and 5 million cells per condition should be used. Again, in cell lines, signals may be achieved with fewer cells (≥ 1000) by labeling with [^{125}I]UdR and detection by autoradiography.
20. Oligonucleosomes will be detected mostly in S and T fractions. B tubes will contain mostly intact DNA, which is poorly solubilized by this technique; for this reason it may be most efficient to run only T, or S and T, fractions.
21. This procedure is intended to visualize the "ladder" pattern of DNA fragments of multiples of 200 bp, achieved by cleavage between nucleosomes. If samples are not contaminated with foreign nucleases (e.g., micrococcal nuclease), the detection of this pattern is highly indicative of apoptosis. It is important to reiterate, however, that because of the problem of recovery and solubilization of intact DNA, the intensity of staining of a ladder does not definitively *quantitate* the amount of apoptosis occurring in a cell population.

References

1. Kerr, J. F. R., Wyllie, A. H., and Currie, A. R. (1972) Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* **26,** 239–257.
2. Kane, D. J., Ord, T., Anton, R., and Bredesen, D. E. (1995) Expression of bcl-2 inhibits necrotic neural cell death. *J. Neurosci. Res.* **40,** 269–275.
3. Squier, M. K., Miller, A. C., Malkinson, A. M., and Cohen, J. J. (1994) Calpain activation in apoptosis. *J. Cell Physiol.* **159,** 229–237.
4. Squier, M. K. and Cohen, J. J. (1997) Calpain, an upstream regulator of thymocyte apoptosis. *J. Immunol.* **158,** 3690–3697.
5. Wyllie, A. H. (1980) Glucocorticoid-induced thymocyte apoptosis is associated with endogenous nuclease activation. *Nature* **284,** 555–556.
6. Kampf, A., Posmantur, R. M., Zhao, X., Schmutzhard, E., Clifton, G. L., and Hayes, R. L. (1997) Mechanisms of calpain proteolysis following traumatic brain injury: Implications for pathology and therapy: Implications for pathology and therapy: A review and update. *J Neurotrauma* **14,** 121–134.
7. Martin, D. and Lenardo, M. (1998) Morphological, biochemical, and flow cytometric assays of apoptosis, in *Current Protocols in Immunology* (Coligan J. E., Kruisbeek A. M., Margulies D. H., Shevach E. M., and Strober W., eds), Wiley, New York, pp. 3.17.1–3.17.39.
8. Dwyer-Nield, L. D., Thompson, J. A., Peljak, G., Squier, M. K., Barker, T. D., Parkinson, A., Cohen, J. J., Dinsdale, D., and Malkinson, A. M. (1998) Selective induction of apoptosis in mouse and human lung epithelial cells by the *tert*-butyl hydroxylated metabolite of butylated hydroxytoluene: A proposed role in tumor promotion. *Toxicology* **130,** 115–127.

9. Sellins, K. S. and Cohen, J. J. (1987) Gene induction by gamma-irradiation leads to DNA fragmentation in lymphocytes. *J. Immunol.* **139,** 3199–3206.
10. Burton, K. (1956) A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62**, 315–323.
11. Fadok, V. A., Voelker, D. R., Campbell, P. A., Cohen, J. J., Bratton, D. L., and Henson, P. M. (1992) Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J. Immunol.* **148,** 2207–2216.
12. van den Eijnde, S. M., Luijsterburg, A. J., Boshart, L., De Zeeuw, C. I., van Dierendonck, J. H., Reutelingsperger, C. P., and Vermeij-Keers, C. (1997) In situ detection of apoptosis during embryogenesis with annexin V: From whole mount to ultrastructure. *Cytometry* **29,** 313–320.