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Chaperonin Protocols

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Purification of Archaeal Chaperonin from *Sulfolobus shibatae*

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1. Introduction

Sulfolobus shibatae is a hyperthermophilic archaeon that was first identified living in acidic geothermal hot springs. This organism grows optimally at pH 3.0–4.0 and 83°C (1), however it grows over the temperature range of 75–85°C. When *S. shibatae* is subjected to higher temperatures (85–90°C) a heat-shock response is observed and the major protein induced is a large ring structure TF55 (2), also called archaeosome (3) or rosettasome (4), which is composed of two different subunits (4), designated α and β (3,4). Apart from the presence of two subunits, another difference between this molecule and the prokaryotic GroEL is the number of subunits per ring. In *S. shibatae*, nine subunits form each ring compared with seven in GroEL.

The similarity of this double ring (2,3) to that of GroEL, together with the fact that the subunits were 60 kDa, it was a heat-shock protein, and it was active in protein folding, suggested that it was the archaeal chaperonin (2) and was similar in function to GroEL. However, comparison of the primary structure showed that both subunits were more closely related to the TCP-1 family of polypeptides, the eukaryotic cytosolic chaperonin (2,4,5). In contrast to the archaeal chaperonin, the eukaryotic cytosolic chaperonin comprises eight sequence-related polypeptides, which also form the characteristic “double doughnut.” The archaeal chaperonin therefore gives us a simplified version of the eukaryotic chaperonin by which it may be possible to determine characteristics of the eukaryotic chaperonin. Archaeal and mammalian cytoplasmic chaperonins are often referred to as Type II chaperonins. The chaperonin from *S. shibatae* is a Type II chaperonin (6) and is related, by primary sequence, to

other chaperonins found in the archaea (for example, 7–9, and others therein). A sequence comparison of this chaperonin with others is given in **Fig. 1**.

We routinely purify this chaperonin by methods commonly used for other chaperonins (**10**, and other chapters in this volume). The final yield of protein can be enhanced by a slight heat shock to the culture (88°C for 30 min). Two main properties, common to most chaperonins, are exploited during its purification, that of its low isoelectric point ($pI = 5.3$) and size (α -subunit = 59.72 kDa; β -subunit = 59.68 kDa, and complex $9\alpha + 9\beta = 1074.6$ kDa).

The chaperonin from the *Sulfolobus* species, with 18 subunits in the oligomer, is the largest of the chaperonins studied to date. Gel-permeation chromatography is therefore a very important step in the purification process. We use Sephacryl high-resolution (HR) resins from Pharmacia (Uppsala, Sweden), which are composite gels made by covalently crosslinking allyl dextran with *N,N,N*;-methylene bis-acrylamide to form a hydrophilic matrix of high-mechanical strength and excellent flow properties.

Purification of the chaperonin from *S. shibatae* involves three chromatographic steps, with concentration and dilution of the sample in between. A detailed discussion of the chromatographic principles used can be found in Chapter 3 of this volume. Detection of the chaperonin is either by native or SDS-PAGE. The three chromatographic steps are:

1. Fast Q-Sepharose anion-exchange chromatography.
2. Gel-filtration chromatography on Sephacryl S-300 HR.
3. Mono Q-Sepharose HR anion-exchange chromatography.

These three steps can produce chaperonin, which is 98–99% pure on a milligram scale in 2–3 d. One interesting aspect of this chaperonin is that when purified, it forms a doublet on a native gel. Initially we thought that this was owing to an “empty” chaperonin ring oligomer and a larger, “loaded,” complex between the chaperonin oligomer and bound substrate. This was not observed for GroEL or other Type I chaperonins. We thought that if it were possible to purify each band, it might give some insight into the mechanism of action of Type II chaperonins.

The two forms are separated by preparative continuous zone native gel electrophoresis, in a preparative cell from Bio-Rad (Hercules, CA). This technique is relatively simple and involves the use of gels of a uniform polyacrylamide concentration in conjunction with a single homogenous buffer system. In such a system, sample is applied to the top of a gel, and separation occurs on the basis of both the charge and the size properties of the proteins being analyzed. In this case, electrophoresis conditions are optimized to maximize the differences between the two forms of the chaperonin from *S. shibatae*. During continuous electrophoresis, protein leaving the bottom of the gel is washed with buffer and

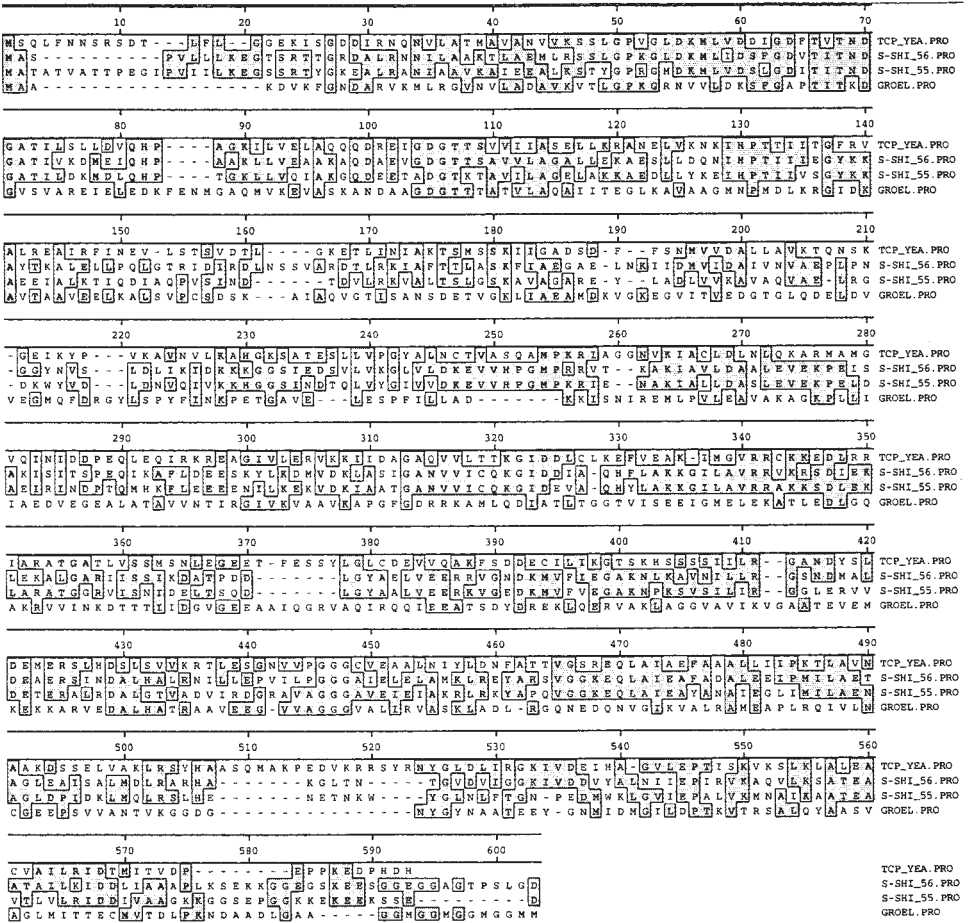


Fig. 1. Sequence comparison of the *Sulfolobus shibatae* chaperonin (S-shi_55; S-shi_56) with Tcp-1 from yeast (TCP_YEA) and GroEL from *E. coli* (GROEL).

collected in a fraction collector, in a manner similar to that used for column chromatography. The two forms can then be studied individually. The high solubility of the chaperonin at neutral pH is an important attribute during this process, since concentration of the chaperonin in a tight band during electrophoresis does not cause precipitation. Sufficient chaperonin from each band can be purified by this method for electron microscopy and circular dichroism studies (3). Since *S. shibatae* is a thermophilic archaea, all steps can be carried out at room temperature. We have not tried to carry out the purification procedure at lower temperatures. However because the chaperonin is stable during

storage at 4°C, it could be inferred that purification at this temperature would not be detrimental to the protein.

2. Materials

Except where noted, all chemicals are purchased from Sigma (St. Louis, MO) and are analytical grade or higher.

2.1. Cell Growth

S. shibatae (DSM strain 5389) was obtained from Grogan et al. (1). Cells were obtained from J. Trent, who maintained the culture in liquid medium in a 150 L fermenter (see Note 1).

1. Liquid culture medium:

- a. 0.1% (w/v) Yeast extract
- b. 0.1% (w/v) Sucrose
- c. Salts: (Weight/L)

9.80 mM (NH ₄) ₂ SO ₄	1.29 g
2.00 mM KH ₂ PO ₄	272 mg
0.48 mM CaCl ₂	52.8 mg
0.74 μM FeCl ₃	119 mg
- d. Trace elements: (Weight/L)

7.5 μM MnCl ₂	0.93 mg
.75 μM ZnSO ₄	0.12 mg
.29 μM CuCl ₂	.038 mg
.12 μM Na ₂ MoO ₂	.021 mg
- e. pH should be adjusted to 3–5 with conc. H₂SO₄.

2.2. Chromatography Buffers

1. Lysis buffer: 50 mM Tris-HCl, pH 7.5, 0.5% Triton X-100 (v/v).
2. Buffer A: 50 mM Tris-HCl, pH 7.5 1 mM EDTA, 1 mM dithiothreitol (DTT), 50 mM NaCl.
3. Buffer B: 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 1 M NaCl.
4. Buffer C: 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 250 mM NaCl.
5. Buffer D: 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 500 mM NaCl.
6. Buffer E: 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM DTT, 250 mM NaCl.
7. Buffer F: 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM DTT, 250 mM NaCl, 50% (v/v) glycerol.

2.3. Fast Q-Chromatography

Q-Sepharose Fast-Flow anion-exchange media are stored in 20% ethanol and must be washed at least four times with 4 vol. of deionized H₂O prior to use. Media are suspended in 5 vol. of buffer A. A 30 × 2.3 cm column is

cleaned, and the resin (90 mL) poured into the column according to the manufacturer's directions. This amount of resin will fill the column to about 7 cm below the top, which allows room for the adapter to be fitted. We find the use of gravity sedimentation sufficient for this process. The column is then packed at a flow rate of 6 mL/min using buffer A. Column equilibration is carried out using at least 10 column volumes of buffer A. Complete equilibrium is obtained when the pH of eluant is the same as the buffer. The column is then ready for use. With care, the column and beads can be used successfully for many months. After use, we always wash the column in a 2 M NaCl solution, followed by water, and store it in a 20% (v/v) ethanol:H₂O solution.

2.4. Gel-Filtration Chromatography

Sephacryl S-300 HR must be washed with deionized, degassed water (at least four times with twice the matrix volume) before column preparation. Several washes are usually required to remove all the "fines" (crushed beads that have a detrimental effect on the flow rate of a column). This matrix is of lower mechanical strength, and care must be taken not to damage the gel beads. The matrix also requires a longer settling time both during washing and column preparation. Prior to column preparation, the gel matrix is generally suspended in 2 vol of buffer C and degassed for 30 min. A 150 × 2.0 cm column is prepared, and the gel poured into the column and allowed to settle by gravity. Approximately 400–420 mL of resin are used, which again allows for an adapter to be fitted to the top of the column. It is also possible to run a gel-filtration column without an adapter, which will increase the effective length of the column. However, we have found that the use of an adapter gives a more uniform sample and eluant application. The column is equilibrated by running the column at 1 mL/min overnight with buffer C. Care should be taken when manipulating this column, since this column matrix is highly sensitive to the introduction of air bubbles. This column is run at room temperature, so all buffers should be equilibrated at this temperature.

2.5. MonoQ HR Chromatography

The final chromatographic step, HR ion-exchange chromatography, is carried out using prepacked columns from Pharmacia. Two different sizes are commonly employed depending on the amount of protein to be applied, MonoQ HR 10/10 (10 × 100 mm) and HR 16/10 (16 × 100 mm). The reactive group on this resin is also a quaternary amine, the same as that in Q-Sepharose. However, the bead size is strictly controlled giving a highly uniform, monodisperse resin. This allows very rapid and HR chromatography.

2.6. Electrophoresis

Screening of the fractions obtained by chromatography is carried out using preformed PHAST gels (Pharmacia): 12.5% for SDS gels and gradient 4–15% for native PAGE. The sample loading buffers are as follows:

5X SDS: 0.5 g SDS, 0.3 g DTT, and 1 mg pyronin Y dissolved in 10 mL 75% glycerol/25% 50 mM Tris-HCl, pH 7.5.

5X Native: 1 mg bromophenol blue dissolved in 10 mL 75% glycerol/25% 50 mM Tris-HCl, pH 7.5.

Alternatively, 12% Lammeli SDS gels (**II**) can be used for screening purposes. GroEL can also be detected on 4 or 6% large-format slab PAGE gels using the protocol described in (**3**) and Chapter 3 of this volume. The staining method described below is suitable for all types of electrophoresis described in this protocol.

1. Staining solution: 10% (v/v) acetic acid, 30% (v/v) methanol, 0.1% Coomassie brilliant blue R250, 60% H₂O (v/v).
2. Destain I: 10% (v/v) acetic acid, 30% (v/v) methanol, 60% H₂O (v/v).
3. Destain II: 10% acetic acid, 10% glycerol, 80% H₂O (v/v) (*see Note 2*).

2.6.1. Preparative Nondenaturing PAGE

Gel solution (4%) caution (*see Note 3*).

1. 10.0 mL 40% acrylamide stock solution.
2. 5 mL Buffer (0.25 M Tris-glycine, pH 8.0).
3. 85 mL H₂O.
4. Degas the solution and add:
 - a. 100 μ L 1 M DTT.
 - b. 50 μ L *N,N,N',N'*-tetramethylethylenediamine (TEMED).
 - c. 500 μ L 10% ammonium persulfate.

For detailed recipes, please refer to Chapter 3 in this volume. Chaperonin forms were separated on 4% (w/v) polyacrylamide gels, cast in the chamber of a Bio-Rad preparative cell model 491. This equipment allows the casting of a vertical gel inside tubular gel formers, forming a polyacrylamide gel tube. We found a gel height of approx 4 cm to be optimal in the separation of the two forms of the chaperonin. The chaperonin sample is applied to the top surface of the gel, the buffer chamber is filled with running buffer, and electrophoresis (25 V/cm; room temperature) is carried out for 30–40 min. The bottom of the tubular gel is washed with running buffer automatically and collected in a chamber at the base of the setup, and flows through a tube leading to a fraction collector. Fraction tubes are usually changed every 30–60 s. Approximately 300–500 μ L elute each minute. These are then concentrated and run on native

gels in order to determine whether the “open” or “closed” form is present (*see Note 4*).

2.7. Protein Concentration

Two methods are employed for the concentration of crude extract or protein solutions during the purification protocol.

1. Nitrogen-pressurized stirred cell (Amicon). For concentrations of large volumes (50–1000 mL), we use the stirred cell according to the manufacturer’s instructions at 50 psi. The use of a membrane with 100-kDa cutoff (Difco [Detroit, MI] or Millipore [Bedford, MA]) allows this to be a fairly rapid procedure and serves as an additional purification step.
2. Centrifugal concentrators: For sample sizes under 50 mL we commonly use centrifugal concentrators; Centricon (2 mL) or Centriprep (15 mL) with 100-kDa cut-off membranes (Amicon). Filtron concentrators have also been used with excellent results (*see Note 5*). Millipore concentrators, a relatively new product, allow the direct visualization of the volume left in the concentrator, which is very useful.

3. Methods

3.1. Cell Growth

1. Successful cell growth can be obtained (with much effort) using benchtop incubators. However, a higher cell density can be achieved in a fermenter (*I*). The cells are grown in liquid culture media at 70°C, and cell growth is monitored by direct counts using a hemocytometer. The cells are harvested after a short heat shock (85°C for 30 min).
2. To ensure that the chaperonin has been induced during this process, an aliquot (1 mL) of culture is removed and spun in an Eppendorf tube in a microcentrifuge to pellet the cells. The supernatant is decanted off, and the resulting cell pellet resuspended in 200 μ L SDS buffer, boiled for 1 min, and after making a series of dilutions using 1X SDS sample buffer, applied to a 12.5% SDS gel. The samples are compared with a purified chaperonin standard, and efficient induction is seen as a large doublet at approx 55 kDa, which can be up to 50% total cell protein (*see Note 6*).

3.2. Cell Harvesting and Extract Preparation

1. After growth in a fermenter, cells are removed from the growth media by centrifugation (26,800g; flowthrough rotor; Sharples 16A). Alternatively, if grown in flasks, they are removed by centrifugation in bottles (5000g, 15 min, GSA rotor). The cell pellet is then gently scraped from the centrifuge bottles, combined, and weighed. A cell yield of 0.5–1.0 g/L culture is normal.
2. The cells are then resuspended in 5x (v/w) lysis buffer. This is stirred gently for 5 min on ice before sonication. Generally, 5 min of sonication on ice, i.e., 1 min interspersed with a 1-min rest, are sufficient for cell disruption.

3. The suspension is centrifuged at 17,000g in a Sorval GSA rotor for 30 min at 15°C, which pellets the cell membranes and other debris.
4. The clarified cell extract, the supernatant, contains the soluble protein from the cell and is applied directly to the Fast Q column. All steps in the purification of *S. shibatae* chaperonin can be carried out at room temperature, since this is “frozen” with respect to the thermophilic proteins.

3.3. Fast Q Chromatography

1. The extract was applied at a flow rate of 6 mL/min to the Fast Q anion-exchange column, which was pre-equilibrated with buffer A. The column was then washed with at least 500 mL buffer A (5 mL/min). *Sulfolobus* extract is very dark and viscous compared to *Escherichia coli* and will form a dark band at the top of the column (see **Note 8**).
2. Proteins are eluted with a 1.7 L linear gradient of 50 mM to 0.5 M NaCl (buffer A and buffer B) and collected in 18-mL fractions. Fractions containing the chaperonin were identified using both 4–15% native PAGE and 12.5% SDS-PAGE (see **Note 7**) using the PHAST system. *S. shibatae* chaperonin elutes between 100 and 220 mM NaCl (**Note 9**).
3. A typical chromatograph is shown in **Fig. 2A**, with the shaded bar representing the fractions containing the chaperonin. Fractions containing the archaeal chaperonin are pooled and concentrated in the stirred cell. This often involves the concentration of 50–200 to 15–20 mL. The reason for this high degree of concentration is because the fact that the resolution of the proteins on the gel-filtration column is dependent on the volume of the sample. Another property of the chaperonins, their high solubility at neutral pH, is an important aspect during this step (**Note 10**).

3.4. Gel-Filtration Chromatography

1. Between 5 and 10 mL of the concentrated chaperonin containing fractions are applied to the gel-filtration column, which has been pre-equilibrated with buffer C. If there is a larger volume of concentrated sample, it must be stored and applied to another column, since loss in resolution occurs if too large a volume is applied to this column. Fifty to 200 mg in a maximum volume of 10 mL (optimal is 5 mL for our 150 × 2 cm column) of protein can successfully be applied, giving very high resolution and separation of the chaperonin oligomer from other components.
2. The column is run using buffer C at a flow rate of 0.5 mL/min with 10 mL fractions being collected. Chaperonin from *S. shibatae* elutes in a sharp peak close to void volume (**Fig. 2B**).
3. Those fractions containing archaeal chaperonin are then identified by 4–15% native PAGE as described above for ion-exchange chromatography with the necessary standards (purified chaperonin) and pooled.
4. The pooled samples are then diluted at least three times with buffer A in preparation for the final anion exchange column (see **Note 11**). The concentration of the archaeal chaperonin can be monitored at this stage, or indeed at any stage in the procedure, by coelectrophoresis of the sample with samples of known concentration.

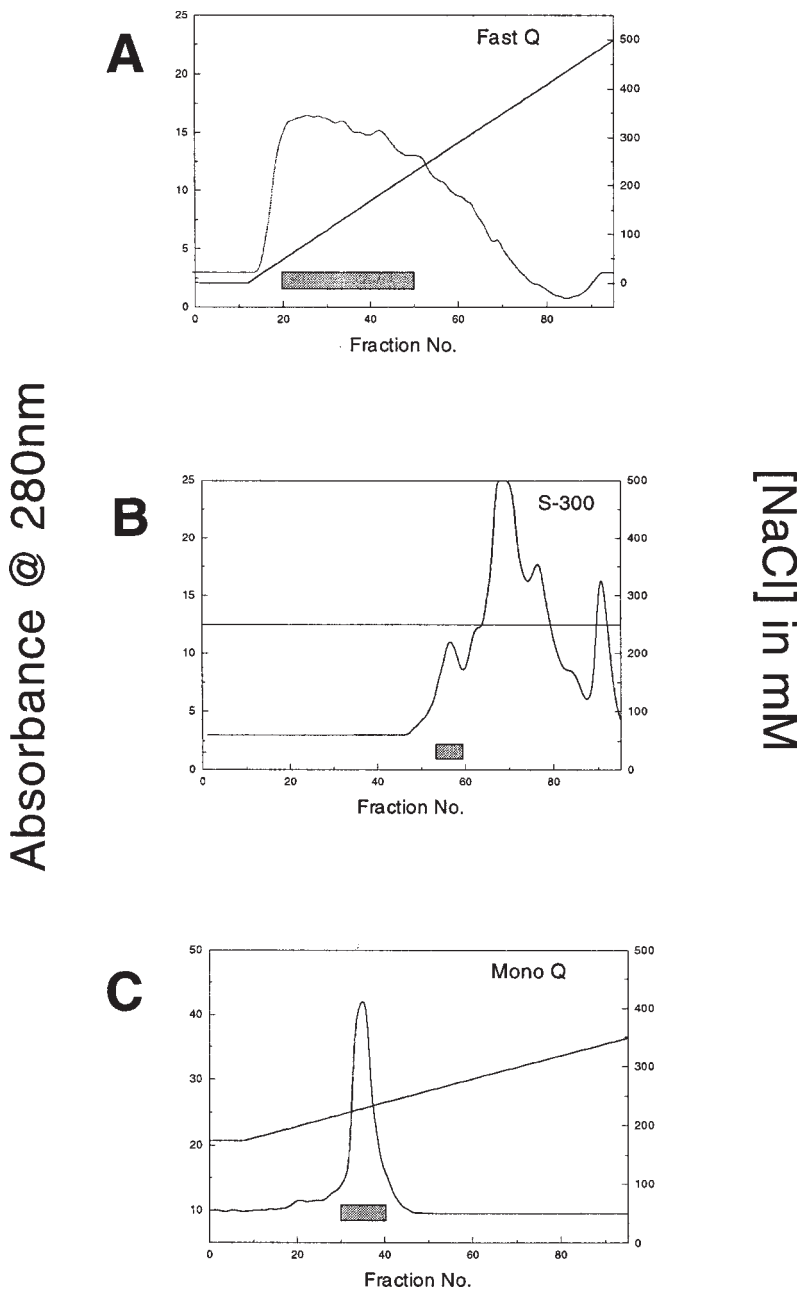


Fig. 2. Chromatographs of the purification of archaeal chaperonin from *S. shibatae*. (A) Fast Q anion-exchange elution profile; (B) S-300 gel-filtration elution profile; (C) MonoQ 10/10 elution profile. The fractions where the chaperonin elutes are indicated by a shaded bar. The void volume of the gel-filtration column is marked with (V).

3.5. MonoQ HR Chromatography

1. The diluted chaperonin-containing fractions are applied to a MonoQ anion-exchange column at 3 mL/min using automated chromatography equipment, e.g., the FPLC system (Pharmacia), which can be preprogrammed. The size of column (either 16/10 or 10/10) is determined by the protein concentration. The maximum loading capacity for the Mono Q 10/10 column is 200 mg/column and for the 16/10, 500 mg/column.
2. After washing with buffer A, a 1 L gradient of 150–350 mM NaCl is run (30% buffer D to 70 % buffer D) at 4 mL/min (HR 10/10) or 6 mL/ min (HR 16/10). The fraction size is usually 10 mL.
3. Fractions containing the chaperonin are identified by running a 1 μ L sample on both 4–15% native PAGE (see **Fig 3A**, lane 1) and 12.5% SDS electrophoresis. A typical chromatograph is shown in **Fig. 2C**. At this stage, the chaperonin is 95–98% pure as demonstrated by two-dimensional (2D) gel electrophoresis and silver staining (**12**).
4. Interestingly, the *S. shibatae* chaperonin has an unusual 2D pattern. Both subunits are clearly seen, and it is clear from the isoelectric focusing (IEF) dimension that charge variants are present, giving eight major spots. The abundance of each spot does change with different preparations of the protein. However, the biological significance of this, if any, is unclear.

3.6. *S. shibatae* Chaperonin Concentration and Storage

1. After identification by electrophoresis, the chaperonin can be concentrated in centrifugal concentrators to concentrations >200 mg/mL (see **Note 10**). We routinely store stocks at this concentration in buffer E. For everyday experimental use, we find that the chaperonin from *S. shibatae* is stable for several weeks when stored in buffer E at room temperature (18–23°C). Longer-term storage at –80°C is carried out by storage in buffer F.
2. The concentration of the chaperonin from *S. shibatae* is determined using an extinction coefficient calculated from the amino acid sequence (**13**). Since the archaeal chaperonin has two subunits with different sequences, two extinction coefficients are calculated. Like GroEL and thermophilic hsp60, the α -subunit has no tryptophan, giving a calculated extinction coefficient of 9200 M^{-1}/cm . However, the β subunit contains one tryptophan and has an extinction coefficient of 32,550 M^{-1}/cm . It is assumed that the subunits are present in 1:1 stoichiometry in the chaperonin complex, giving a complex extinction coefficient of 374,670 M^{-1}/cm ($[9 \times \alpha] + [9 \times \beta]$). The effect of this on purification is discussed in **Note 9**.

3.7. Preparative PAGE: Separation of the Two Forms of the Archaeal Chaperonin

1. The purified chaperonin runs as a doublet in native gels. To separate the doublet, native one-dimensional (1D) PAGE was optimized by examining a range of gel

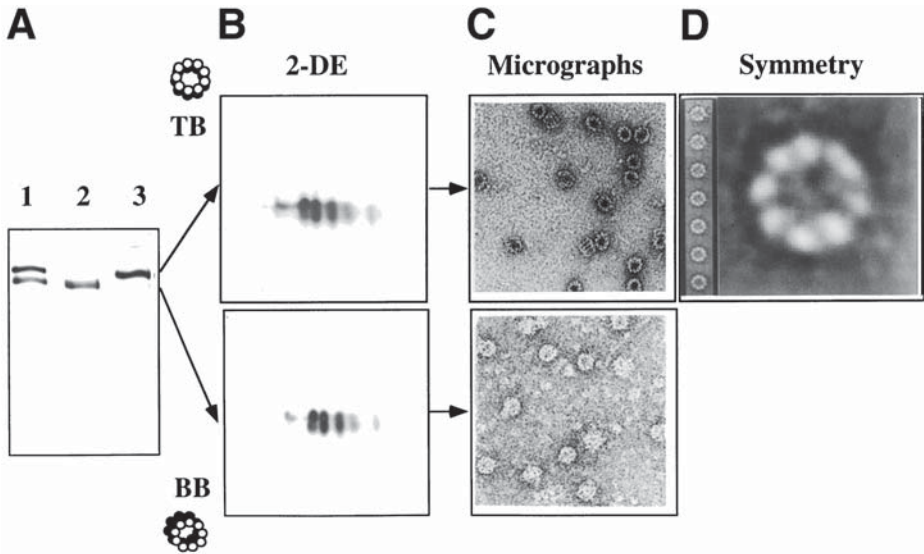


Fig. 3. Separation of the chaperonin from *S. shibatae* using native preparative PAGE (A) and subsequent 2D PAGE (B) and EM studies (C and D). (A) Lane 1: pure chaperonin obtained from a freshly grown culture of *S. shibatae* (20 μ g of pure protein applied) Lane 2: purified bottom band (BB; closed complex). Lane 3: purified top band (TB; open complex). Protein bands are stained with silver. (B) 2-DE panel; Bands were excised from native PAGE, electroeluted, and run on denaturing 2D gels (IEF = 4–10; slab = 10–17% gradient SDS-PAGE, pH 8.0). Both complexes show identical subunit composition, and no other protein components are seen on the gels. Proteins are stained with silver. (C) Micrographs panel: The two chaperonin complexes show different structures, the TB being “open,” and the BB being “closed.” (D) Symmetry panel: after rotational averaging, the chaperonin from *S. shibatae* has clear ninefold symmetry.

concentrations, buffers, and electrophoresis conditions. For the best result, two aspects must be considered, the separation between the two bands and the resolution, i.e., the sharpness of each band. Using conventional analytical PAGE, it is found that electrophoresis using 4% acrylamide gels gives the best separation between the two forms with the maximum resolution. These conditions are therefore used to purify the two bands in the native preparative electrophoresis cell.

- The gel is run at 25 V/cm, and 500 mL fractions are collected as described in **Subheading 2.6.1**. Each fraction is concentrated on Centricon (Amicon) centrifugal concentrators (volume = 2 μ L; 100-kDa cutoff membrane) to ~30 μ L. The chaperonin containing fractions are identified by electrophoresis using the PHAST system and 4–15 % native gels. Using this method, we obtained 5–6 mg of each form, pure “top band” and pure “bottom band” from 25 mg of starting material (i.e., the “doublet”).

3. Further analysis shows that the difference in mobility is owing to a difference in conformation of the chaperonin molecule (**3**), not to the presence of protein substrates. Electron microscopy shows that the top band is ring-like with a definite cavity in the center and is termed the “open complex.” The bottom band showed no defined cavity and is termed the “closed complex.” **Figure 3** (B-D) shows the analysis of the open and closed forms. CD analysis confirmed that there was a difference in secondary structure (**3**).

4. Notes

1. *Sulfolobus* cells were grown in liquid media (as described in **Subheading 3.**) pH 3.5 at 70°C in a custom made 150 L fermenter (J. Trent, personal communication). The doubling time of the *Sulfolobus* strain is slow compared to most eubacteria (approx 2–3 h). Therefore cell growth must be monitored over several days. This was effectively carried out by direct visualization on a hemocytometer slide. However, this can also be monitored spectrophotometrically. Cells are harvested at late-log phase (A_{650} 0.4–0.6), which is normally reached in about 30–36 h.
2. Since the PHAST preformed gels have a plastic backing, glycerol is added to the final destain (Destain II) to prevent the polyacrylamide from peeling off this support. If gels are prepared by the user, the Destain II step should be omitted. This is particularly important if the gels are to be dried in a vacuum drier, because the glycerol will prevent the gel from drying in the machine.
3. Since acrylamide is a neurotoxin and listed carcinogen, the preparation of acrylamide solutions should be carried out with great care. The chemicals should be weighed in a hood designated for carcinogen use, and gloves, eye protection, and lab coat should be worn when handling the material. The monomer solution is also hazardous and should be handled carefully using the same precautions. Once polymerized, the gel is not considered such a hazard and can be discarded in the normal waste. The experimenter should contact their chemical hygienists regarding the safety procedures for acrylamide use and disposal in their own establishments.
4. The purification of the “open” and “closed” forms of the chaperonin can also be carried out by electroelution. We have used this method with reasonable success. However, although the purity is good, the yield is much lower than using preparative native PAGE. The technique involves running preparative 1D slab gels, excising the bands and placing them in dialysis tubing (9000 MWCO, Amika, Columbia, MD) in an appropriate buffer. We normally use the same as that used for electrophoresis (11.25 mM Tris-glycine, pH 8.0). The dialysis tubing is placed in a small horizontal electrophoresis device (we commonly use the minigel apparatus from Bio-Rad) and electrophoresed for 30 min at 50 V. The protein in the gel slice elutes into the buffer solution inside the dialysis tubing. After this time, the solution is removed, and the protein is concentrated in Centricon 30K (Amicon) centrifugal concentrators.
5. Recently, various companies have produced centrifugal concentrators with 300-kDa cutoff membranes. In our hands we found that 10–20% of chaperonin passes

through these membranes, and continued to use 100-kDa membranes for concentration purposes.

6. We have found that ensuring good induction of the chaperonin proteins by heat shock is an important step in the purification process. It avoids wasting time and resources when looking for a “lost” protein, which in reality was not induced properly.
7. The use of both “native” and SDS-PAGE to screen the fractions for pure chaperonin may seem superfluous. However, one of the main challenges in chaperonin purification is ensuring that the chaperonin is free of bound substrate proteins. Native page, which shows the high-molecular-weight complex, allows us to ensure that the chaperonin is and that it has not disassembled into subunits. SDS-PAGE, on the other hand, allows us to check for the presence of other polypeptides, which might be tightly bound to the chaperonin. Both these techniques therefore contribute useful information.
8. Careful washing and regeneration of the column can usually remove this brown layer. If it cannot be adequately cleaned in place, the column adapter can be taken off, the dark layer removed using a Pasteur pipet, the adapter replaced, and the column reused.
9. The very low extinction coefficient of chaperonins in general creates a significant problem during chromatography. For example, a protein with a “normal” extinction coefficient (i.e. $1 \text{ mg/mL} = 1 A_{280}$), is readily observed on a chart recorder during column chromatography as an increase in absorbance or a peak. This peak should be huge if one considers the amount of chaperonin in the sample. However with a 1 mg/mL solution having an A_{280} of 0.35, only a relatively small peak is observed. We have often found milligrams of “lost” chaperonin by running gels through the whole gradient and not relying on the chart recorder.
10. The chaperonin from *S. shibatae* is an extremely soluble protein in neutral buffers. We routinely concentrate the protein to $>200 \text{ mg/mL}$. The maximum concentration achieved to date is 350 mg/mL in buffer E.
11. A step that is often forgotten is the dilution of the fractions from the gel-filtration column. The running buffer for this column contains 250 mM NaCl. We know from anion-exchange chromatography that the chaperonin elutes between $100\text{--}220 \text{ mM}$ NaCl. If applied directly to the column without dilution, the chaperonin will not bind to the resin and can be recovered in the flowthrough.

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Purification of Hsp60 from *Thermus thermophilus*

Elsie Quaite-Randall and Andrzej Joachimiak

1. Introduction

Thermus thermophilus is an eubacteria that grows optimally at 75°C and was initially discovered in geothermal springs (1). Surprisingly, even though the proteins of this organism are constantly subjected to high temperatures and must therefore have evolved to be highly thermostable, this thermophilic bacteria expresses large amounts of heat-shock proteins (2–4). In addition, it was shown that the Hsp60 chaperonin of this organism can be induced by a small heat shock (80°C for 1 h). This thermophilic chaperonin has been cloned and over-expressed in *Escherichia coli* (3,4). The amino acid sequence of Hsp60 from *T. thermophilus* is highly homologous to the GroEL chaperonin from *E. coli* (52% identity) as shown in Fig. 1. It is similar to GroEL also in its structure, as seen by electron microscopy, in that it also comprises two rings of seven identical subunits (5,6). Each subunit has a mol-wt of approx 57 kDa, making the tetradecameric complex 800 kDa.

This chaperonin can be purified by methods similar to those for many other chaperonins. This chapter describes the method that we use successfully in our lab. The initial important step is to subject the cell culture to a slight heat shock. This induces the production of Hsp60 (and its cochaperonin Hsp10) to a great extent and gives a highly enriched soluble protein fraction for the chromatography steps. Two main properties, common to most chaperonins, are exploited during its purification, that of its low isoelectric point ($pI = 5.1$) and large size.

There are few differences between Hsp60 from *T. thermophilus* and GroEL from *E. coli*, the most obvious being their thermal stability. Use of a thermophilic chaperonin in protein-folding experiments allows for the folding mechanisms of thermally denatured mesophilic proteins to be studied. The thermophilic chaperonin is also a useful tool in the investigation of the refolding of thermo-

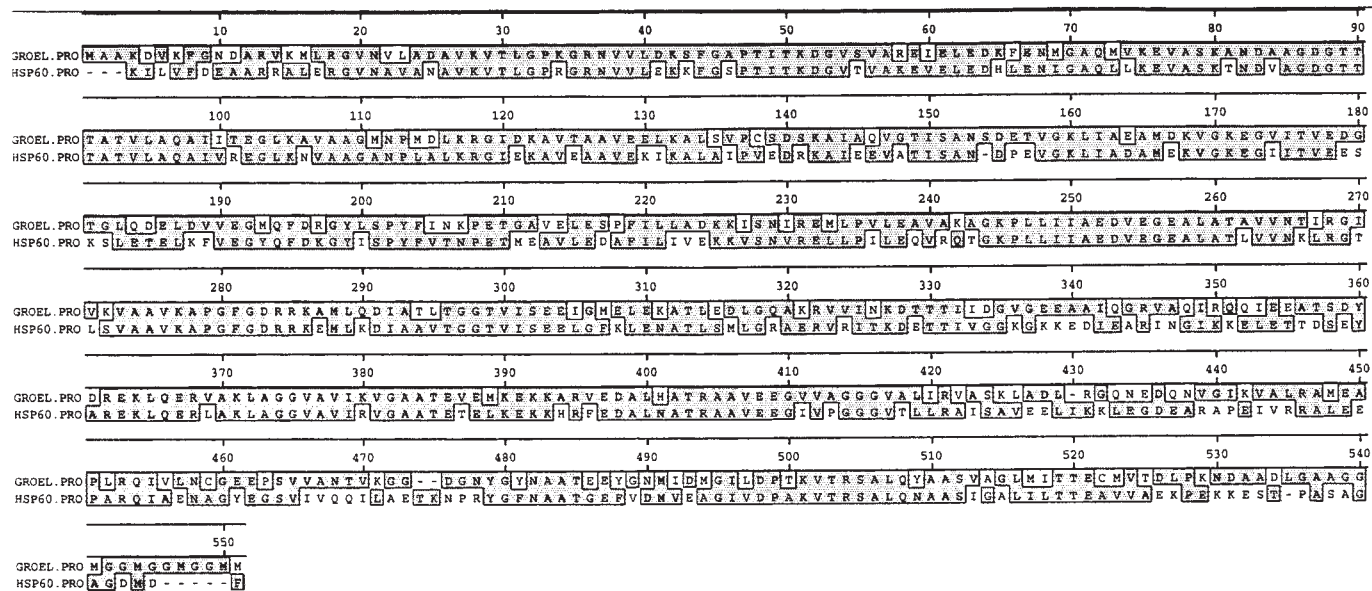


Fig. 1. Sequence comparison of *E. coli* GroEL and Hsp60 from *Thermus thermophilus*. Identical residues are shaded.

philic enzymes. These enzymes have become extremely important in the biotechnology industry, and thermophilic chaperonin allows folding pathways of these enzymes to be investigated. GroEL is not useful in most of these studies, since it is no longer active at temperatures at which thermophilic enzyme activity is measured. At present, it is not known whether structural differences in the chaperonin complex account for the differences in thermal stability. This awaits the solution of the crystal structure of Hsp60 from *T. thermophilus*.

Purification of *T. thermophilus* Hsp60 can be achieved by two different methods. Recombinant strains have been produced (3,4) and Hsp60 can be successfully purified using protocols developed for GroEL (Chapter 3) (see **Note 1**). Alternatively, Hsp60 can easily be purified directly from the *T. thermophilus* strain. This chapter describes the growth methods for *T. thermophilus* and purification of Hsp60 directly from the organism.

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Purification of Hsp60 from *T. thermophilus* involves the same three chromatographic steps as described for GroEL, with concentration and dilution of the sample in between. Detection of the chaperonin is either by native or SDS-PAGE. The three chromatographic steps are:

1. Fast Q-sepharose anion-exchange chromatography.
2. Gel-filtration chromatography on Sephacryl S-300 high resolution.
3. Mono Q-Sepharose HR anion-exchange chromatography.

These three steps can produce Hsp60, which is 98–99% pure on a milligram scale in 2–3 d. Protein prepared by this method is of sufficient quality to produce crystals for X-ray crystallography.

2. Materials

Except where noted, all chemicals are purchased from Sigma (Sigma, St. Louis, MO) and are analytical grade or higher.

2.1. Cell Growth

1. The strain we used is *T. thermophilus* HB8 (ATCC 27634).
2. Growth medium is Castenholtz TYE medium. This is a complex medium and is made from three stock solutions: Nitsch's trace elements, Castenholtz salts, and 1% TYE.
 - a. Nitsch's trace elements: Can be filter-sterilized and stored for several months at room temperature.

- | | |
|--------------------------------------|---------|
| H ₂ SO ₄ | 0.5 mL |
| Mn SO ₄ | 2.2 g |
| Zn SO ₄ | 0.5 g |
| H ₃ BO ₃ | 0.016 g |
| CuSO ₄ | 0.025 g |
| Na ₂ MoO ₄ | 0.025 g |
| CoCl ₂ ·6H ₂ O | 0.046 g |
| Distilled H ₂ O | to 1 L |
- b. Catenholtz salts, 2X: Prepare fresh for each preparation
- | | |
|--------------------------------------|---------|
| Nitrilotriacetic acid | 0.2 g |
| Nitsch's trace elements | 2.0 mL |
| FeCl ₃ (0.3%) | 2.0 mL |
| CaSO ₄ ·2H ₂ O | 0.12 g |
| MgSO ₄ ·7H ₂ O | 0.2 g |
| NaCl | 0.016 g |
| KNO ₃ | 0.21 g |
| NaNO ₃ | 1.4 g |
| Na ₂ HPO ₄ | 0.22 g |
| Distilled H ₂ O | to 1 L |
- c. 1% TYE: Prepare fresh for each preparation
- | | |
|----------------------------|--------|
| Tryptone (Difco 0123) | 10 g |
| Yeast extract | 10 g |
| Distilled H ₂ O | to 1 L |

Mix aseptically five parts of 2X Castenholtz salts with one part 1% TYE and four parts distilled water. Final pH of the complete medium should be adjusted with NaOH to 7.6. We normally make 10 L of culture for each protein preparation.

2.2. Buffers

All buffers are freshly made and degassed and filtered just prior to chromatography. Purification of *T. thermophilus* is carried out at room temperature, since this is a thermophilic organism and room temperature is considered frozen. We have not tried purification at 4°C. However, since the chaperonin can be stored at 4°C with no detrimental effects, one would assume that the whole purification could be carried out at a lower temperature if necessary. In this case, all buffers should be equilibrated accordingly. It is important that the dithiothreitol (DTT) solution is prepared and added to the buffers just before chromatography, because this compound is unstable and is oxidized by oxygen dissolved in the buffers.

1. Buffer A: 50 mM Tris-HCl, pH 7.5, 1 mM ethylenediamine tetra-acetic acid (EDTA), 1 mM DTT, 50 mM NaCl.
2. Buffer B: 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 1 M NaCl.

3. Buffer C: 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 250 mM NaCl, 20% (v/v) ethanol.
4. Buffer D: 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 500 mM NaCl.
5. Buffer E: 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM DTT, 250 mM NaCl.
6. Buffer F: 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM DTT, 250 mM NaCl, 50% (v/v) glycerol.
7. Washing buffer: 50 mM sodium phosphate buffer, pH 7.0.

2.3. Fast Q Chromatography

Q-Sepharose Fast-Flow anion-exchange media is stored in 20% ethanol, and must be washed at least four times with 4 vol of deionized H₂O prior to use. Media are suspended in 5 vol of buffer A. A 30 × 2.3 cm column is cleaned, and the resin (90 mL) poured into the column according to the manufacturer's directions. This amount of resin will fill the column to about 7 cm below the top, which allows room for the adapter to be fitted. We find the use of gravity sedimentation sufficient for this process. The column is then packed at a flow rate of 6 mL/min using buffer A. Column equilibration is carried out using at least 10 column volumes of buffer A. Complete equilibrium is obtained when the pH of eluant is the same as the buffer. The column is then ready for use. With care, the column and beads can be used successfully for many months. After use, we always wash the column in a 2 M NaCl solution, followed by water, and store it in a 20% (v/v) ethanol:H₂O solution.

2.4. Gel-Filtration Chromatography

Sephacryl S-300 HR is washed with water (at least four times in 2X matrix volume) before column preparation. Several washes are usually required to remove all the "fines" (crushed beads that have a detrimental effect on the flow rate of a column). This matrix is of lower mechanical strength, and care must be taken not to damage the gel beads. The matrix also requires a longer settling time both during washing and column preparation. Prior to column preparation, the gel matrix is generally suspended in 2 vol of Buffer C and degassed for 30 min. A 150 × 2.0 cm column is prepared, and the gel poured into the column and allowed to settle by gravity. Approximately 400–420 mL of resin are used, which again allows for an adapter to be fitted to the top of the column. It is also possible to run a gel-filtration column without an adapter, which will increase the effective length of the column. However, we have found that the use of an adapter gives a more uniform sample application and elution. The column is equilibrated by running the column at 1 mL/min overnight with buffer C. Care should be taken when manipulating this column, since this column matrix is highly sensitive to the introduction of air bubbles. This column is run at room temperatures, so all buffers should be equilibrated at this temperature.

2.5. MonoQ HR Chromatography

The final chromatographic step, HR ion-exchange chromatography, is carried out using prepacked columns from Pharmacia (Uppsala, Sweden). Two different sizes are commonly employed, depending on the amount of protein to be applied, MonoQ high resolution 10/10 (10 × 100 mm) and HR 16/10 (16 × 100 mm). The reactive group on this resin is also a quaternary amine, the same as that in Q-Sepharose. However, the bead size is strictly controlled giving a highly uniform, monodisperse resin. This allows very rapid and HR chromatography.

2.6. Electrophoresis

Screening of the fractions obtained by chromatography is carried out using preformed PHAST gels (Pharmacia): 12.5 % for SDS gels and gradient 4–15% for native PAGE. Sample loading buffers are:

5X SDS gel buffer: 0.5 g SDS, 0.3 g DTT, and 1 mg pyronin Y dissolved in 10 mL 75% glycerol/25% 50mM Tris-HCl, pH 7.5.

5X Native gel buffer: 1 mg bromophenol blue dissolved in 10 mL 75% glycerol/25% 50mM Tris-HCl, pH 7.5.

Alternatively, 12% Laemmli SDS gels (7) can be used for screening purposes. GroEL can also be detected on 4 or 6% large-format slab PAGE gels using the protocol described in (8) and Chapter 3 in this volume.

1. Staining solution: 10% (v/v) acetic acid, 30% (v/v) methanol, 0.1% Commaissie brilliant blue R250, 60% H₂O (v/v).
2. Destain I: 10% (v/v) acetic acid, 30% (v/v) methanol, 60% H₂O (v/v).
3. Destain II: 10% acetic acid, 10% glycerol, 60% H₂O (v/v) (*see Note 2*).

2.7. Protein Concentration

Two methods are employed for the concentration of crude extract or protein solutions during the purification protocol.

1. Nitrogen-pressurized stirred cell (Amicon, Danuers, MA). For concentration of large volumes, 50–1000 mL, we use the stirred cell under pressure of 50 psi according to the manufacturer's instructions. The use of a membrane with 100-kDa cutoff (Difco [Detroit, MI] or Millipore [Bedford, MA]) allows this to be a fairly rapid procedure and serves as an additional purification step.
2. Centrifugal concentrators: For sample sizes <50 mL, we commonly use centrifugal concentrators; Centricon (2 mL) or Centriprep (15 mL) with 100-kDa cutoff membranes (Amicon). Filtron concentrators have also been used with excellent results (*see Note 3*). Millipore concentrators, a relatively new product, allow the direct visualization of the volume left in the concentrator, which is very useful.

3. Methods

3.1. Cell Growth

1. Ten liters of TYE media is made and 1 L of media placed in each of 2.5 L flasks. The use of flasks with molded “baffles” increases aeration and allows faster cell growth. Two 250 mL flasks with 100 mL of media are also included for the “overnight culture” that serves as inoculum.
2. After autoclaving, the media are allowed to cool. The 100 mL cultures are allowed to equilibrate at 75°C, and overnight cultures are started by inoculating (aseptically) the flasks with a single bacterial colony, or with a scraping of frozen HB8 cells, which are stored in a -80°C freezer. These are placed in a shaker at 250 rpm and allowed to grow overnight at 75°C. The culture is grown until the late-log phase (looks cloudy, $OD_{600} = 2-3$, cell density of approx 1×10^9 cells/mL).
3. The 10 flasks containing the media for the production culture are pre-equilibrated to 75°C (see **Note 4**), and are inoculated with 5–10 mL of the fresh overnight culture. The OD_{600} is measured every 30 min, using the uninoculated TYE medium as a blank. The cells are grown until the culture reaches an OD_{600} of >1.0, which takes about 6 h. Then the temperature in the shaker is increased to 80°C, and the cultures are then further incubated for 1 h.
4. At this point, you should ensure that the heat shock has induced Hsp60 production. An aliquot (1 mL) of culture is removed and spun in an Eppendorf tube in a microcentrifuge to pellet the cells. The supernatant is decanted off and the resulting cell pellet resuspended in 200 μ L SDS buffer, boiled for 1 min, and after making a dilution series with 1X SDS loading buffer, 1 mL is applied to a 12.5% SDS gel. The samples are compared with an Hsp60 standard, and efficient heat-shock induction is seen as a large band at 60 kDa, which can be up to 50% total cell protein (see **Note 5**).

3.2. Cell Harvesting and Extract Preparation

1. The culture (which should look cloudy) is allowed to cool to room temperature, is poured into centrifuge bottles (6×250 mL), and is spun at 5000g in a Sorval GSA rotor at 4°C for 10 min. After this time, the media (which should be clear) are poured off, another 250 mL of culture added to the same flask, and the centrifugation repeated. The clear media are again decanted off. This process is repeated until all the culture has been centrifuged, and all the cells harvested. The cells are washed once by resuspending the cells in 50 mM sodium phosphate buffer, pH 7.0, and pelleting the cells as described above.
2. The cell pellet is then gently scraped from the centrifuge bottles, combined, and weighed. A cell yield of 1–2 g/L culture is normal. The cells are then resuspended in 5 vol of buffer A. This is stirred gently for 5 min on ice before sonication. No lysozyme is added to the suspension of *T. thermophilus* cells, since they lyse very easily. Generally, 5 min of sonication on ice, i.e., 1 min interspersed with a 1 min rest, are sufficient for cell disruption.

3. This suspension is then centrifuged at 17,000g in a Sorval GSA rotor for 30 min at 4°C which pellets the cell membranes and other debris. The clarified cell extract, the supernatant, contains the soluble protein from the cell and is applied directly to the Fast Q column. All steps in the purification of *T. thermophilus* Hsp60 can be carried out at room temperature, since this is “frozen” with respect to the thermophilic proteins.

3.3. Fast Q Chromatography

1. The extract is applied at a flow rate of 6 ml/min to the Fast Q anion-exchange column, which was pre-equilibrated at room temperature with buffer A. The column is washed with 500 mL buffer A (5 mL/min), and proteins are eluted with a linear gradient (1.7 L) of 50 mM to 1 M NaCl (buffer A and buffer B).
2. Fractions (18 mL) containing Hsp60 were identified using both 4–15% native PAGE and 12.5% SDS-PAGE (see **Note 6**) using the PHAST system. Generally good visualization of the proteins is obtained by removing 8 µL from every third fraction, mixed with 2 µL loading dye, and 1 mL of this applied to the PHAST gel.
3. It is important in all the chromatographic steps to check the entire gradient, since the low extinction coefficient of the chaperonins can make them difficult to detect (see **Note 7**). *T. thermophilus* Hsp60 elutes between 140 and 220 mM NaCl. A typical chromatograph is shown in **Fig. 2A**, where the shaded bar represents the position of the Hsp60.
4. Fractions containing the Hsp60 are pooled and concentrated from 50–200 to 15–20 mL. The reason for this high degree of concentration is the fact that the resolution of the proteins on the gel-filtration column is dependent on the volume of the sample. Another property of the chaperonins, their high solubility at neutral pH, is an important aspect during this step (see **Note 8**).

3.4. Gel-Filtration Chromatography

1. Between 5–10 mL of the concentrated Hsp60 containing fractions are applied to the gel-filtration column (150 × 2 cm) which has been pre-equilibrated with buffer C. If there is a larger volume of concentrated Hsp60 extract, it must be stored and applied to a second gel-filtration column, since the resolution is related to the sample volume. Fifty to 200 mg of protein can successfully be applied to this column (optimal sample size = 5 mL; maximum = 10 mL), giving very HR and separation of the Hsp60 oligomer from other components.
2. The column is run using buffer C at a flow rate of 0.5 mL/min, fraction size 10 mL. Hsp60 from *T. thermophilus* elutes in a sharp peak close to void volume. A typical chromatograph is shown in **Fig. 2B**, with the shaded bar again showing the fractions containing Hsp60.
3. Those fractions containing Hsp60 are then identified by 4–15 % native PAGE as described above using standards, and pooled. The pool is then diluted at least three times with buffer A in preparation for the final anion-exchange column (see **Note 9**). If required, the concentration of Hsp60 at this time, or indeed at any time during the purification, can be determined by coelectrophoresis of the sample with pure Hsp60 samples of known concentration.

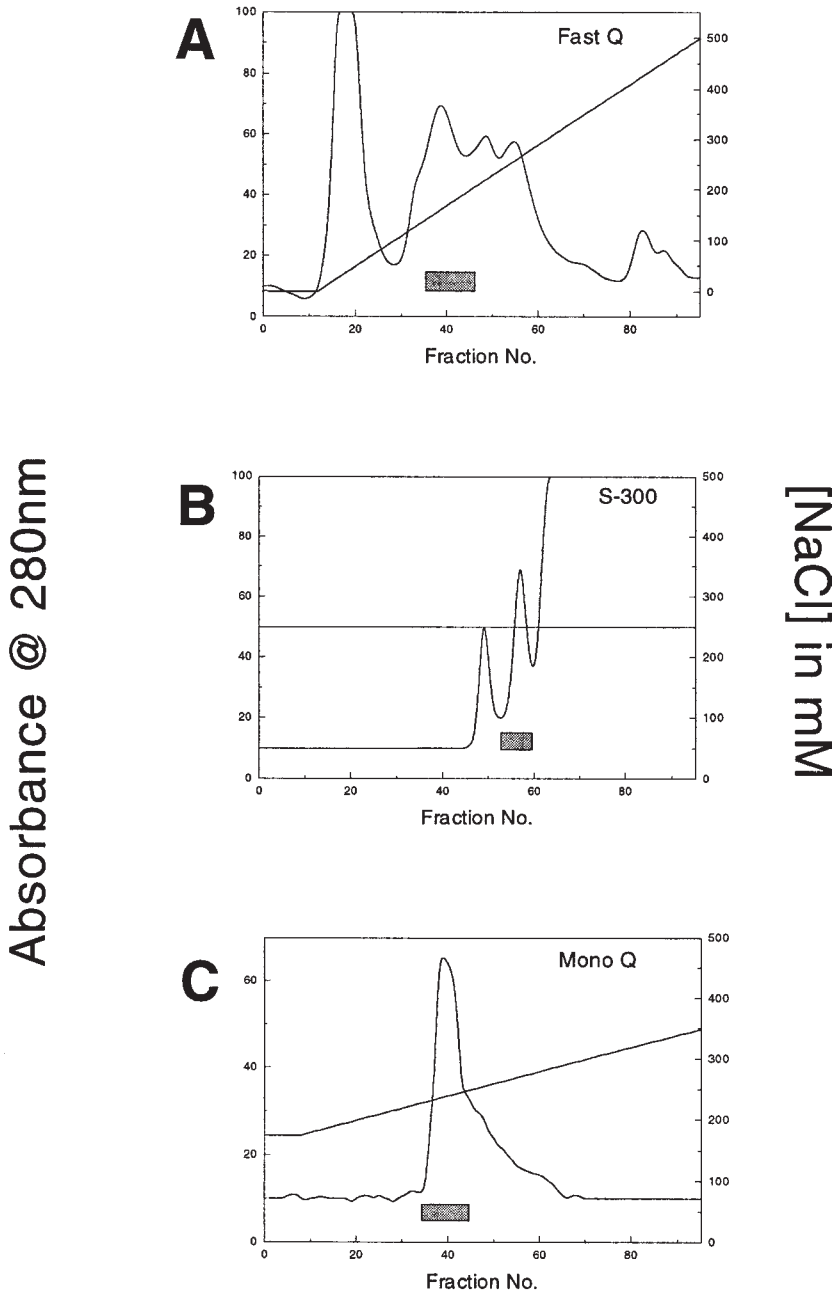


Fig. 2. Chromatographs from the purification of *T. thermophilus* Hsp60. (A) Fast Q anion-exchange elution profile; (B) S-300 gel-filtration elution profile; (C) MonoQ 10/10 elution profile. The elution position of Hsp60 is indicated by a shaded bar.

3.5. MonoQ HR Chromatography

1. The diluted Hsp 60 containing fractions are applied to a MonoQ anion-exchange column at 3 mL/min (*see Note 9*). The column size (either 16/10 or 10/10) is determined by the protein concentration. The maximum loading capacity for the Mono Q 10/10 column is 200 mg/column and for the 16/10, 500 mg/column.
2. After washing the column with buffer A at 3 mL/min for four column volumes, a 1-L gradient of 150–500 mM NaCl is run (30% buffer D to 100% buffer D) at 4 mL/min (HR 10/10) or 6 mL/min (HR 16/10).
3. Fractions containing Hsp60 are identified by both 4–15% native PAGE and 12.5% SDS electrophoresis as described for the other chromatography steps. A typical chromatograph is shown in **Fig. 2C**. At this stage, the Hsp 60 is 95–98% pure as judged by two-dimensional (2D) gel electrophoresis and silver staining (**9**). A summary of the purification steps of Hsp60 from *T. thermophilus* is shown in **Table 1**.

3.6. Comparison of Thermophilic Chaperonins with GroEL

A comparison of chaperonins from different sources is shown in **Fig. 3**. This figure compares different properties of thermophilic chaperonins. GroEL is included as a standard.

1. Panel A shows a native gel of all the chaperonins. No standards are included, since we have not as yet found suitable-size standards for this molecular-weight range. From this it is seen that the two thermophilic Type II chaperonins (*Thermococcus littoralis* [**1**] and *Sulfolobus shibatae* [**2**]) are the slowest migrating, and in marked contrast to all others, that from *S. shibatae* is a doublet (for more details *see Chapter 1*, this vol.). Thermophilic Hsp 60 (**3**) migrates more slowly than its mesophilic counterpart, GroEL (**4**).
2. Panel B shows micrographs of all the thermophilic chaperonins. It demonstrates the differences in symmetry; *T. littoralis* has eight subunits in each ring, *S. shibatae* has nine, and *T. thermophilus* has seven (as has GroEL).
3. Panel C shows the 2D patterns of the purified thermophilic chaperonins (**1–3**) and compares them to GroEL (**4**). In all cases isoelectric focusing (IEF) is along the top with the acidic end at the left, and the second dimension (10–17% SDS) is from top to bottom. From these patterns, it is clear that thermophilic chaperonins (in particular Type II chaperonins) have greater charge variability than GroEL.
4. The last panel (D) is a cartoon showing the relative positions of all the chaperonins after running all purified chaperonins together and individually in comigration studies with standards.

3.7. *T. thermophilus* Hsp60 Concentration and Storage

1. After identification of Hsp60 by electrophoresis, the protein can be concentrated in buffer E in centrifugal concentrators to concentrations >200 mg/mL (*see Note 8*). We routinely store Hsp60 stocks at this concentration. For everyday experimental, use we find that Hsp60 from *T. thermophilus* is stable for several weeks when stored in buffer E at room temperature (18–23°C). Longer term storage at –80°C is carried out by storage in buffer F.

Table 1
Summary of Total Yield of Hsp60 from *T. thermophilus*
(46.2 g Cell Paste)

	Volume, mL	A ₂₈₀	Hsp60, mg _a
Crude extract	210	672	92
Fast Q	130	114	81.5
S- 300	35	13.6	74.2
MonoQ	44	8.1	62.3

^aEstimated from gel electrophoresis.

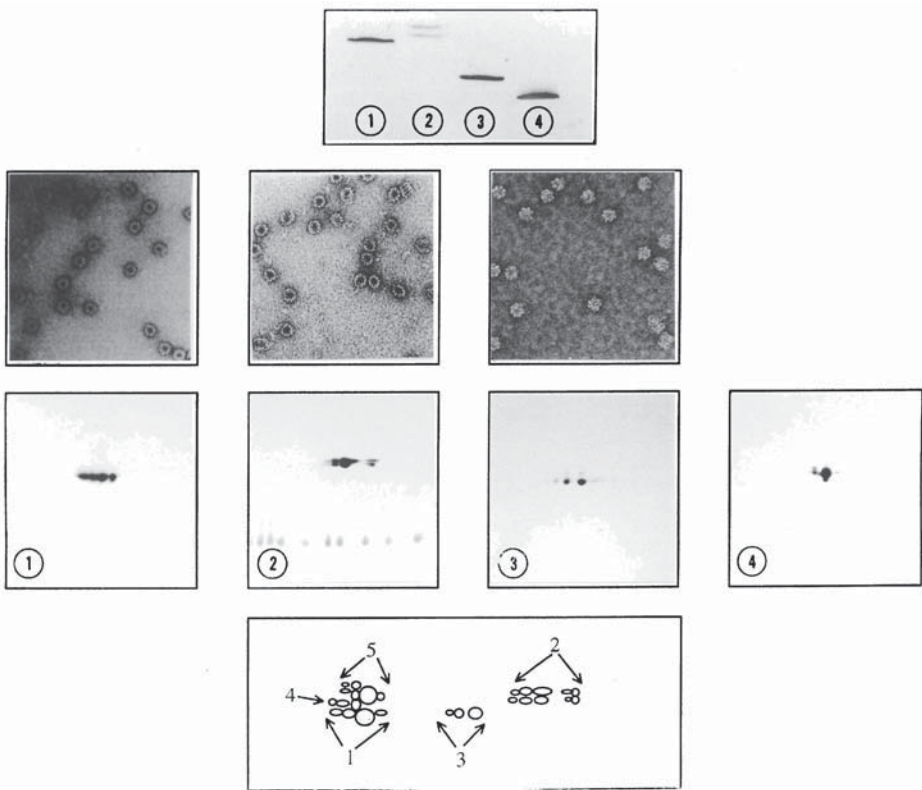


Fig. 3. Comparison of thermophilic chaperonin with GroEL. For a detailed discussion, see **Subheading 3.6**. (A) Native gel of purified chaperonins. (B) Electron-micrographs of thermophilic chaperonins. (C) 2D gel patterns of the different chaperonins. (D) Comigration studies. The numbering scheme is as follows (1) *T. litoralis*, (2) *S. shibatae*, (3) *T. thermophilus*, (4) *E. coli*, (5) *Pyrococcus furiosus*.

2. The concentration of Hsp60 from *T. thermophilus* is determined using an extinction coefficient calculated from the amino acid sequence (**10**). Like GroEL, thermophilic Hsp60 contains no tryptophan. It has also fewer tyrosines and therefore a correspondingly lower extinction coefficient, which is calculated to be $7680 \text{ M}^{-1}/\text{cm}/\text{monomer}$. The effect of this on purification is discussed in **Note 7**.

4. Notes

1. The slight difference in the pI of GroEL (4.74) and *T. thermophilus* Hsp60 (5.1) allows the separation of recombinant Hsp60 from GroEL after expression of Hsp60 in *E. coli* by careful adjustment of the salt gradient during anion-exchange chromatography.
2. Since the PHAST preformed gels have a plastic backing, glycerol is added to the final destain (Destain II) to prevent the polyacrylamide from peeling off this support. If gels are prepared by the user, the Destain II step should be omitted. This is particularly important if the gels are to be dried in a vacuum drier, since the glycerol will prevent the gel from drying in the machine.
3. Recently, various companies have produced centrifugal concentrators with 300-kDa cutoff membranes. In our hands, we find that 10–20% of Hsp60 passes through these membranes, and continue to use 100-kDa membranes for concentration purposes.
4. Efficient equilibration of the large flasks at 75°C can be achieved by placing the large flasks containing the media in the shaker during growth of the overnight culture.
5. We have found that ensuring good induction of the Hsp60 proteins by heat shock is an important step in the purification process. It avoids wasting time and resources when looking for a “lost” protein, which in reality was not induced properly.
6. The use of both “native” and SDS-PAGE to screen the fractions for Hsp60 may seem superfluous. However, one of the main challenges in chaperonin purification is ensuring that the chaperonin is free of bound substrate proteins. Native page, which shows the high-molecular-weight complex, allows us to ensure that the chaperonin is tetradecameric and that it has not disassembled into subunits. SDS-PAGE, on the other hand, allows us to check for the presence of other polypeptides, which might be tightly bound to the chaperonin. Both these techniques, therefore, contribute useful information.
7. The very low extinction coefficient of Hsp60 creates a significant problem during chromatography. For example, a “normal” protein, with a normal extinction coefficient (i.e., $1 \text{ mg/mL} = 1 A_{280}$), is readily observed on a chart recorder during column chromatography as an increase in absorbance or a peak. This peak should be huge if one considers the amount of Hsp60 in the heat-shocked cell. However, with a 1 mg/mL solution having an A_{280} of 0.10, a small peak is observed. We have often found milligrams of “lost” chaperonin by running gels through the whole gradient and not relying on the chart recorder.
8. *T. thermophilus*. Hsp60 is an extremely soluble protein in neutral buffers. We routinely concentrate the protein to 200 mg/mL . The maximum concentration achieved to date is 312 mg/mL .

9. A step, which is often forgotten, is the dilution of the fractions from the gel-filtration column. The running buffer for this column contains 250 mM NaCl. We know from anion-exchange chromatography that Hsp60 elutes between 200 and 250 mM NaCl. If applied directly to the column without dilution the Hsp60 will not bind to the resin, but can be recovered from the flowthrough.

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Purification of GroEL from an Overproducing *E. coli* Strain

Elsie Quaite-Randall and Andrzej Joachimiak

1. Introduction

Molecular chaperones are a class of abundant and ubiquitous proteins, which assist and accelerate protein folding in the cell. Classification of these proteins is continually changing owing to advances in our understanding of how these molecules function. One major group, the chaperonins (**I**), are defined as large, multimeric proteins that assemble into a double ring (or “double-doughnut”) structure. The crystal structure of the chaperonin GroEL from *Escherichia coli* (**2**) showed the 14 subunits arranged in two rings of 7 subunits. Each subunit has three distinct domains, the equatorial domain (through which the rings are joined), a hinge region (which facilitates movement), and the apical domain (where the unfolded protein substrate binds). There are many models for the mechanism of action of this molecule (for recent reviews, *see* **3**), however it is generally accepted that the unfolded protein binds to the apical domain and is sequestered inside the cavity of the chaperonin owing to the binding of the cochaperonin, GroES, to the same sites on the apical domains. Release of the protein from the chaperonin occurs after ATP hydrolysis. Chaperonins are broadly further classified into two other groups based on source and sequence comparisons: Type I, which comprises bacterial, mitochondrial, and chloroplast chaperonins, and Type II, which comprises the archaeal and eukaryotic cytoplasmic chaperonins. All chaperonins have a subunit size of approx 60 kDa. GroEL has become the most examined and standard chaperonin in the field of chaperone-mediated protein folding. This chapter describes a purification protocol by which it is possible to obtain pure GroEL from an overproducing strain of *E. coli*. Several such strains have been produced in many laboratories and new constructs

of GroEL, and its homologs can be generated easily using standard molecular biology techniques. Two main properties of the active chaperonin are exploited during its purification, that of its low isoelectric point ($pI = 4.7$) and large size (14×57 kDa).

1. Exploitation of pI is carried out using ion exchange chromatography. This chromatography technique depends on the reversible binding of charged solute molecules to an immobilized ion-exchange group of opposite charge. A complex mixture of proteins is applied to a column containing the desired ion-exchange resin. Depending on the pI of the protein, the pH of the solvent, and salt concentration, some proteins will bind to the column, but others not. Bound proteins are removed from the column by making the elution conditions unfavorable for ionic bonding of the solute molecules (change in pH or salt concentration). Most often the release of a protein from the matrix is achieved by the introduction of salt into the solute. Different proteins are released from the matrix at different salt concentrations. By introducing a salt concentration gradient to the matrix, protein molecules are released from the column in the order of their strengths of binding, with the most weakly bound proteins being eluted first. *E. coli* GroEL chaperonin, with a pI of 4.74, is one of the most acidic proteins in the bacterium. In the buffer (pH 7.5) used for purification, it will therefore be negatively charged and will bind strongly to a positively charged matrix. We find that the Q-Sepharose anion-exchange matrix is an excellent fast flow ion-exchange media for the purification of GroEL. The strong ion-exchange matrix has a quaternary amine as the active group, and shows high capacity, physical strength, excellent flow properties, and can be reused many times after regeneration.
2. Exploitation of chaperonin size is achieved by another chromatographic technique known as gel-permeation chromatography or gel filtration. In this technique, protein molecules are separated in solution according to differences in their sizes as they pass through a column packed with chromatographic media. In gels made for gel-permeation chromatography, the pore size is carefully controlled, and the matrix is chosen for its chemical and physical stability and inertness. The pore size is an important factor, since this will determine which size of molecule can fit inside the beads. Molecules that can diffuse into the beads will be slower moving down the column than larger molecules, which will interact less with the beads and move more quickly down the column. Larger molecules therefore leave the column first followed by the smaller molecules in order of their sizes. The relationship between the elution volume and the protein molecular weight is approximately logarithmic. GroEL is a homotetradecamer of ~57 kDa subunits giving a native size of 800 kDa. This is one of the largest protein molecules in the cell, making gel-filtration a powerful step in the purification process. We use Sephacryl high resolution (HR) resins, which are composite gels made by covalently crosslinking allyl dextran with *N,N,N'*-methylene *bis*-acrylamide to form a hydrophilic matrix of high mechanical strength and excellent flow properties.

Purification of recombinant GroEL from *E. coli* involves three chromatographic steps, with concentration and dilution of the sample in between. Detection of the chaperonin is either by native or SDS-PAGE, and comparison with molecular-weight standards. The three chromatographic steps are:

1. Fast Q-Sepharose anion-exchange chromatography.
2. Gel-filtration chromatography on Sephacryl S-300 HR (Pharmacia, Uppsala, Sweden).
3. MonoQ-Sepharose HR anion-exchange chromatography.

These three steps can produce GroEL that is at least 98–99% pure on a milligram scale in 2–3 d.

2. Materials

Except where noted, all chemicals are from Sigma (St. Louis, MO) and are analytical grade or higher.

2.1. Cell Growth

1. Strain: *E. coli* strain DH5 was transformed using pTric 99 containing the GroE operon under control of the IQ repressor and is amp⁺. This was a kind gift from A. Horwich (2) and will be referred to in this chapter as the IQ strain.
2. Growth media 2XTY: 16 g tryptone, 10 g yeast extract, 5 g NaCl 2.5 mL 1 N NaOH/L. We normally use 10 L for one protein preparation and make the media in 5 L batches. It is important to check the pH using indicator paper. It should be pH 7.0–7.5.
3. Ampicillin stock: 50 mg/mL in H₂O.
4. 1 M (238 mg/mL) Isopropyl β-D thiogalactopyranoside (IPTG) stock solution in H₂O.
5. Lysozyme stock solution: 10 mg/mL.
6. 50 mM Sodium phosphate buffer, pH 7.0.
7. 1 M DTT (dithiothreitol) in H₂O: 154 mg/mL.

2.2. Buffers

All buffers are freshly made, degassed, and filtered just prior to purification. We normally carry out the chromatographic steps at room temperature. Therefore, the buffers are not prechilled. However, if chromatography is to be carried out at 4°C, the buffers should be equilibrated at this temperature. It is important that the DTT solution is prepared and added to the buffers just before chromatography, because this compound is unstable and is oxidized by oxygen dissolved in the buffers.

1. Buffer A: 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 50 mM NaCl.
2. Buffer B: 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 1 M NaCl.
3. Buffer C: 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 250 mM NaCl, 20% (v/v) ethanol.

4. Buffer D: 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 500 mM NaCl.
5. Buffer E: 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM DTT, 250 mM NaCl.
6. Buffer F: 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM DTT, 250 mM NaCl, 50% (v/v) glycerol.

2.3. Fast Q Chromatography

Q-Sepharose Fast-Flow anion-exchange media are stored in 20% ethanol and must be washed at least four times with 4 vol of deionized H₂O prior to use. Media are suspended in 5 vol of buffer A. A 30 × 2.3 cm column is cleaned, and the resin (90 mL) poured into the column according to the manufacturer's directions. This amount of resin will fill the column to about 7 cm below the top, which allows room for the adapter to be fitted. We find the use of gravity sedimentation sufficient for this process. The column is then packed at a flow rate of 6 mL/min using buffer A. Column equilibration is carried out using at least 10 column volumes of buffer A. Complete equilibrium is obtained when the pH of eluant is the same as the buffer. The column is then ready for use. With care, the column and beads can be used successfully for many months. After use, we always wash the column in a 2 M NaCl solution, followed by water, and store it in a 20% (v/v) ethanol, H₂O solution, at room temperature.

2.4. Gel-Filtration Chromatography

Sephacryl S-300 HR is washed with water (at least four times in 2X matrix volume) before column preparation. Several washes are usually required to remove all the "fines" (crushed beads that have a detrimental effect on the flow rate of a column). This matrix is of lower mechanical strength, and care must be taken not to damage the gel beads. The matrix also requires a longer settling time both during washing and column preparation. Prior to column preparation, the gel matrix is generally suspended in 2 vol of buffer C and degassed for 30 min. A 150 × 2.0 cm column is prepared, and the gel poured into the column and allowed to settle by gravity. Approximately 400–420 mL of resin is used, which again allows for an adapter to be fitted to the top of the column. It is also possible to run a gel-filtration column without an adapter, which will increase the effective length of the column. However, we have found that the use of an adapter gives a more uniform sample and eluant application. The column is equilibrated by running the column at 1 mL/min overnight with buffer C. Care should be taken when manipulating this column, since this column matrix is highly sensitive to the introduction of air bubbles. This column is run at room temperature, so all buffers should be equilibrated at this temperature.

2.5. MonoQ HR Chromatography

The final chromatographic step, high resolution ion-exchange chromatography, is carried out using prepacked columns from Pharmacia (Uppsala, Swe-

den). Two different sizes are commonly employed, depending on the amount of protein to be applied, MonoQ HR 10/10 (10 × 100 mm) and HR 16/10 (16 × 100 mm). The reactive group on this resin is also a quaternary amine, the same as that in Q-Sepharose. However, the bead size is strictly controlled giving a highly uniform, monodisperse resin. This allows very rapid and high resolution chromatography.

2.6. Electrophoresis

Screening of the fractions obtained by chromatography is carried out using preformed PHAST gels (Pharmacia): 12.5 % for SDS gels and gradient 4–15% for native PAGE. Alternatively, 12% Lammeli SDS gels (4) can be used for screening purposes. Sample loading buffers are:

5X SDS: 0.5 g SDS, 0.3 g DTT, and 1 mg pyroninY dissolved in 10 mL 75% glycerol/25% 50 mM Tris-HCl (v/v), pH 7.5

5X Native: 1 mg bromophenol blue dissolved in 10 mL 75% glycerol/25% Tris-HCl (50 mM) (v/v), pH 7.5

GroEL can also be detected on 4 or 6% large-format slab PAGE gels using the protocol described below and in **ref. (5)**.

40% Acrylamide stock solution: **caution** (see **Note 1**)

394.7 g *N*-methyl acrylamide.

5.3 g *Bis*-acrylamide.

Dissolve in H₂O and adjust final volume to 1 L.

Filter through a 2- μ m disposable filter from Millipore.

Buffer (20x): 0.25 M Tris-Glycine pH 8.0

4% Gel solution 6% Gel solution

10.0 mL 40% acrylamide stock solution 15.0 mL 40% acrylamide stock solution

5 mL buffer (0.25 M Tris-glycine, pH 8.0) 5 mL buffer (0.25 M Tris-glycine, pH 8.0)

85 mL H₂O 80 mL H₂O

Degas the solution and add 100 μ L 1 M DTT, 50 μ L *N,N,N',N'*-Tetramethylethylene diamine (TEMED), and 500 μ L 10% ammonium persulfate.

This is a continuous electrophoresis system with the same buffer being used for both the separating gel and the running buffer. Early attempts at using stacking gels proved to produce no better resolution over a range of different salt and buffer conditions. We use a 16 × 16 cm gel format with 1-mm thick spacers in a vertical electrophoresis setup from Hoefer (Pharmacia, Piscataway, NJ). Whether to use 4 or 6% gels is a matter of individual preference. Four percent gels run very fast (total run time = 1 h at 400 V). However they are very difficult to handle. Six percent gels are much easier to handle, and run in about

90 min at 400 V. Complex formation between GroEL and its cochaperonin GroES and indeed other protein substrates, are often more easily observed on 4% gels. However, they can also be visualized on 6% gels. The following staining method can be used for all gel electrophoreses described in the protocol.

1. Staining solution: 10% (v/v) acetic acid, 30% (v/v) methanol, 0.1% Coomassie brilliant blue R250, 60% H₂O (v/v).
2. Destain I: 10% (v/v) acetic acid, 30% (v/v) methanol, 60% H₂O (v/v).
3. Destain II: 10% acetic acid, 10% glycerol, 80% H₂O (v/v) (*see Note 2*).

2.7. Protein Concentration

Two methods are employed for the concentration of crude extract or protein solutions during the purification protocol.

1. Nitrogen-pressurized stirred cell (Amicon): For concentration of large volumes, 50–1000 mL, we use the stirred cell according to the manufacturer's instructions at a pressure of 50 psi. The use of a membrane with 100-kDa cutoff (Difco or Millipore) allows this to be a fairly rapid procedure and serves as an additional purification step.
2. Centrifugal concentrators: For sample sizes under 50 ml we commonly use centrifugal concentrators, Centricon (2 mL) or Centriprep (15 mL) with a 100-kDa cutoff membranes (Amicon). Filtron concentrators have also been used with excellent results (*see Note 3*). Millipore concentrators, a relatively new product, allow the direct visualization of the volume left in the concentrator, which is very useful.

3. Methods

3.1. Cell Growth

1. Approximately 1 L of media is placed in each of the 10 2.5-L flasks. The use of flasks with molded "baffles" increase aeration and allows faster cell growth. Two 250 mL flasks with 100 mL of media are also included for the "overnight culture" that serves as inoculum.
2. The flasks are autoclaved on the "wet cycle" for 20 min at 130°C, and the media removed and allowed to cool. The overproducing GroEL strain is ampicillin-resistant. Therefore, after cooling to room temperature, 1.6 mL of ampicillin stock solution (final concentration is 80 µg/mL) is added to each liter of media. Similarly, 160 µL of ampicillin stock are added to the 100-mL media for the overnight inoculum (final concentration is 80 µg/mL).
3. The two 100-mL overnight cultures are started by inoculating (aseptically) the media with a single freshly grown transformed bacterial colony or with a scraping of frozen cells, which are stored in a -80°C freezer. The cultures are placed in a shaker at 37°C, shaken at 200 rpm, and allowed to grow overnight. The culture is grown until the late-log phase (looks cloudy, cell density of approx 1×10^9 cells/mL; OD₆₀₀ = 2–3).

4. The production culture (10 L) pre-equilibrated to 37°C is then inoculated with 5–10 mL of the fresh overnight culture. The OD₆₀₀ is measured every 30 min, using the uninoculated 2xTY medium as a blank. The *GroE* operon in this strain is induced by IPTG and when the cells have reached an OD₆₀₀ of 0.4–0.6, 1 mM IPTG (final concentration) is added to the culture. The cultures are then further incubated until the cells stop growing (absorption at OD₆₀₀ reaches plateau (approx 2.0 OD₆₀₀)). Usually it takes about 3–4 h, after which the cultures are placed on ice, ready for cell harvesting.
5. It is advisable to check induction of the GroEL during fermentation. An aliquot (1 mL) of culture 2 h after IPTG induction is removed, and cells are pelleted in an Eppendorf tube in a microcentrifuge. An uninduced sample should be included as a control. The supernatant is decanted off, and the resulting cell pellet resuspended in 200 µL of 5xSDS buffer, boiled for 1 min, and applied (at several concentrations) to a 12.5% SDS gel. Efficient induction is seen as a large GroEL band at 60 kDa, which can be up to 70% total cell protein (see **Note 4**). **Figure 1** shows a two-dimensional (2D) gel of GroEL induction in *E. coli*.

3.2. Cell Harvesting and Extract Preparation

1. During cell harvesting and extract preparation, it is important to keep everything at 4°C to reduce the effects of proteases in the sample. The prechilled culture containing the cells (which should look cloudy) is poured into centrifuge bottles (6 × 250 mL), and spun at 5000g in a Sorval GSA rotor at 4°C for 10 min. After this time, the media (which should be clear) are decanted off, another 250 mL of culture added to the same flask, and the centrifugation repeated. This process is repeated until all the culture has been centrifuged and all the cells have been harvested. The pellet is washed once by resuspending the cells in 50 mM sodium phosphate buffer, pH 7.0, and pelleting the cells as described above.
2. The cell pellet is then gently scraped from the centrifuge bottles, combined, and weighed. Typically, the cell yield is 1–3 g/L of culture. The cells are then resuspended in 5 vol of (v/w) buffer A, and 1 mL/g cells of stock lysozyme solution is added. This is stirred gently for 5 min on ice before sonication (note: solution becomes viscous). Generally, 2.5 min of sonication on ice, i.e., 5 × 0.5 min interspersed with a 1 min cooling period, are sufficient for complete cell disruption.
3. This suspension is then centrifuged at 17,000g in a Sorval GSA rotor for 30 min at 4°C, which pellets the cell membranes and other debris. The clarified cell extract, the supernatant, contains the soluble protein from the cell and is applied directly to the Fast Q-Sepharose column.
4. If desired, an aliquot for electrophoresis can be taken at this stage in order to determine the effectiveness of the purification stages. The concentration of GroEL at this step and in subsequent steps can be determined by visual assessment after coelectrophoresis of the sample with standards of known concentration.

3.3. Fast Q Chromatography

1. The extract is applied at a flow rate of 6 mL/min to the Fast Q anion-exchange column, which was pre-equilibrated with buffer A.

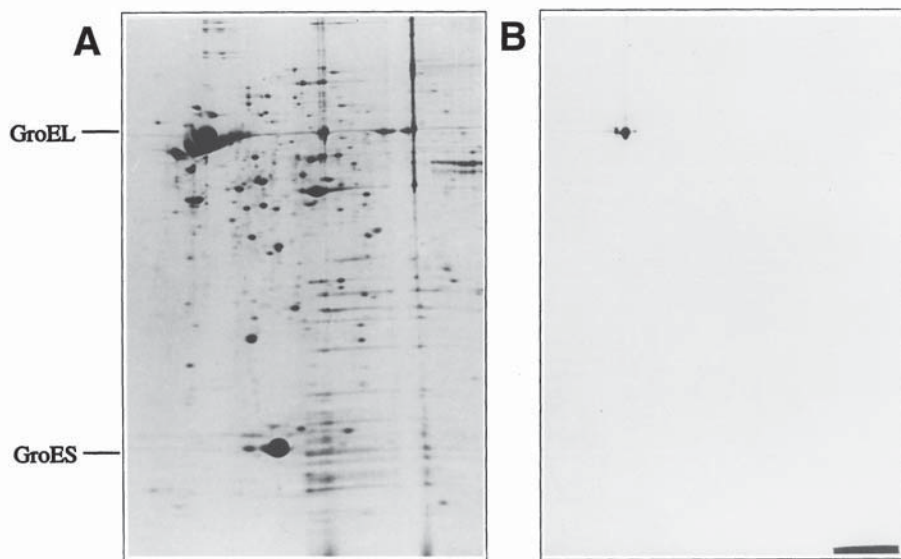


Fig. 1. (A) This shows a 2D gel stained with Coomassie brilliant blue of the clarified cell extract from *E. coli* demonstrating the induction of GroEL. (B) This shows a gel, run under the same conditions, of the purified GroEL. In brief, protein samples were mixed with an equal volume of 9 M urea, 4% (v/v) Nonident P40, 2% 2-mercaptoethanol, and 2% ampholytes (pH 9.0–11.0). The first-dimension isoelectric focusing (IEF) was carried out using 40-cm rod gels containing 50%, pH 3.0–10.0, and 50%, pH 5.0–7.0, ampholytes from Bio-Rad (Hercules, CA). 2D SDS electrophoresis was run in slab gels containing a 10–17% linear gradient of acrylamide, pH 8.0, according to Lammeli (4). In both cases, IEF is from left to right, and SDS is from top to bottom.

2. The column is washed with 500 mL of buffer A (5 mL/min), and proteins are eluted with a linear gradient of 50 mM to 1 M NaCl. The total gradient volume is 1.7 L with 95 fractions of 18 mL each being collected throughout.
3. Fractions containing GroEL were identified using both 4–15% native PAGE and 12.5% SDS-PAGE (see Note 5) using the PHAST system. Eight microliters of fraction are mixed with 2 μ L of loading buffer and 1 μ L of this is applied to the PHAST gel. This is sufficient to detect the protein. GroEL elutes between 200 and 250 mM NaCl (see Note 6).
4. Fractions containing GroEL are pooled and concentrated in the nitrogen-pressurized stirred cell from about 50 to 200 mL to approx to 15–20 mL. This is possible because GroEL is highly soluble at neutral pH (see Note 7). The reason for this high degree of concentration is because of the fact that the resolution of the proteins on the gel-filtration column is dependent on the volume of the sample.

3.4. Gel-Filtration Chromatography

1. Even though there may be 20 mL of concentrated extract, only 5–10 mL are applied to the gel-filtration column (150 × 2.0 cm), which we use. Any remaining extract is saved, and a second gel-filtration step is carried out. Typically, 500 mg of protein can successfully be applied to this column, giving very high resolution and separation of GroEL from other components.
2. The column is pre-equilibrated with buffer C, which contains 20% ethanol. In comparative runs, including and omitting alcohols, it was found that buffer C produced the purest GroEL. It is thought that the ethanol is effective in releasing GroEL-bound proteins/peptides, leading to a much cleaner preparation. At present, the underlying reason for this is not clearly understood.
3. The column is run using buffer C at a flow rate of 0.5 mL/min, and fractions of 5 mL are collected. Chaperonin elutes in a sharp peak close to void volume. Those fractions containing GroEL are identified by 4–15% native PAGE (8 μ L sample, 2 μ L loading buffer, and 1 μ L applied to gel) and pooled.
4. The pooled sample is diluted with buffer A three times in preparation for the final anion exchange column (*see Note 8*). It is important to check fractions carefully, since the low extinction coefficient means that the chart recorder may not detect the peak of GroEL (*see Note 6*).

3.5. MonoQ HR Chromatography

1. The diluted GroEL containing fractions are reapplied to a MonoQ anion-exchange column at 3 mL/min using automated chromatography equipment, e.g., the FPLC system (Pharmacia), which can be preprogrammed. The size of column (either 16/10 or 10/10) is determined by the amount of protein. The maximum loading capacity for the MonoQ 10/10 column is approx 200 mg/column and for the 16/10, 500 mg/column.
2. After washing with buffer A (3x column volume), a 1 L gradient of 150–500 mM NaCl is at 4 mL/min (HR 10/10) or 6 mL/min (HR 16/10) and 10-mL fractions are collected.
3. Fractions containing GroEL are identified by both 4–15% native PAGE and 12.5% SDS electrophoresis (*see Note 5*). At this stage, the GroEL is 98–99% pure as judged by 2D gel electrophoresis and silver staining (**6**).

A summary of the purification steps is shown in **Table 1** and **Fig. 1B** shows a 2D gel of the purified GroEL.

3.6. GroEL Concentration and Storage

Pure GroEL can be concentrated in centrifugal concentrators to concentrations >200 mg/mL in buffer E. We routinely store GroEL stocks at this concentration. The concentration of GroEL is determined using A_{280} measurement with an extinction coefficient calculated from the amino acid sequence (**7**). Since GroEL contains no tryptophan the extinction coefficient is low and was

Table 1
Summary of Total Yield of GroEL from *E. coli*
(50 g Cell Paste)

	Volume, mL	A ₂₈₀	GroEL, mg
Crude extract	300	800	150
Fast Q	150	175	124
S-300	50	26.3	106
MonoQ	70	12.5	96.7

calculated to be 9320 M⁻¹/cm. The effect of this property on purification is discussed in **Note 6**. For everyday experimental use, we find that GroEL is stable for several weeks when stored in high concentrations in buffer E at 4°C. Longer-term storage at -80°C is carried out by storage in buffer F.

4. Notes

1. Since acrylamide is a neurotoxin and listed carcinogen, the preparation of acrylamide solutions should be carried out with great care. The chemicals should be weighed in a hood designated for carcinogen use, and gloves, eye protection, and lab coat should be worn when handling the material. The monomer solution is also hazardous and should be handled carefully using the same precautions. Once polymerized, the gel is normally not considered such a hazard and can be discarded in the normal waste. Experimenters should contact their chemical hygienists regarding the safety procedures for acrylamide use and disposal in their own establishments.
2. Since the PHAST preformed gels have a plastic backing, glycerol is added to the final destain (Destain II) to prevent the polyacrylamide from peeling off the plastic backing. If gels are prepared by the user, the Destain II step should be omitted. This is particularly important if the gels are to be dried in a vacuum drier, because the glycerol will prevent the gel from drying.
3. Recently, various companies have produced centrifugal concentrators with 300-kDa cutoff membranes. In our hands, we find that 10–20% of GroEL passes through these membranes, and therefore, we continue to use 100-kDa membranes for concentration of chaperonins.
4. We have found that ensuring good induction of GroEL (or indeed any of the recombinant proteins we study in our lab) is an important step in the purification process. It avoids wasting time and resources when looking for a “lost” protein, which in reality was not induced properly.
5. The use of both native and SDS-PAGE to screen the fractions for GroEL may seem superfluous. However, one of the main challenges in chaperonin purification is ensuring that the chaperonin is free of bound substrate proteins. Native PAGE, which shows the high-molecular-weight complex, allows us to ensure

that the chaperonin is tetradecameric and that it has not unassembled into subunits. SDS-PAGE, on the other hand, allows us to check for the presence of other polypeptides, which might be closely bound to the chaperonin. Both of these techniques therefore contribute useful information.

6. The very low extinction coefficient of GroEL creates a significant problem during chromatography. For example, a "normal" protein with a normal extinction coefficient (i.e., $1 \text{ mg/mL} = 1 A_{280}$), is readily observed on a chart recorder during column chromatography as an increase in absorbance or a peak. This peak should be large if one considers the amount of GroEL in the induced cell. However, with a 1 mg/mL solution having an A_{280} of 0.12, only a small peak is observed. We have often found milligrams of "lost" GroEL by running gels through the whole gradient and not relying on the output from the chart recorder.
7. GroEL is an extremely soluble protein in all buffers used in our protocol. We routinely concentrate the protein to 200 mg/mL . The maximum concentration achieved to date is 285 mg/mL .
8. A step that is often forgotten is the dilution of the fractions from the gel-filtration column. The running buffer for this column contains 250 mM NaCl . We know from anion-exchange chromatography that GroEL elutes between 200 and 250 mM NaCl . If applied directly to the column without dilution, the GroEL will not bind to the resin, but can be recovered from the flowthrough.

Acknowledgments

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Purification of GroES from an Overproducing *E. coli* Strain

Elsie Quaite-Randall and Andrzej Joachimiak

1. Introduction

Of the Hsp10/cpn10 family of molecular chaperones, GroES, the cochaperonin or “release factor” from *Escherichia coli* is the best characterized. Since GroEL and GroES are on the same operon in *E. coli* (1), they are usually overexpressed together in the same strain (see Note 1). We commonly purify GroES from the same cells used for GroEL purification (2).

GroES is an oligomer comprising seven identical subunits arranged in a ring (3). Unlike GroEL, where size is an important separation characteristic, the monomers have a molecular weight of 10,387 Dalton, making the oligomer 72,709 Dalton, which is in the size range of many cellular proteins. The gel-filtration step, which is so powerful in the purification of tetradecameric chaperonins, is therefore not of great benefit in the purification of the heptameric cochaperonin, GroES. The method described in this chapter for the purification of GroES from an overproducing strain of *E. coli* involves several steps, which depend on the *pI* of the protein and utilize anion-exchange chromatography.

This chromatography technique depends on the reversible binding of charged solute molecules to an immobilized ion-exchange group of opposite charge. A complex mixture of proteins is applied to a column containing the desired ion-exchange resin. Depending on the *pI* of the protein, the pH of the solvent, and salt concentration, some proteins will bind to the column, but others will not. Bound proteins are removed from the column by making the elution conditions unfavorable for ionic bonding of the solute molecules (change in pH or salt concentration). Release of a protein from the matrix is achieved by the introduction of salt into the solute. Different proteins are

released from the matrix at different salt concentrations. By introducing a salt concentration gradient to the matrix, protein molecules are released from the column in the order of their strengths of binding, with the weakest bound proteins being eluted first. GroES, with a *pI* of 5.08, is an acidic protein. In the buffer used for purification. It will therefore be negatively charged and will bind strongly to a positively charged matrix. We find that Q-Sepharose anion-exchange matrix (Pharmacia, Uppsala, Sweden) is an excellent fast-flow ion-exchange media for the purification of *E. coli* GroES. The strong ion-exchange matrix has a quaternary amine as the active group and shows high capacity, physical strength, excellent flow properties, and can be reused many times after regeneration

Purification of recombinant GroES from *E. coli* involves three chromatographic steps, with concentration and dilution stages in between. Detection of the chaperonin is either by native or SDS-PAGE. The three chromatographic steps are:

1. Fast Q-sepharose anion exchange chromatography (pH 7.5)
2. MonoQ-sepharose HR anion exchange chromatography (pH 7.5)
3. MonoQ-sepharose HR anion exchange chromatography (pH 5.3)

These three steps can produce GroES, which is 98–99% pure on a milligram scale in 2–3 d.

2. Materials

Except where noted, all chemicals are from Sigma (St. Louis, MO) and are analytical grade or higher.

2.1. Cell Growth

1. Strain: *E. coli* strain DH5 was transformed using pTric 99 containing the GroE operon under the control of the IQ repressor and is amp^r. This was a kind gift from A. Horwich (4), and is referred to in this chapter as IQ strain.
2. Growth media is 2x TY media. For 1 L: 16 g tryptone, 10 g yeast extract, 5 g NaCl 2.5 mL 1 N NaOH. It is important to check the pH using indicator paper. It should be pH 7.0–7.5.
3. Ampicillin stock is 50 mg/mL in H₂O.
4. 1 M (238 mg/mL) Isopropyl β-D thiogalactopyranoside (IPTG) stock solution in H₂O.
5. Lysozyme stock solution: 10 mg/mL.
6. 50 mM sodium phosphate buffer, pH 7.0.
7. 1 M dithiothreitol (DTT) in H₂O.

2.2. Buffers

All buffers should be freshly made, degassed, and filtered just prior to purification. GroES purification is normally carried out at room temperature, so

the buffers and columns should be equilibrated at this temperature. No detrimental effect is found if GroES purification must take place at 4°C. However, the buffers must be equilibrated accordingly. DTT is always added just before the chromatography is run, since this compound is unstable and easily oxidized by oxygen dissolved in the buffers.

1. Buffer A: 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT.
2. Buffer B: 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 1 M NaCl.
3. Buffer C: 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 250 mM NaCl.
4. Buffer D: 20 mM Imidazole pH 5.3.
5. Buffer E: 20 mM Imidazole pH 5.3, 500 mM NaCl.
6. Buffer F: 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM DTT, 100 mM NaCl.
7. Buffer G: 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM DTT, 100 mM NaCl, 50% (v/v) glycerol.

2.3. Fast Q chromatography

Q-Sepharose Fast-Flow anion-exchange media are available in bulk form from Pharmacia. This media are stored in 20% ethanol and must be washed in deionized H₂O before use. Media are suspended in 5 vol of buffer A. A 30 × 2.3 cm column is cleaned, and the resin poured into the column according to the manufacture's directions. We find the use of gravity sedimentation sufficient for this process. The column is then packed at a flow rate of 6 mL/min using buffer A. Column equilibration is carried out using at least 10 column volumes of buffer A. Complete equilibrium is obtained when the pH of eluant is the same as the buffer. The column is then ready for use.

2.4. MonoQ HR chromatography

The second and third chromatographic steps are identical in that they use high resolution ion exchange chromatography. In this case, the separation criterion is that of pH. Chromatography is carried out using prepacked columns from Pharmacia. Two different sizes are commonly employed, MonoQ HR 10/10 (10 × 100 mm) and HR 16/10 (16 × 100 mm). The reactive group on this resin is also a quaternary amine, the same as that in Q-Sepharose. However, the bead size is strictly controlled giving an extremely uniform, monodisperse resin, allowing rapid and high resolution separations to be performed.

2.5. Electrophoresis

Screening of the fractions obtained by chromatography is carried out using the PHAST system from Pharmacia. The preformed PHAST gels used are 20% for SDS gel electrophoresis. If the experimenter has no access to a PHAST system, the same result can be achieved using 20% Laemmli gels (5) running in any standard laboratory electrophoresis equipment. It may also be necessary

to run native gels to ensure that the oligomer and not just the monomer is purified. In this case, adequate resolution between the heptamer and monomer can be seen using 8–25% native PHAST gels. However, better resolution is obtained with 15% native PAGE gels run in large-gel format.

1. Staining solution: 10% (v/v) acetic acid, 30% (v/v) methanol, 0.1% Coomassie brilliant blue R250 60% H₂O (v/v).
2. Destain I: 10% (v/v) acetic acid, 30% (v/v) methanol, 60% H₂O (v/v).
3. Destain II: 10% acetic acid, 10% glycerol (*see Note 2*), 80% H₂O (v/v).

2.6 Protein Concentration

Two methods are employed for the concentration of extract and protein solutions during the purification protocol.

1. Nitrogen-pressurized stirred cell (Amicon, Beverly, MA): For concentration of large volumes, 50–1000 mL, we use the stirred cell according to the manufacturer's instructions. The use of a membrane with 10-kDa (Difco, Detroit, MI or Millipore, Bedford, MA) cutoff allows this to be a fairly rapid procedure.
2. Centrifugal concentrators: For sample sizes <50 mL, we commonly use centrifugal concentrators; Centricon (2 mL) and Centriprep (15 mL) with 10-kDa cutoff membranes (Amicon). Filtron concentrators (Pall Filtron, Northborough, MA) have also been used with excellent effects. Millipore concentrators, a relatively new product, allow the direct visualization of the volume left in the concentrator, which is very useful (*see Note 3*).

3. Methods

3.1. Cell Growth

Successful cell growth can be obtained using bench top incubators and is identical to that described in (2).

1. The 2X TY media are made in batches of 5 L, the pH is corrected, and 1 L of media placed in 2.5 L flasks. The use of flasks with molded "baffles" increase aeration and allows faster cell growth. Two 250 mL flasks with 100 mL of media are also included for the "overnight culture" that serves as inoculum.
2. The flasks are autoclaved on the "wet cycle" for 20 min at 130°C, and the media removed and allowed to cool. The IQ OF strain is ampicillin-resistant. Therefore, after cooling to room temperature, 1.6 mL of ampicillin stock solution (final concentration is 80 mg/mL) is added to each liter of media. Similarly, 160 µL of ampicillin stock are added to the 100-mL media for the overnight inoculum (final concentration is 80 µg/mL).
3. Overnight cultures are started by inoculating (aseptically) the media with a single freshly grown bacterial colony, or with a scraping of frozen IQ OF cells, which are stored in a –80°C freezer. The cultures are placed in a shaker at 37°C, shaken at 200 rpm and allowed to grow overnight. The culture is grown until the late-log phase (looks cloudy, cell density of approx. 1×10^9 cells/mL).

4. The production cultures (2.5 L flasks) pre-equilibrated to 37°C are then inoculated with 5–10 mL of the fresh overnight culture. The OD₆₀₀ is measured every 30 min, using the uninoculated 2x TY medium as a blank. The *GroE* operon in this strain is induced by IPTG, and when the cells have reached an OD₆₀₀ of 0.4–0.6, 1 mL/L of stock IPTG is added to the culture (final concentration 1 mM). The cultures are then further incubated until the cells stop growing (absorption at OD₆₀₀ reaches plateau (approx. 2.0 OD₆₀₀). Usually it takes about 3–4 h, after which the cultures are placed on ice, ready for cell harvesting.
5. It is advisable to check induction of the GroES during fermentation. An aliquot (1 mL) of culture 2 h after IPTG induction is removed and spun in an Eppendorf tube in a microcentrifuge to pellet the cells. An uninduced sample should be included as a control. The supernatant is decanted off, and the resulting cell pellet resuspended in 200 µL of 5x SDS buffer, boiled for 1 min, and applied (at several concentrations) to a 12.5% SDS gel. Efficient induction is seen as a large GroES band, which can be up to 70% total cell protein (see **Note 4**). A two-dimensional (2D) gel of GroEL/GroES induction in *E. coli* is shown in Chapter 3 in this volume (2).

3.2. Cell Harvesting and Extract Preparation

1. The culture containing the cells (which should look cloudy) is poured into centrifuge bottles (6 × 250 mL), and spun at 5000g in a Sorval GSA rotor at 4°C for 30 min. After this time, the media (which should be clear) are poured off, another 250 mL of culture added to the same flask, and the centrifugation repeated. The clear media are again decanted off. This process is repeated until all the culture has been centrifuged and all the cells harvested.
2. The cell pellet is then gently scraped from the centrifuge bottles, combined, and weighed. A cell yield of 1–3 g/L culture is normal. The cells are then resuspended in 5X (v/w) buffer A, and 1 mL/g cells of stock lysozyme solution added. This is stirred gently for 5 min on ice before sonication. Generally, 5 min of sonication, i.e., 1 min interspersed with a 1 min rest, are sufficient for cell disruption. This suspension is then centrifuged at 17,000g in a Sorval GSA rotor for 30 min at 4°C, which pellets the cell membranes and other debris. The clarified cell extract, the supernatant, contains the soluble protein from the cell and is applied directly to the Fast Q column.

3.3. Fast Q chromatography

1. The extract is applied at a flow rate of 6 mL/min to the Fast Q anion-exchange column, which was pre-equilibrated with buffer A.
2. The column was then washed with 500 mL buffer A (5 mL/min) and proteins are eluted with a linear gradient of 50 mM to 1 M NaCl (100% buffer A to 100% buffer B).
3. Fractions containing GroES are identified using 20% SDS-PAGE on the PHAST system. GroES elutes between 80–150 mM NaCl. **Figure 1** shows the gels

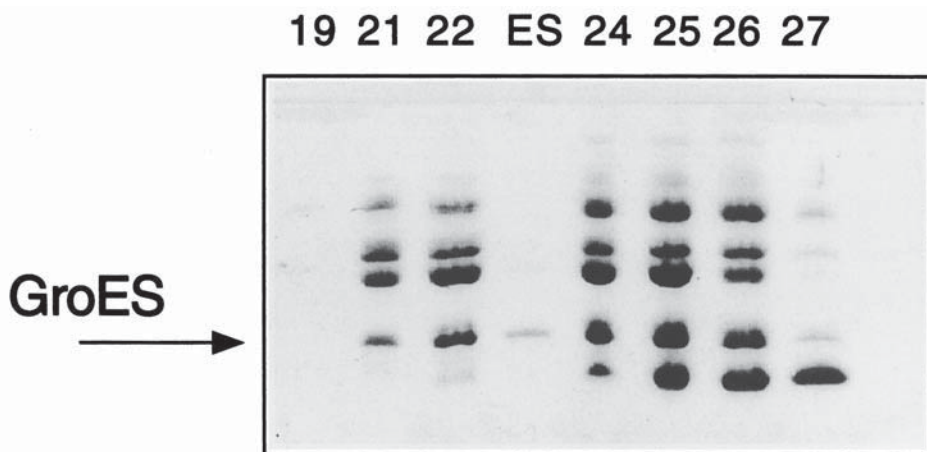


Fig. 1. This gel shows the abundance and position of GroES in fractions obtained after the first Fast Q column. During this chromatographic step, GroES is eluted in a wide band in salt concentrations of 80–150 mM NaCl.

through the GroES-containing fractions from the Fast Q column. Fractions containing GroES are pooled and concentrated on the stirred cell.

3.4. MonoQ HR chromatography

1. The diluted GroES-containing fractions were applied to a MonoQ anion exchange column freshly equilibrated with buffer A. This step is normally carried out on automated chromatography equipment, e.g., the FPLC system (Pharmacia), which can be pre-programmed. The size of column (either 16/10 or 10/10) is determined by the protein concentration. The maximum loading capacity for the MonoQ 10/10 column is 200 mg/column, and for the 16/10, 500 mg/column.
2. After washing with buffer A, a gradient of 0–250 mM NaCl is run (100% buffer A to 100% buffer C) at 4 mL/min (HR 10/10) or 6 mL/min (HR 16/10).
3. Fractions containing GroES are identified by 20% SDS-PAGE. At this stage, the GroES is judged to be about 60–70% pure as determined by Coomassie-stained SDS gels. The purpose of this second column is to separate those proteins that elute under low salt concentrations, i.e., 0–250 mM from others. We find this an excellent step before the final column, which if omitted, makes it difficult to obtain highly purified GroES.
4. The fractions containing GroES from this column are pooled and concentrated on a stirred-cell (30 kDa) membrane. The final purification step is dependent on a change in pH and after concentration the protein is diluted (*see Note 5*) with imidazole buffer, pH 5.3. It is very important that the protein solution reaches this pH, since it is just above the *pI* of GroES. Chromatography at this pH enables GroES to be separated from the remaining contaminating proteins. We have tried

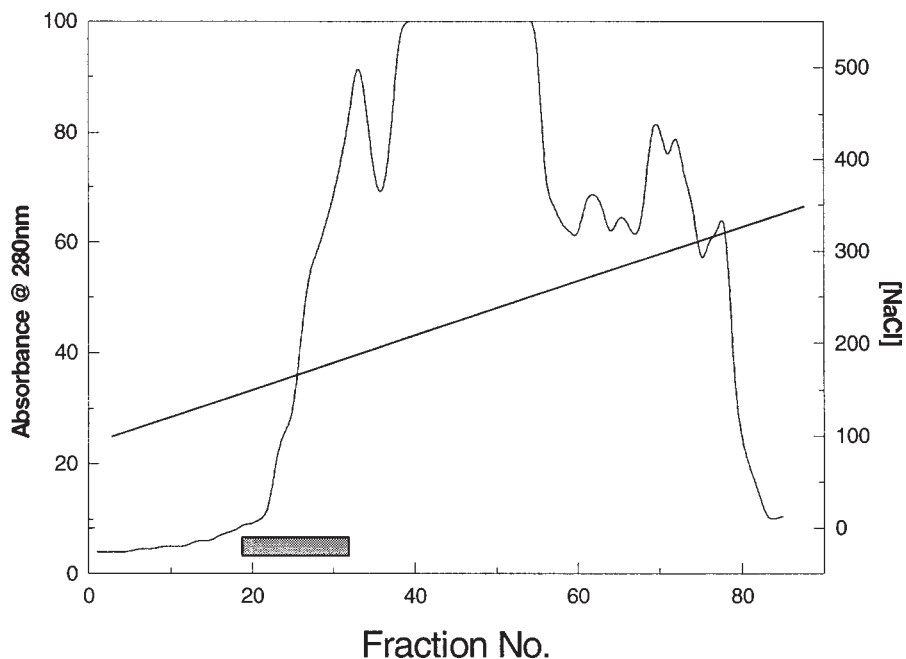


Fig. 2. This chromatograph shows a typical run of the final MonoQ column (pH 5.3). The position of GroES is shown by the shaded bar. This corresponds approximately to fractions 19–34. GroES elutes at between 120 and 170 mM NaCl under these chromatography conditions.

many different pHs and buffer systems for this step. However, none proved as successful as the imidazole buffer system described in this procedure.

5. After dilution (10x v/v), the sample is applied to a MonoQ 10/10 column which has been equilibrated with buffer D. A 240 mL gradient of 100–350 mM NaCl (20–70% buffer E) is run. **Figure 2** shows a chromatograph from this column, and **Figure 3** shows the purified GroES using 20% PHAST gels. As can be seen from the gels, GroES is 95–98% pure after this column. This can be further evaluated by 2D electrophoresis and silver staining (6).

3.6. GroES Concentration and Storage

1. After identification of GroES by electrophoresis, the protein can be concentrated in centrifugal concentrators to concentrations >100 mg/mL. We routinely store GroES stocks at this concentration.
2. The concentration of GroES is determined using an extinction coefficient calculated from the amino acid sequence (7). Since GroES contains no tryptophan, this is low and was calculated to be $1280 M^{-1}/\text{cm}^{-1}$. The effect of this on purification is discussed in **Note 6**.

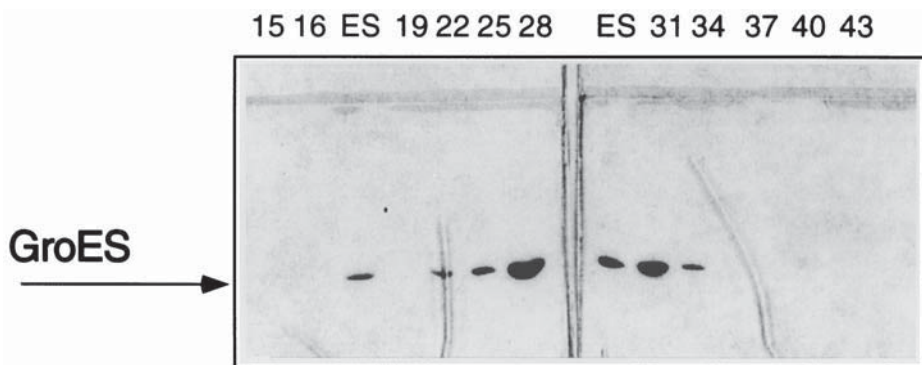


Fig. 3. A 20% PHAST gel showing the pure GroES fractions from the final chromatography step.

3. For everyday experimental use, we find that GroES is stable for several weeks when stored in buffer F at 4°C. Longer-term storage at -80°C is carried out by storage in buffer G.
4. Using the purification method described above we routinely obtain 30–40 mg of GroES from 20 g of overproducing cells in 2–3 d.

4. Notes

1. We have cloned and overexpressed the GroEL and GroES homologs from the thermophilic bacterium *Thermus thermophilus* (8). It was found that the yield of the tetradecameric Hsp60 was reduced when the cochaperonin was not coexpressed. However, it was easier to purify the thermophilic hsp10 when it was overexpressed alone.
2. Since the PHAST preformed gels have a plastic backing, glycerol is added to the final destain (Destain II) to prevent the polyacrylamide from peeling off the plastic backing. If gels are prepared by the user, the second step should be omitted. This is particularly important if the gels are to be dried in a vacuum drier, because the glycerol will prevent the gel from drying in the machine.
3. The use of 10-kDa cutoff membranes for GroES concentration makes this step particularly slow. It is possible to speed up this step by using 30- or 50-kDa cutoff membranes. However, care should be taken that GroES is not passing through these membranes. This can be carried out by testing the concentrate and flowthrough for the presence of GroES. The presence of both the heptamer and/or the monomer can be detected using native PAGE.
4. We have found that ensuring good induction of GroES (or indeed any of the recombinant proteins we study in our lab) is an important step in the purification process. It avoids wasting time and resources when looking for a “lost” protein, which in reality was not induced properly.

5. The concentration and dilution steps between the columns are very important. Since GroES elutes at a low salt concentration, care must be taken to ensure that the salt concentration is low enough to enable binding to the MonoQ column. If not, the GroES is eluted in the flowthrough. It is particularly important when diluting the concentrated sample with the imidazole buffer for the final column. This involves a change in pH as well as reduction in salt concentration. It is therefore important to check the pH of the sample before application to the imidazole-equilibrated MonoQ column.
6. The very low extinction coefficient of GroES creates a significant problem during chromatography. For example, a “normal” protein with a normal extinction coefficient (i.e., $1 \text{ mg/mL} = 1 A_{280}$), is readily observed on a chart recorder during column chromatography as an increase in absorbance or a peak. This peak should be huge if one considers the amount of GroEL in the induced cell. However, with a 1 mg/mL solution having a A_{280} of 0.123, a small peak is observed. We have often found milligrams of GroES, which were not detected by the chart recorder, by running gels through the whole gradient.

Acknowledgments

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Purification of the Gp31 Co-chaperonin of Bacteriophage T4

Saskia M. van der Vies

1. Introduction

Gp31 from bacteriophage T4 is a specialized co-chaperonin, which, in a complex with the host GroEL chaperonin, facilitates the folding of the T4 major capsid protein Gp23 during bacteriophage morphogenesis (1,2). The GroEL protein from *Escherichia coli* (*E. coli*) is a component of the GroEL/GroES chaperonin folding complex, which assists the correct folding of a variety of unrelated polypeptides in vivo and in vitro (reviewed in refs. 3–5). Gp31 is functionally related to GroES, since it can substitute for the *E. coli* cochaperonin in a variety of protein-folding reaction both in vitro and in vivo (2). The interaction of Gp31 with the host GroEL chaperonin is most likely similar to that of GroES as judged by cryo-EM side views (Chen et al., personal communications), in vitro binding studies, and amino acid sequence comparison (2,6,7). Despite this functional similarity, bacteriophage T4 cannot propagate in the absence of Gp31, indicating that the Gp31 protein possesses distinct properties that are absent in GroES (8–10). The 2.3-Å crystal shows that the tertiary and quaternary structures of the Gp31 heptamer are similar to those of GroES, despite the low amino acid sequence identity between the two proteins (14%). However, a series of structural adaptations are observed that may increase the size and hydrophilicity of the so-called Anfinsen cage, the enclosed cavity in the GroEL/GroES complex in which the polypeptide folds. Although other interpretations are possible, the simplest explanation for this adaptation is that the Gp23 capsid polypeptide is too large to fit into the GroEL/GroES complex (11). With a molecular mass of 56 kDa, Gp23 is among the largest polypeptide known to utilize the chaperonin system.

Here is described a method for the isolation of the Gp31 cochaperonin to aid further in vitro analysis. Because the amount of Gp31 that can be purified from *E. coli* cells infected with bacteriophage T4 is low, the protein is produced from a high copy number plasmid that contains gene *31* under the control of a strong inducible promoter. Gp31 is naturally stable in *E. coli* and large amounts of soluble protein can therefore be obtained (see Fig. 1). Once conditions have been established for the production of these large quantities, the isolation of Gp31 is straightforward. A unique set of distinct Gp31 properties is utilized for the isolation of the protein from *E. coli*. Since Gp31 possesses no known genuine biological activity, the protein is identified on the basis of its characteristic migration in an SDS polyacrylamide gel.

2. Materials

1. *E. coli* strain MC1009 containing a plasmid that carries gene *31* from bacteriophage T4 under the transcriptional control of the inducible *ara* promoter (12,13).
2. LB medium: 10 g Bacto-tryptone, 5 g yeast extract, and 5 g NaCl/L of H₂O.
Dissolve the reagents in 800 mL of H₂O, and adjust the pH to 7.0 with 5 N NaOH. Make up to 1 L with H₂O, and sterilize by autoclaving for 20 min at 15 lb/sq. in. on a liquid cycle.
3. LB agar: 1% (w/v) agar in LB medium. Before autoclaving add the agar to the LB medium (see item 2). After autoclaving, mix the solution gently to dissolve the agar evenly. Be careful, since the fluid may be overheated and may start to boil when swirled. Allow the agar to cool to about 45°C, and use immediately or store the bottle in a 45°C water bath until use. Add antibiotic just before making the plates.
4. LB agar plates: These are made by pouring 30–35 mL of the melted LB agar into a Petri dish with a diameter of 90 mm. Once the agar has set, the plates can be used immediately or stored inverted at 4°C.
5. Glycerol buffer: 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM β-mercaptoethanol and 15% (v/v) glycerol. This buffer is prepared using stock solutions of 1 M Tris-HCl, pH 7.5, 5 M NaCl, 80% (v/v) glycerol and 0.5 M Na₂EDTA, pH 8.0. The β-mercaptoethanol should be added just before use.
6. Buffer A: 20 mM Tris-HCl, pH 7.7, 200 mM NaCl, 2 mM EDTA, and 5 mM β-mercaptoethanol. Prepare the buffer from stock solutions of 1 M Tris-HCl, pH 7.7, 5 M NaCl, and 0.5 M EDTA, pH 8.0. The β-mercaptoethanol should be added just before use.
7. Bacteriophage T4 lysozyme, ribonuclease A (RNase), deoxyribonuclease (DNase), and phenylmethylsulfonyl fluoride (PMSF) are of the highest analytical grade available. Take extra care when handling the protease inhibitor PMSF, since it is highly toxic.
8. Streptomycin sulfate solution: 25% (w/v). Make the solution fresh by dissolving 25 g of streptomycin in H₂O to a final volume of 100 mL.

9. Buffer B: 20 mM Tris-HCl, pH 7.7, 1 mM EDTA, and 5 mM β -mercaptoethanol. The buffer is prepared using stock solution of 1 M Tris-HCl, pH 7.7, and 0.5 M Na₂EDTA, pH 8.0, whereas β -mercaptoethanol should be added just before use.
10. Buffer B/0.2 M NaCl: 20 mM Tris-HCl, pH 7.7, 0.2 M NaCl, 1 mM EDTA, and 5 mM β -mercaptoethanol. The buffer is prepared using stock solution of 1 M Tris-HCl, pH 7.7, 0.5 M EDTA, pH 8.0, and 5M NaCl, but β -mercaptoethanol should be added just before use.
11. Buffer B/0.55 M NaCl: 20 mM Tris-HCl, pH 7.7, 0.55 M NaCl, 1 mM EDTA, and 5 mM β -mercaptoethanol. The buffer is prepared using stock solution of 1 M Tris-HCl, pH 7.7, 0.5 M Na₂EDTA, pH 8.0, and 5 M NaCl, whereas β -mercaptoethanol should be added just before use.
12. Buffer C: 20 mM Na-phosphate buffer, pH 6.8, 1 mM EDTA, and 5 mM β -mercaptoethanol. Prepare the buffer by mixing a solution of 0.02 M Na₂HPO₄ and 0.02 M NaH₂PO₄ until the pH is 6.8. Add EDTA from a stock solution of 0.5 M Na₂EDTA, pH 8.0, and β -mercaptoethanol just before use.
13. Buffer D: 20 mM Na-phosphate buffer, pH 7.4, containing ammonium sulfate at a concentration of 30% saturation. Prepare the buffer by mixing a solution of 0.02 M Na₂HPO₄ and 0.02 M NaH₂PO₄ until the pH is 7.4. Add solid ammonium sulfate to 30% saturation: 17.6 g to 100 mL.
14. Storage buffer: 50 mM Tris-HCl, pH 7.7, 10% glycerol, 1 mM dithiothreitol (DTT), 1 mM EDTA. This buffer can be prepared using stock solutions of 1 M Tris-HCl, pH 7.7, 5 M NaCl, 80% (v/v) glycerol, and 0.5 M Na₂EDTA, pH 8.0.
15. Inducer solution: 10% (w/v) L-arabinose. Dissolve 10 g of L-arabinose in 100 mL H₂O. Sterilize the solution by filtration. Autoclaving can be used, but tends to cause some caramelization.
16. Column matrices: Q-Sepharose and phenyl sepharose from Sigma (St. Louis, MO) and Hydroxyapatite (HA):*HA Biogel.HTP from Bio-Rad (Hercules, CA) (130-0420).
17. Dialysis tubing: Dialysis tubing is available in a variety of sizes. Use one that does not allow molecules larger than 15,000–20,000 Dalton to pass through.

3. Methods

3.1. Determination of Conditions for High-Level Expression of Gp31 in *E. coli*

It is essential first to determine the optimal conditions under which the transformed cells produce large amounts of Gp31 before starting the purification procedure (**Subheading 3.3.**). The concentration of arabinose needed to synthesize the maximum amount of Gp31 inside the *E. coli* cell needs to be determined and requires the bacteriophage gene 31 to be present on a plasmid under the control of the inducible arabinose (*ara*) promoter. For bacterial strains, such as MC1009, which are deleted for *ara* genes, synthesis reaches maximal levels on induction with 0.001% (w/v) L-arabinose (**12,13**), whereas the strain that ferments arabinose need 1% of L-arabinose for full induction (data not

shown). The bacterial growth stage at the time of induction does not seem to be a critical parameter (data not shown).

1. Streak an *E. coli* strain MC1009 containing a plasmid carrying the bacteriophage T4 gene 31 under the transcriptional control of the *ara* promoter out on an LB agar plate (see **Subheading 2., item 4.**) that contains the required antibiotics, and incubate the plate overnight at 37°C to obtain individual colonies.
2. Use an individual colony to inoculate 5 mL of LB medium (see **Subheading 2., item 1**) plus antibiotic in a 25-mL flask. Incubate the culture overnight at 37°C on a shaking platform.
3. Determine the optical density of the overnight culture at 600 nm (OD₆₀₀) (see **Note 1**).
4. Add an aliquot of the overnight culture to 200 mL of LB medium plus antibiotic in a 1000-mL flask so that OD₆₀₀ is about 0.08, and grow the cells at 37°C while shaking until OD at 600 nm is about 0.8 (takes about 4 h) (see **Note 2**).
5. Transfer 10 mL of the culture to a 50-mL flask (6 flasks in total), and add L-arabinose (see **Subheading 2., item 14**) to final concentration of:
 - a. 0% (Control).
 - b. 0.00005% (w/v).
 - c. 0.00010% (w/v).
 - d. 0.00050% (w/v).
 - e. 0.00100% (w/v).
 - f. 0.00200% (w/v).
6. Incubate the cultures overnight at 37°C with shaking.
7. Transfer an aliquot of 1 mL of the O/N cultures to an Eppendorf centrifuge tube and harvest the cells by centrifugation in an Eppendorf centrifuge for 5 min at 8000g and room temperature.
8. Resuspend the pellet in 100 mL H₂O.
9. Add 100 µL 2X SDS-PAGE loading buffer.
10. Incubate the samples in a heating block at 80°C for about 3 min. Mix the solution on a Vortex, and incubate again for another 3 min.
11. Remove cell debris and membranes by centrifugation in an Eppendorf centrifuge for 5 min at 10,000g and room temperature.
12. Transfer the supernatant to a fresh Eppendorf tube, and analyze 5 µL of the sample by electrophoresis on a 15% polyacrylamide gel containing β-mercaptoethanol and SDS. If the supernatant is viscous, sonicate for 30 s at room temperature, and repeat the centrifugation as above (see **Step 11**).
13. Stain the proteins in the gel with Coomassie brilliant blue (see **Note 3**).

A result of such an experiment is shown in **Fig. 1**.

3.2. Large-Scale Synthesis of the Gp31 Co-chaperonin

The bacteria can easily be grown in large conical flasks on an orbital shaking platform, and no special equipment or requirements are needed. This is a simple method, but has always worked well for me.

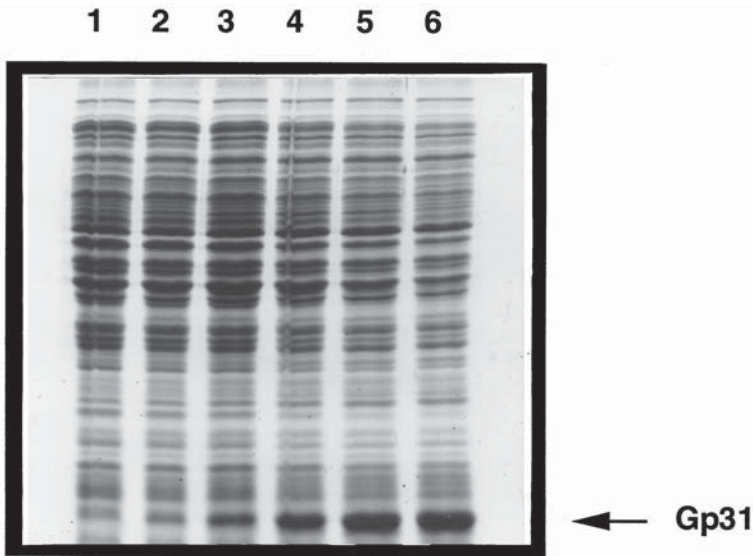


Fig. 1. A 15% polyacrylamide gel with Coomassie-stained proteins from *E. coli* extracts prepared from strain MC1009 containing bacteriophage gene 31 on a plasmid under the control of the *ara* promoter. The arrow indicates the position of Gp31 in the gel. Different concentrations of L-arabinose are shown as follows: 1. 0%; 2. 0.00005%; 3. 0.00010%; 4. 0.00050% ; 5. 0.00100%, and 6. 0.00200%. For further experimental details *see text* under **Subheading 3.1**.

1. Streak *E. coli* strain MC1009 that contains a plasmid carrying the bacteriophage T4 gene 31 under the transcriptional control of the *ara* promoter out on a fresh and dried LB plate (*see Subheading 2., Items 2–4*) that contains the required antibiotics.
2. Incubate the plate at 37°C overnight.
3. Inoculate 10 mL of LB medium (in a 50-mL flask) containing the required antibiotics with an individual colony from the overnight plate, and grow the cells overnight at 37°C. This culture can be started when individual colonies are clearly visible on the overnight plate, generally in the afternoon.
4. The next day, use 5 mL of the overnight culture to inoculate 500 mL of LB medium plus the required antibiotics in a 2-L flask. Grow the cells overnight at 37°C. The same day, sterilize 5-L flasks (6 in total) that contain 2 L of LB medium each as described under **Subheading 2., Item 2**.
5. Determine the OD of the overnight cell culture at 600 nm.
6. Add the required antibiotics plus an aliquot of the overnight culture to each of the 5-L flasks so that the final OD₆₀₀ is about 0.1 (about 60 mL).
7. Place the flasks on an orbital shaking platform and grow the cells at 37°C. Measure the OD₆₀₀ until it is about 0.9 (will take about 4–5 h) (*see Note 2*).

8. Add L-arabinose to each flask to give a final concentration as determined in the small-scale experiment (*see Subheading 3.1.*) and grow the cells overnight at 37°C.
9. Harvest the cells by centrifugation using a Sorvall RC-3B centrifuge with a 6 × 1 L swing-out rotor for 40 min at 3000g and 4°C.
10. Pool the cell pellets and wash them 2x in a glycerol-buffer (*see Subheading 2., Item 5.*)
11. The cells can either be used immediately for purification of Gp31 or can be stored at -80°C.

The yield is normally about 50 g of wet cell paste. Analyze a small amount of the cells by SDS-PAGE as described under **Subheading 3.1.**, and further. The amount of Gp31 should be similar to that observed on the polyacrylamide gel from the small-scale experiment (*see Subheading 3.1.* and **Fig. 1**).

3.3. Purification of the Gp31 Co-chaperonin

This protocol has been modified from the one described by Castillo and Black (**14**) and contains various standardized biochemical techniques. For additional information, refs. (**15,16**) can be consulted. Unless otherwise stated, all handlings are performed at 0°C.

3.3.1. Preparation of the Cell-Free Extract

1. Thaw the frozen *E. coli* cell paste (40–50 g) and suspend in buffer A (*see Subheading 2., Item 6*) to a total volume of about 250 mL (*see Note 4*).
2. Add 10 mg lysozyme, 10 mg DNase, 10 mg RNase and 10 mg PMSF (*see Subheading 2., Item 8*).
3. Bring the cell suspension quickly to 30°C (water bath), and incubate for 15 min with occasional mixing (cells may start to lyse).
4. Transfer the cell suspension to a beaker (precooled on ice), and brake the cell mechanically by passing the cell suspension twice through a French Press at 16,000 psi and 4°C. The color change of the suspension from light brown to clear darker brown indicates that the cells have been broken.
5. Remove the cell debris by centrifugation for 50 min at 20,000g and 4°C.
6. Pour the supernatant carefully into a measuring cylinder, and determine the volume.
7. Transfer the cell-free extract to a clean glass beaker, and keep on ice.

When using the shorter purification method, load the soluble protein extract onto a Q-Sepharose column as described in **Subheading 3.3.4.1**.

3.3.2. Streptomycin Sulfate Precipitation

Streptomycin is an antibiotic that interacts with ribosomes. Addition of a solution of streptomycin sulfate to extracts precipitates ribonuclear proteins and clarifies the extract.

1. Add an aliquot (5% of the volume of the cell-free extract) of the freshly prepared streptomycin sulfate solution (*see Subheading 2., Item 8*) to the cell-free extract. Stir constantly.
2. Leave the solution on ice for 15 min without stirring.
3. Remove the precipitated proteins by centrifugation for 40 min at 20,000g and 4°C.
4. Pour the supernatant carefully into a measuring cylinder, and determine the volume and the protein concentration (Bradford assay kit) using bovine serum albumin (BSA) as a standard.

3.3.3. Ammonium Sulfate Fractionation

1. Transfer the solution to a glass beaker that is placed in an ice-water mixture (0°C). If needed adjust the protein concentration to 14 mg/mL by adding cold buffer A.
2. Add solid ammonium sulphate under constant stirring to a final concentration of 35% saturation (*see Note 5*).
3. When the ammonium sulphate is dissolved (check carefully by lifting the glass beaker and inspecting the bottom), leave the suspension on ice for 30 min without stirring. Determine the pH, and if necessary, adjust with 1 M NaOH or HCl to pH 7.7.
4. Remove the precipitated proteins by centrifugation for 40 min at 20,000g and 4°C.
5. Transfer the supernatant to a precooled beaker (on ice). Add ammonium sulfate under constant stirring to a final concentration of 70% saturation (*see Note 5*). Determine the pH, and if necessary, adjust with 1 M NaOH or HCl to pH 7.7.
6. When the ammonium sulfate is dissolved, leave the suspension on ice for 30 min without stirring. *See* remark under **step 3**.
7. Harvest the precipitated proteins by centrifugation for 40 min at 18,000g and 4°C.
8. Remove the supernatant and suspend the pellet in buffer B (*see Subheading 2., item 8*).
9. Dialyze the protein solution against buffer B overnight at 4°C. Change the buffer at least 1x (*see Note 6*).

3.3.4. Column Chromatography

The Gp31 purification method described here is rather long, but will produce very (>99.9%) pure protein that we have used successfully for crystallization (*II*). However, when the cell-free extract is loaded directly onto a Q-Sepharose column, thus omitting **Subheadings 3.3.1.–3.3.3.**, good quality Gp31 will also be obtained. If not available, prepare the columns the day before usage to allow sufficient time for equilibration. A combination of different matrices is utilized for adsorption and release of proteins in order to separate Gp31 from the rest of the *E. coli* proteins. All handlings are performed at 4°C unless otherwise indicated.

3.3.4.1. Q-SEPHAROSE CHROMATOGRAPHY

Q-Sepharose is a strong anion-exchange adsorbent that exploits the different net charges on proteins at a given pH and interacts with the proteins mainly through electrostatic interaction.

1. Clear the dialyzed protein solution by centrifugation for 10 min at 10,000g and 4°C.
2. Load the supernatant on a Q-Sepharose column (height = 20 cm and diameter = 2.6 cm; column volume is approx. 100 mL), which has been equilibrated in buffer B. Use a peristaltic pump that gives a flow rate of about 1.0 mL/min.
3. Wash column with 3 column volumes of buffer B, until the OD₂₈₀ is zero.
4. Elute proteins with a gradient of 0.2–0.55 M NaCl in buffer B (**Subheading 2., items 10 and 11**). The total volume of the gradient should be 8–10 times the volume of the column. The exact volume depends of course on the exact size of the column, but the total volume of the gradient is generally between 800 and 1000 mL.
5. Collect fractions of 10 min (about 10 mL) each. The elution takes about 15 h, and it is thus recommended to do this during the night.
6. Mix the protein solution in each tube gently using a Vortex. If the tubes are too full and good mixing is impossible, cover the tubes with parafilm, and mix gently by head-over-head turning.
7. Analyze 2.5 µL of each fraction on a 15% SDS polyacrylamide gel to determine which fractions contain Gp31 (molecular mass is about 12 kDa). Stain the proteins in the gel with Coomassie brilliant blue (*see Note 3*). Gp31 elutes from the column at around 0.35 M NaCl.
8. Pool the fractions that contain Gp31, and dialyze the protein sample against buffer C (**Subheading 2., item 12**) overnight at 4°C. Change the buffer at least 1X (*see Note 6*).

3.3.4.2. HYDROXYAPATITE CHROMATOGRAPHY

Hydroxyapatite is the name for the crystalline form of calcium hydroxyphosphate. Unlike the ion-exchange matrices (like Q-Sepharose), it does not have a readily explainable mode of action, although it is generally thought that both electrostatic and polar interactions are important for adsorption to the “calcium phosphate gel.”

1. Clear the dialyzed protein by centrifugation for 10 min at 10,000g and 4°C.
2. Load the supernatant onto a Hydroxyapatite (HA) column (about 100 mL, height = 20 cm and diameter = 2.6 cm), which has been equilibrated with buffer C using a peristaltic pump that provides a flow rate of about 1.0 mL/min.
3. Wash the column with 3 column volumes of buffer C, until the OD₂₈₀ is zero.
4. Elute the proteins during the night using a gradient of 0.02–0.3 M Na-phosphate, pH 6.8, with a total volume of approx. 800 mL (*see comments under Subheading 3.3.4.1., step 4*). Collect fractions at about 11 min (about 11 mL) each.

5. Mix the protein solution in each tube gently using a Vortex, and analyze 2.5 μL of each fraction on a 15% SDS polyacrylamide gel. Stain the proteins with Coomassie brilliant blue (*see Note 3*). Gp31 elutes from the column with a peak around 0.18 *M* Na-phosphate.
6. Pool the Gp31-containing fractions, and precipitate the protein(s) with ammonium sulfate (70% saturation as described under **Subheading 3.3.3, step 5**). Resuspend the pellet in 20 *mM* Na-phosphate, pH 7.4., and dialyze against buffer D (*see Subheading 2., item 13*) overnight at 4°C. Change the buffer at least 1X (*see Note 6*).

3.3.4.3. PHENYL SEPHAROSE CHROMATOGRAPHY

This type of matrix allows separation based on the hydrophobicity and hydrophilicity of proteins, and is often used toward the end of a purification procedure.

1. Clear the dialyzed protein by centrifugation for 10 min at 10,000*g* and 4°C.
2. Load the supernatant onto a phenyl Sepharose column (volume = about 20 mL, height = 12 cm, diameter = 1.5 cm) that has been equilibrated in buffer D using a peristaltic pump at about 1.0 mL/min.
3. Wash column with 3 column volumes of buffer D, until the OD₂₈₀ is zero.
4. Elute the proteins with a gradient of 30–0% ammonium sulfate-saturated solution in buffer D, and collect fractions at about 3 min (about 3 mL) each. Total volume of the gradient is generally about 250 mL (*see comments under Subheading 3.3.4.1., step 4*).
5. Mix the protein in each the tube using a Vortex, and analyze 2.5 μL of the fractions on a 15% SDS polyacrylamide gel. Stain the proteins with Coomassie brilliant blue (*see Note 3*).
6. Pool the fractions that contain pure Gp31, precipitate the protein with ammonium sulfate (70% saturation as described under **Subheading 3.3.3., step 5**). The suspension can be stored at 4°C. Alternatively, dialyze the protein solution against the storage buffer (**Subheading 2., item 4**), freeze aliquots in liquid nitrogen and store the purified protein at –80°C.

The purified Gp31 protein is stable for more than 1 yr under either condition. The yield is generally between 35 and 50 mg of highly pure protein, but can vary quite a bit, since the starting material does not always have the same high amount of Gp31. In addition, after each column chromatography step, a selected number of fractions containing Gp31 are chosen. The selection is of course a personal choice and thus a determining factor for the final yield. However, the author has found this purification procedure to be very reproducible, and once established in a lab, a high protein yield and purity can be obtained.

4. Notes

1. When measuring the OD₆₀₀ of overnight cell cultures, it is important to remember that most spectrophotometers do not show a linear response at high optical densities. Typically overnight cultures are diluted 10- 20-fold into LB medium before measuring the OD.
2. Remember that the bacteria are growing exponential and that the OD₆₀₀ will change rapidly. Therefore, check the OD frequently in order to make sure the OD of the culture does not go much beyond 0.8.
3. Stain the gel in a dilute solution of Coomassie brilliant blue during the night. The long time will allow small amounts of protein to stain to saturation without the polyacrylamide gel becoming dark blue. Destain the gel the next day in destain solution. Change the destain solution every 20–30 min until the gel is white (normally three to four times). When the gel is white and the proteins are visible, do not leave the gel in the destain solution, because small amounts of protein may otherwise lose their color. Dry the gel either on Whatmann 3MM paper or in between cellophane. This way, a high contrast is obtained allowing the visualization of small amounts of protein (impurities) in the presence of a large amount of almost pure protein. The author always uses this staining method, since it gives clear results and a high-quality protein profile.
4. It is convenient to remove the cell paste from the –80°C freezer and to leave it at 4°C the night before the purification. It saves time, and the author has not observed any proteolytic degradation when pre-thawing the cells.
5. If the ammonium sulfate is in big chunks, grind it in a mortar with the use of pestle. Fine ammonium sulfate dissolves rapidly, thus avoiding local high-salt concentrations, which can lead to unwanted local denaturation of proteins.
6. Complete removal of the endogenous salt from a sample will not occur in one dialysis, because at equilibrium, what was originally in the dialysis bag is now distributed throughout the buffer and the dialysis bag. If the volume of the buffer is 50 times that of the bag, then at best, a 51-fold dilution can be achieved. To speed up the dialysis process, stirring of the buffer and movement of the dialysis bag should occur. A well-mixed system can reach more than 90% equilibrium within 2–3 h. This information is sometimes provided by the manufacturer.

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Removing Trace Fluorescent Contaminants from GroEL Preparations

Frank Weber

1. Introduction

Many applications require GroEL in a highly purified form (e.g., *see Chapter 10*). Since the GroEL sequence does not contain any tryptophan, the detection of tryptophan fluorescence in a preparation of GroEL can be used as a measurement of purity. Following the standard purification procedure as described in Chapter 3, the purified GroEL may show significant tryptophan fluorescence, which has been suggested to be owing to peptides bound to GroEL. An accurate spectroscopic quantification of the GroEL concentration or applications of GroEL in fluorescence measurements require further purification in order to remove traces of tryptophan fluorescence.

Several methods have been used for this purpose. GroEL can be disassembled into monomers (*see Chapter 7*) and further purified under denaturing conditions (1–3). The purified monomers are reassembled to the GroEL tetradecamer (*see Chapter 7*). Another procedure involves the treatment of GroEL with 20% methanol and gel filtration (4). The protocol described here (5,6) allows the removal of tryptophan containing impurities under native conditions on a reactive red 120-agarose dye column.

2. Materials

1. Buffer A: 20 mM MOPS-NaOH, pH 7.2, 10 mM Mg-acetate.
2. 50 mL Reactive red 120 agarose (Sigma, St. Louis, MO, Type 3000-CL, ca. no.R0503).
3. 1 mL GroEL at a concentration of 10 mg/mL in buffer A (*see Note 1*).

3. Method

1. Fill an Econo column (Bio-Rad, Hercules, CA; 0.5 cm diameter, 30 cm high) with reactive red 120 agarose resin. Carry out the following procedure at 4°C.
2. Equilibrate the column with 10 column volumes of buffer A by means of a peristaltic pump at a flow rate of 0.5 mL/min.
3. Apply 1 mL of 10 mg/mL GroEL in buffer A onto the column, and let the sample enter the gel bed by gravity. Add 1 mL of buffer A, and let enter the gel bed by gravity.
4. Lock the column, and incubate for 15 min.
5. Elute GroEL in buffer A by means of a peristaltic pump at a flow rate of 0.5 mL/min, and collect 0.5 mL fractions.
6. Check aliquots of the fractions for GroEL on a 12% SDS-PAGE and for tryptophan fluorescence in a fluorometer using an excitation wavelength of 295 nm (monitor from 310 to 400 nm) (*see Note 2*).
7. Combine tryptophan fluorescence free fractions (*see Note 3*).

4. Notes

1. Purified GroEL can be transferred to buffer A by gel filtration, e.g., on a NAP-10 column (Pharmacia).
2. The first GroEL-containing fractions eluted from the column do not contain detectable tryptophan fluorescence. Later fractions may contain increasing amounts of impurities.
3. Tryptophan fluorescence clean preparations of GroEL may still contain impurities without tryptophans.

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Assembly and Disassembly of GroEL and GroES Complexes

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1. Introduction

The assembly of chaperonin subunits into heptameric ring structures is a hallmark common to this group of proteins. In GroEL two of these rings are symmetrically stacked, resulting in a tetradecameric double-ring cylinder which is the functional form of the chaperonin. In this structure, unfolded substrate proteins bind to the apical subunit domains at the inner surface of the cylinder, whereas adenosine 5'-triphosphate (ATP) binding and hydrolysis occur at the level of the central subunit domains (1). GroES subunits assemble into a single heptameric ring, which can bind asymmetrically on top of a GroEL ring (2). Interestingly, the intracellular assembly of GroEL-type chaperonins appears to be a chaperone-dependent process itself and requires functional pre-formed chaperonin complexes (3). The intracellular folding pathway of GroES has not been analyzed, but in vitro the complex is able to assemble spontaneously from monomeric subunits (4,5). Cell-free assembly of GroEL in a Mg-ATP-dependent manner, starting from monomeric subunits, has been established, too (6,7). Like with GroES, in vitro assembly of GroEL is a reversible process, and depending on the conditions, dissociation of complexes can occur. The formation of these oligomeric structures poses thus an interesting folding problem per se. However, in addition to providing an interesting model system for folding and assembly studies, controlled (dis)assembly of chaperonins has also been found to be helpful in generating highly purified chaperonin complexes (8). A common problem during purification of GroEL is its contamination with bound substrate proteins, which are difficult to remove by conventional purification steps (see Chapter 3). Disassembly of GroEL into folded monomers and subsequent reassembly can result in functional chaperonin complexes,

which are largely devoid of bound substrates. In a different application, site-directed mutagenesis studies of GroEL, the readiness with which the complex dissociates and reassembles, has been assayed to obtain information about the role of certain amino acid residues in the stability of the complex and in its oligomerization (9,10).

As described below, the best-characterized method to dissociate and reassemble chaperonin complexes reversibly involves treatment with the chaotropic agent urea. **Subheading 3.1.** provides a procedure for reversible unfolding and refolding of GroES heptamers; **Subheading 3.2.** is a protocol for dissociation and reassembly of GroEL tetradecamers. Several assays are available to analyze the assembly state of GroES and GroEL. They are mostly based on structural features that are different for monomeric and oligomeric complexes, including changes in tyrosine fluorescence (neither GroES nor GroEL from *Escherichia coli* contains tryptophan residues), fluorescence anisotropy, gel-sizing chromatography, or sedimentation velocity analysis (4–7). In this chapter, the assay described for GroES is based on differential elution of heptamers and monomers on a gel-sizing column. For GroEL, the assay is migration of oligomeric and monomeric forms as distinct bands on native polyacrylamide gels. Protocols for functional assays, which can be used to test whether the reassembled complexes have chaperonin activity, can be found in other chapters of this volume (Chapters 11 and 12).

2. Materials

2.1. Unfolding and Refolding of GroES

1. Buffer A: 50 mM Tris-HCl, pH 7.5, 100 mM NaCl.
2. GroES protein, purified as described in Chapter 4, and stored in aliquots at -80°C .
3. 8 M urea solution in 50 mM Tris, pH 7.5 (see **Note 1**).
4. Dialysis cassettes (Pierce, Rockford, IL), ready-to-use, with a capacity of 0.1–0.5 mL and a 3500-Da mol-wt cutoff (see **Note 2**).
5. Gel-sizing column Superdex 75 HR 10/30 (Pharmacia, Uppsala, Sweden) (see **Note 3**), and FPLC apparatus with fraction collector, UV-monitor (280 nm), and recorder.
6. Gel-filtration low-mol-wt calibration set (Pharmacia), consisting of ribonuclease A (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), and blue dextran (~2 MDa).
7. 5X stock solution of SDS sample buffer: 312.5 mM Tris-HCl, pH 6.8, 50% (w/v) glycerol, 25% (v/v) β -mercaptoethanol, 10% (w/v) sodium dodecyl sulfate (SDS), 0.08% (w/v) bromophenol blue.

2.2. Dissociation and Reassembly of GroEL Complexes

1. Buffer A: 50 mM Tris-HCl, pH 7.5, 80 mM KCl, 1 mM dithiothreitol (DTT), 0.1 mM ethylene diamine tetraacetic acid (EDTA).

2. Disodium ATP: A 100 mM solution in buffer A without DTT, stored in aliquots at -20°C (see **Note 4**).
3. Magnesium chloride stock solution (1 M) in distilled water.
4. GroEL protein, purified as described in Chapter 3, stored in aliquots at -80°C .
5. 8 M urea solution in buffer A (see **Note 1**).
6. Gel-sizing desalting columns Sephadex G-50, 0.9×2.0 cm (NICK columns, Pharmacia).
7. 5X stock solution of sample buffer for native gel: 50% (w/v) glycerol, 0.08% (w/v) bromophenol blue.

3. Methods

3.1 Unfolding and Refolding of GroES

1. Mix GroES with the 8 M urea solution, so that the final protein concentration is 2 mg/mL (200- μM monomeric subunits; see **Note 5**) and the final urea concentration is 6 M. Set up two samples, each with a volume of 200 μL . Incubate both samples for 2 h at 25°C .
2. To initiate refolding, inject one of the unfolded GroES samples into the small dialysis cassette, and dialyze it at room temperature against a 200-fold volume of buffer A for a total of 4 h. Stir the reaction, and exchange the buffer solution once after 2 h. Keep the undialyzed sample in the meantime at room temperature.
3. Equilibrate the Superdex 75 column with 3 column volumes (72 mL) of buffer A, and calibrate it with a 200- μL mixture of the size-standards. Start elution with a flow-rate of 0.75 mL/min using a Pharmacia FPLC apparatus, and monitor the elution progress with a UV recorder at 280 nm. Total elution volume is 20 mL.
4. After washing the column with 1 vol of buffer A, apply the dialyzed GroES sample in a volume of 200–250 μL , run the column under identical conditions as described above, and collect 1-mL fractions. There should be a major absorbance peak at an elution volume corresponding to an ~ 75 -kDa protein. In a second run, load the undialyzed GroES sample (**step 1**, corresponding to monomeric GroES) after mixing with 100 μL buffer A, and elute as described above (see **Note 6**).
5. Load 20- μL aliquots of the fractions onto suitable SDS-polyacrylamide gels for electrophoresis (see **Note 7** and **Fig. 1**), and stain the gels after the run.

3.2 Dissociation and Reassembly of GroEL Complexes

1. Prepare three samples of GroEL (4.8 mg/mL, 80 μM monomeric subunits) mixed with 8 M urea and buffer A, so that the final concentrations of the chaotropic agent are 3, 3.5, and 4 M, respectively. The volume of each sample should be 100 μL or less.
2. Incubate the samples for 30 min at room temperature (see **Note 8**).
3. Equilibrate the NICK desalting columns with 3 mL buffer A. Apply the samples onto the columns to remove the urea. Add 400 μL buffer A, and let it enter into the gel bed. Then place a test tube under the column, elute the monomeric GroEL subunits with 400 μL buffer A, and collect the samples.

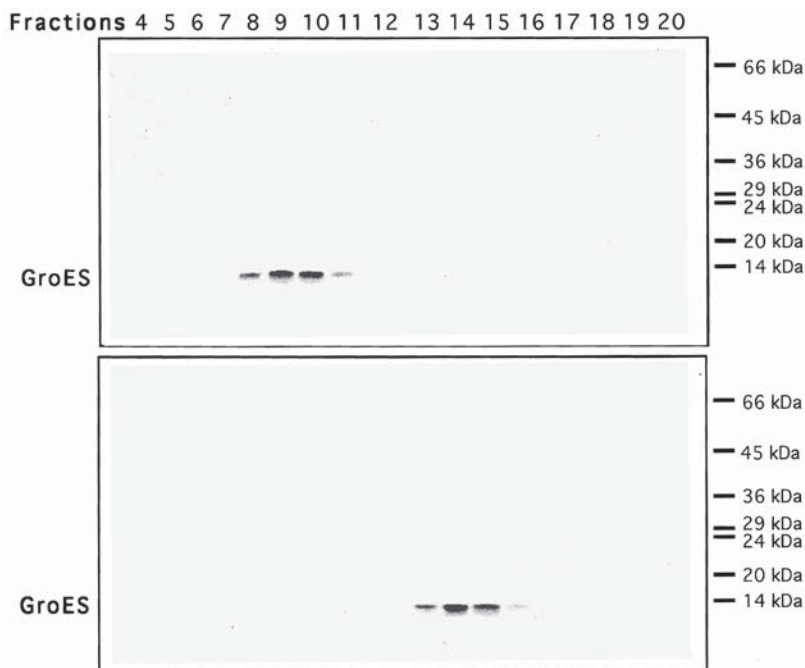


Fig. 1. Assembled (upper panel) and monomeric (lower panel) GroES. Superdex 75 fractions were analyzed by SDS-PAGE. After electrophoresis, gels were stained with Coomassie blue.

4. Take 50 μL from each sample, add Mg-chloride (5 mM) and ATP (2 mM), and incubate for 90 min at room temperature to allow reassembly to occur (*see Note 9*).
5. Add sample buffer, and load aliquots (5–15 μL) of the samples on 6% native polyacrylamide gels (*see Note 10*). Also load aliquots of the samples that did not receive Mg-ATP and an aliquot of native GroEL that was not treated with urea. Stain gels after the run with Coomassie blue.

4. Notes

1. The urea solution should be prepared freshly, as during long-term storage, it may decompose to form cyanate, which reacts with amino and thiol groups. On incubation with proteins, this could lead to carbamylated species, which are inactive and incompetent for reassembly.
2. For dialysis of GroES, the molecular weight cutoff has to be below 10 kDa, in order to retain the monomeric GroES subunits. For dialysis of GroEL, cutoffs below 50 kDa are required. Instead of dialysis cassettes, which are convenient for small samples, also regular dialysis tubing can be used.
3. Any gel-sizing column that gives resolution of proteins in the mol-wt range 5–100 kDa can be used in place of the Superdex 75 column. To obtain good separation between

monomeric and heptameric GroES, flow rates, fraction sizes, and elution volumes have to be adjusted depending on the respective column dimension and resin.

4. The ATP stock solution must be buffered at a neutral pH to prevent spontaneous hydrolysis to adenosine diphosphate.
5. For reassembly studies, the GroES monomer concentration should be at least 1 μM , since at lower concentrations, increasing amounts of the monomeric form are observed under equilibrium conditions, even in the absence of urea.
6. Even though during the column run in buffer A refolding conditions are established, the kinetics of GroES reassembly are so slow that most of the protein stays monomeric during the rapid column run (26 min) and elutes at the corresponding position (11 kDa).
7. In order to visualize the GroES band at 11 kDa, high-percentage SDS polyacrylamide gels have to be used, e.g., 12.5% acrylamide/1% bis-acrylamide Bio-Rad minigels. An example is shown in **Fig. 1**. Analysis of fractions by SDS-polyacrylamide gel electrophoresis (PAGE) is optional, as the elution position on the calibrated gel-sizing column already gives the required information about the assembly status of GroES.
8. The efficiency of dissociation is affected by a number of parameters. Lower temperature (4°C) or binding of nucleotides (Mg-ATP) to GroEL destabilize the complex. On the other hand, GroES, bound substrate proteins, or magnesium ions in the absence of nucleotides tend to stabilize the tetradecamer. Therefore, the actual urea concentration at which disassembly occurs depends on the incubation conditions and on the purity of the chaperonin preparation. In this context, it is noted that the GroEL protein used for incubation with urea should not contain glycerol as a stabilizer. In the above-described protocol, three different urea concentrations are tested. To generate folded monomeric GroEL subunits that are capable for reassembly, the lowest urea concentration that initially leads to disassembly should be used, since complete denaturation of GroEL subunits and loss of secondary structure, which could be caused by higher urea concentrations, make the reassembly process less efficient.
9. Up to 90% efficiency for reassembly can be achieved after prolonged incubations at room temperature. As for disassembly, several parameters are critical for a good yield. Mg-ATP is essential for reassembly, probably because binding or subsequent hydrolysis of the nucleotide induces conformational changes in the GroEL subunits, converting them into an assembly-competent conformation. ADP or nonhydrolyzable ATP analogs are far less efficient in that respect. Thus, ATP at the same time destabilizes GroEL complexes and promotes their formation. GroES/Mg-ATP (but not GroES/Mg-ADP), or unfolded substrate proteins are also positive effectors for reassembly. However, the two most critical parameters are temperature and concentration of the monomers. The diluted monomeric GroEL samples, obtained after elution of the urea-denatured GroEL from the desalting columns, are at a concentration of 5–20 μM , which is the optimal range. Reassembly takes only place above 4°C, most likely because the ATP-induced conformational changes cannot occur at the low temperature.

10. Various native gel systems can be used, including fixed percentage gels and 4–20% gradient gels. One possible system that is easy to set up consists of a 6% acrylamide/0.6% bis-acrylamide gel, prepared analogously to SDS-polyacrylamide gels, but without SDS. Likewise, the running buffer does not contain SDS. Assembled GroEL does not enter far into low-percentage native gels and stays close to the top. Monomeric GroEL migrates farther and may appear as a somewhat fuzzy band.

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GroEL/GroES Interaction Assayed by Protease Protection

Jörg Martin

1. Introduction

The GroEL double-ring cylinder is a compact structure that in the presence of nucleotides is resistant to proteolysis by proteinase K. Only the last 16 carboxy-terminal residues of each subunit are cleaved (**1,2**), as determined by mass spectrometry. These residues are part of a Gly-Met-rich tail, which is buried inside the cylindrical chaperonin cavity at the level of the equatorial subunit domains (**3**). The truncated GroEL (EL Δ C), in which these 16 residues are missing, forms an intact oligomer, can bind nucleotides, GroES, and unfolded proteins, and is functional as a chaperonin in mediating protein refolding. A notable difference, however, is its reduced ATPase activity, which is only 25% that of wild-type GroEL (**1**). Two properties of the GroEL oligomer permit proteinase K to attack the carboxy-termini of the chaperonin subunits. First, owing to their flexibility, the tails can reach out of the cylinder cavity, as shown by the fact that a carboxy-terminally His-tagged GroEL is able to bind to Ni-NTA affinity columns (**4**). Second, proteinase K (29 kDa) is small enough to enter the cavity and to exert its proteolytic activity there.

This is not possible when GroES binds on top of the GroEL cylinder. The chaperonin cofactor effectively seals off the cavity of the GroEL ring with which it is associated. As a consequence, proteinase K cannot gain access to the tails of the seven GroEL subunits in this ring. The opposite GroEL ring, however, which has no bound GroES, is still susceptible to proteolysis, and the tails of these seven subunits can be cleaved (*see Note 1*). Thus, proteolysis of GroEL in the presence of GroES results in two bands of equal intensity after analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-

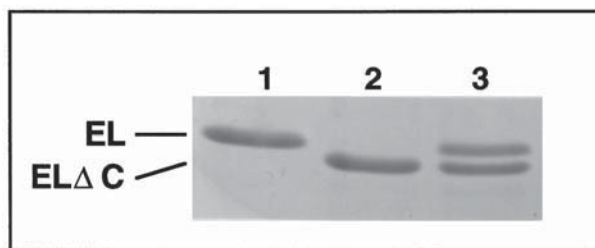


Fig. 1. Protease protection of GroEL carboxy-termini by GroES. Shown in lane 1 is a GroEL band at 58 kDa on a Coomassie blue-stained SDS-polyacrylamide minigel (7.5% acrylamide/0.2% bis-acrylamide). The band at 56 kDa in lane 2 corresponds to truncated GroEL subunits (EL Δ C), generated by proteinase K treatment of the chaperonin. Lane 3: Binding of GroES on top of GroEL protects the adjacent chaperonin ring from attack by the protease (upper band). Therefore, only the last 16 residues from the 7 subunits in the opposite ring (lower band, EL Δ C) are removed.

PAGE) (**Fig. 1**, lane 3). The upper band represents the intact subunits of the GroEL ring which is protected by bound GroES, and the lower band represents the cleaved subunits of the opposite ring.

In the past, the protease protection assay has been used in its basic form to demonstrate that GroEL and GroES interact in equimolar ratios to form asymmetric complexes (**1**), and to probe for effects of chaperonin substrates on the dissociation rates of nucleotides and GroES from GroEL during the chaperonin reaction cycle. In a more sophisticated approach, the assay was integrated in experiments that investigated whether GroES could (re)bind to the same GroEL toroid from which release occurred during the preceding round of Adenosine 5'-triphosphate (ATP) hydrolysis in a reaction cycle, or whether it had to (re)bind to the opposite toroid (**2**). Furthermore, it was used to demonstrate that simultaneous binding of a second GroES is not required for a functional cycle (**4**). Other applications may include the analysis of binding parameters of GroEL/GroES interactions, the influence of chaperonin substrate proteins on the stability of GroEL/GroES complexes, and testing for functionality of purified GroES and GroEL oligomers.

2. Materials

1. Buffer A: 25 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7.2, 50 mM KCl, 5 mM MgCl₂.
2. Adenosine 5'-diphosphate (ADP): A 100-mM solution in buffer A, stored in aliquots at -20°C.
3. Proteinase K (1 mg/mL) in water, stored in aliquots at -20°C.
4. Phenylmethylsulfonyl fluoride (PMSF): A 100-mM solution in ethanol, stored at -20°C (*see Note 2*).

5. GroEL and GroES proteins (*see also Note 3*), purified as described in Chapters 3 and 4, stored in aliquots at -80°C .
6. 5X stock solution of SDS sample buffer: 312.5 mM Tris-HCl, pH 6.8, 50% (w/v) glycerol, 25% (v/v) β -mercaptoethanol, 10% (w/v) SDS, 0.08% (w/v) bromophenol blue.

3. Method

1. Prepare three samples of 0.12 μM GroEL in buffer A plus 1 mM ADP (*see Note 4*) in a volume of 20 μL . Add 0.6 μM GroES to sample 3 (*see Note 5*), and incubate all three samples for 15 min at 25°C .
2. Add proteinase K (final concentration 10 $\mu\text{g}/\text{mL}$) to samples 2 and 3, and incubate all three samples for 10 min at 25°C .
3. To inhibit the protease, add 1 mM PMSF to all samples, and incubate for 5 min at 25°C .
4. To prepare samples for SDS-PAGE, add 5 μL 5X sample buffer, and heat them for 3 min at 95°C .
5. Load the samples onto a suitable SDS-polyacrylamide gel for electrophoresis (*see Note 6*), and stain the gel after the run with Coomassie blue. The band pattern should look like that shown in **Fig. 1**.

4. Notes

1. In the presence of elevated magnesium concentrations (10 mM or higher), symmetric GroEL/GroES complexes can be formed, because negative cooperativity between the two GroEL rings is reduced. These complexes are not very stable, and it is not possible to obtain full protection of the GroEL carboxy-termini after treatment with proteinase K.
2. PMSF, a toxic serine protease inhibitor that binds covalently to proteinase K, is unstable in aqueous solutions.
3. Although the assay has been originally established with the *Escherichia coli* proteins GroEL and GroES, it may be applied also to other members of the chaperonin family, since their primary and tertiary structures have been highly conserved in evolution, including in many cases, the carboxy-terminal flexible tails.
4. Lower concentrations of ADP, as little as 80 μM , are sufficient to generate stable GroEL/GroES complexes. The assay works also with other nucleotides than ADP, such as ATP or nonhydrolyzable ATP analogs. However, ADP is preferable, since in the presence of ATP or analogs, GroES is not as tightly bound as with ADP, which may result in weaker protection. Similarly, GroEL/GroES complexes formed in the presence of MgADP are more stable at room temperature than they are at 4°C .
5. If an excess of GroES over GroEL is used, also the free chaperonin cofactor will be cleaved by proteinase K in its mobile loop region, resulting in 7-kDa and 3-kDa fragments. GroEL-bound GroES is protected from proteolytic attack.

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Determination of Chaperonin Activity In Vivo

Saskia M. van der Vies and Pete A. Lund

1. Introduction

During the last 10 years, much effort has been made to unravel the molecular mechanism by which the chaperonins, in particular GroEL and GroES proteins from *Escherichia coli* (*E. coli*), facilitate the correct folding of a variety of unrelated polypeptides. Most of these studies have been carried out using purified proteins for in vitro analysis. Although we have learned a great deal about the remarkable manner by which the *E. coli* chaperonin machine facilitates polypeptide folding (reviewed in refs. **1–3**), ultimately it is the activity of the chaperonins in their biological context (inside the cell) that is of the most interest. Despite the difficulty of studying these proteins in their natural environment, there are a couple of reports that address this question (**4,5**). In this chapter, we describe assays that can be used to determine whether or not different chaperonin proteins (either mutant forms of GroEL and GroES or “putative” chaperonins from other organisms) are able to function in *E. coli*. The advantage of using *E. coli* for such studies is:

1. Strains of *E. coli* carrying different mutations in chaperonin genes are available.
2. *E. coli* physiology is well understood.
3. *E. coli* is an easy organism to handle in the laboratory, including for molecular and genetic manipulations.

1.1. History of the *E. coli* Chaperonins

The chaperonin class of proteins was discovered during studies on the assembly of the photosynthetic CO₂-fixing enzyme ribulose-1.5-bis-phosphate carboxylase-oxygenase (rubisco) (**6,7**). This hexadecameric enzyme is composed of an equal amount of chloroplast-encoded large subunits and nuclear-encoded small subunits, and its assembly was proposed to require the activity

of another chloroplast protein called the rubisco subunit binding protein (RSBP) (8). Sequence comparisons of the RSBP revealed an amino acid similarity of 50% to two bacterial proteins of unknown function. One, an unpublished gene sequence from *E. coli* (*groEL*, kindly provided by R. W. Hendrix), had been identified in studies of mutants of *E. coli* that were unable to support the growth of bacteriophages λ and T4, and was interpreted as a host factor required for the assembly of the bacteriophage head structure (reviewed in 9). In a detailed analysis Kochan and Murialdo (10) subsequently showed the *groEL* gene product (GroEL) is required for the correct assembly of the λ head-tail connector, a donut-like dodecamer structure of 12 identical λ B polypeptides onto which the prohead is assembled (reviewed in 11). The observed high degree of amino acid sequence similarity between the RSBP and GroEL, and the implied similar function led to the suggestion that these two proteins comprise one distinct class of the larger family of molecular chaperones, since their function met the criteria suggested for molecular chaperones (12 and reviewed in 13,14)

In the original studies aimed at the identification of *E. coli* host factors that were essential for the growth of bacteriophage λ and T4, three classes of mutants were isolated. The *groN* and *groP* class (where *gro* stands for growth) represent mutations in host proteins that are involved in the transcription and replication of the bacteriophage genome. The third class, called *groE*, represents a class of mutants that prevents the formation of bacteriophage particles, but allows normal injection and replication of DNA, gene expression, and cell lysis (15,16). The *groE* locus was shown to be part of the *E. coli* heat-shock operon and is composed of two genes called *groES* (S for small) and *groEL* (L for large). Mutations in either the *groEL* or the *groES* gene exhibit similar phenotypes with respect to λ head morphogenesis (17). Tilly and Georgopoulos showed subsequently that mutations in *groES* could be suppressed by mutations in *groEL*, suggesting that the two chaperonin proteins interact functionally in vivo (18).

Some *groEL* mutants were also unable to support the growth of the unrelated bacteriophage, T4. As for bacteriophage λ , the absence of a functional GroEL protein results in the formation of abnormal head structures (reviewed in 19). Unlike any other bacteriophage known, T4 possesses a phage-encoded chaperonin protein, Gp31, that is functionally related to GroES, but with unique properties that are absolutely required for the correct folding of Gp23, the major capsid protein of bacteriophage T4 (20–22 and references in chapter 5 “Gp31”).

Some of the *groE* mutants displayed an additional phenotype, i.e., they had lost the ability to grow at elevated temperatures. *E. coli*, like all organisms, has evolved to live in a particular temperature niche (from approx. 15 to 42°C),

and the overall structure and stability of the cellular proteins are maintained within this range. However, organisms, such as *E. coli*, have no means of regulating their body temperature, which thus changes according to the external temperature. Elevated temperatures and other forms of cellular stress can cause protein denaturation of both newly-synthesized as well as pre-existing proteins, which results in aggregation into non-functional conformations (reviewed in **23**). Moreover, under normal growth conditions, newly-synthesized polypeptides still have the tendency to form nonfunctional conformations, a result of their interaction with other cellular proteins. Chaperonins help minimize protein aggregation both at normal and elevated temperatures, and their synthesis is induced upon increased temperature (**6,13,14,24** and references therein). The chaperonins are required for bacterial growth at all temperatures (**25**).

The ability of a strain carrying a mutant chaperonin gene to survive at high temperature depends on the severity of the defect in the mutated protein. For example, a strain expressing the GroEL (E191G) and GroES (G23D) proteins is temperature-sensitive and fails to grow at 42°C, whereas the GroEL (A383T) mutant grows normally at 42°C, but does not support the growth of bacteriophage λ (reviewed in **26**). This in turn implies that either the formation of the λ head structure requires more chaperonin activity than the prevention of protein aggregation at 42°C, or that a specific interaction is required for λ head assembly, but not for growth at elevated temperature.

This chapter describes methods for determining whether a given chaperonin protein is able to function in vivo in *E. coli*. The methods rely on the same basic premise, i.e., the *E. coli* chaperonins, GroEL and GroES, are essential for growth of the *E. coli* cell at all temperatures and for the growth of certain bacteriophages. The ability of mutated or heterologous chaperonin proteins to support the growth of *E. coli* or/and to allow bacteriophage growth in the absence of normal chaperonin function is a measure of their in vivo activity. Because of the essential nature of the chaperonin proteins, chaperonin activity can only be tested in so-called conditional mutant chaperonin strains or in a strain where it is possible to remove or inactivate the chromosomal chaperonin gene after a potential complementing protein has been expressed from a plasmid.

For the purpose of the following protocols, we assume that the gene for the relevant chaperonin mutant or homolog is already present on a plasmid under the control of an inducible promoter and that the protein can be expressed in an *E. coli* wild-type strain, e.g., TG1 or B178. This should be determined through analysis of *E. coli* protein cell extracts by electrophoresis on polyacrylamide gels (for example, see Chapter 5, **Subheading 3.1.** and **Fig. 1**), immunoblotting and ideally by confirming the N-terminal sequence of the expressed protein. Some discussion about which plasmids and promoters can be used and considerations for successful expression of the protein is to be found in **Note 1**).

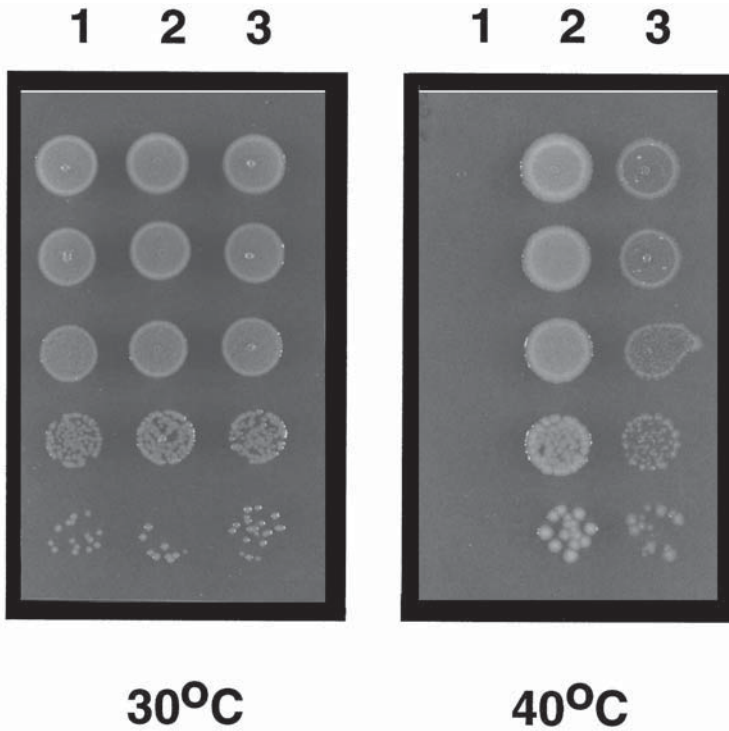


Fig. 1. Complementation of the GroES (G24D) mutant strain by the Gp31 protein from bacteriophage T4. Aliquots of 10-fold serial dilution on LB agar plates are shown. The mutant strain transformed with the vector (1), a plasmid containing the *groES* gene (2), and a plasmid containing the bacteriophage T4 gene 31 (3). Temperatures at which the plates have been incubated are indicated.

2. Materials

1. Strains: To test chaperonin mutants and homolog proteins, we routinely use strains that have been derived from the widely used *E. coli* hosts TG1 and B178 by P1 transduction of the mutant chaperonin alleles as follows:

Strain	Transduced allele	Recipient host	Mutation	Growth at elevated temperature	Reference for allele
SF103*	<i>groEL44</i>	TG1	E191G	No	(27)
SV1	Wild type	B178	No mutation	Yes	
SV5	<i>groEL515</i>	B178	A383T	Yes	(27)
SV7	<i>groES42</i>	B178	G23D	No	(28)
SV8	<i>groES619</i>	B178	G24D	No	(28)

All strains are resistant to tetracycline. SV1, SV2, SV7, and SV8 are isogenic strains. *E. coli* strain B and B178 (or SV1) are recommended for the growth of bacteriophages T4D0 and λ (b2cI), respectively. *E. coli* strain AI90/pBAD50 is the host strain for growing bacteriophage P1 and grows only in the presence of in 0.2% D-arabinose (29). We routinely grow this strain also in the presence of 50 $\mu\text{g}/\text{mL}$ kanamycin to avoid contamination. Notice that the SF and all the SV strains are Rec+, which means that recombination between plasmid and chromosome can and does take place. Thus double crossover or gene conversion events may occur between the *groEL* genes on the plasmid and the chromosome to give a wild-type copy of *groEL*, which will therefore allow normal growth. Although they happen, such events are relatively rare and can easily be distinguished from true complementation, which occurs much more frequently.

2. LB medium: 10 g Bacto-tryptone, 5 g yeast extract, and 5 g NaCl/L of deionized H₂O. Dissolve the reagents in 800 mL of H₂O, and adjust the pH to 7.0 with 5 N NaOH. Make up to 1 L with H₂O, and sterilize by autoclaving for 20 min at 15 lb/sq. in. on a liquid cycle.
3. LB agar: 1% (w/v) agar in LB medium. Before autoclaving, add the agar to the LB medium (**item 2**). After autoclaving, mix the solution gently to dissolve the agar evenly. Be careful, since the fluid may be overheated and may start to boil when swirled. Allow the agar to cool to about 45°C, and use immediately or store the bottle in a 45°C water bath until use. Add antibiotic just before making the plates.
4. LB agar plates: These are made by pouring 30–35 mL of the melted LB agar into a Petri dish with a diameter of 90 mm. Once the agar has set, the plates can be used immediately or stored inverted at 4°C.
5. LB agar/kanamycin plates: These are made as described in **item 4**, but just after before pouring the plates, kanamycin is added from a stock solution (**item 11**) either to a final concentration of 10 or 50 $\mu\text{g}/\text{mL}$.
6. LB soft agar: 0.6 and 0.7% (w/v) agar in LB medium. Prepare aliquots of 250 mL as described under **item 3**. The sterilized soft agar can be stored solid at room temperature for 1 yr or melted in a water bath of 45°C for usage up to 2 wk.
7. TE buffer: 0.01 M Tris-HCl, pH 8.0, and 0.001 M Na₂EDTA. Dissolve the Tris and Na₂EDTA in H₂O, and adjust the pH to 8.0 with HCl. Sterilize aliquots of 100–250 mL (**item 2**). The sterilized TE buffer can be stored indefinitely at room temperature.
8. λ -diluent: 0.01M Tris-HCl, pH 7.8, and 0.008 M MgSO₄. Sterilize aliquots of 100–250 mL (**item 2**). Sterilized λ -diluent can be stored indefinitely at room temperature.
9. TSG: 0.01 M Tris-HCl, pH 7.4–7.5, 0.15 M NaCl, and 0.03% gelatine. Dissolve the Tris and NaCl, and adjust the pH with HCl. Dissolve the gelatine in about 25 mL of H₂O by heating in the microwave. Add the dissolved gelatine to the buffer and autoclave 100 mL aliquots as described in **item 2**. Store at room temperature.
10. Inducer/repressor solutions: 20% (w/v) L-arabinose and 20% (w/v) glucose. Dissolve 20 g of glucose or L-arabinose in 100 mL H₂O. Sterilize the solution by filtration. Autoclaving can be used, but tends to cause some caramelization (more so for glucose than for arabinose).

11. Antibiotics: kanamycin, tetracycline, and ampicillin. Kanamycin and ampicillin are made at 50 mg/mL of H₂O. Tetracycline is dissolved in ethanol at a concentration of 10 mg/mL. Kanamycin and ampicillin solutions are filter-sterilized using a 0.2- μ m filter. All antibiotic solutions are stored at -20°C. Ampicillin is used at a final concentration of 100 μ g/mL, but kanamycin is used either at 50 or at 10 μ g/mL.
12. Maltose solution: 20% (w/v) maltose. Dissolve 20 g of glucose or maltose in 100 mL H₂O. Sterilize the solution by filtration. Store at room temperature
13. 1 M MgSO₄. Dissolve MgSO₄ in H₂O to a final concentration of 1 M. Store at room temperature.
14. Chloroform.
15. Salt solution: 0.1 M MgSO₄ and 0.005 M CaCl₂. Dissolve MgSO₄ and CaCl₂ in H₂O to a final concentration of 0.1 and 0.005 M, respectively. Sterilize aliquots of 100–250 mL (**item 2**) stored at room temperature.

3. Method

3.1. Introduction

The methods described here will determine whether or not a given chaperonin protein is able to function in vivo in *E. coli*. Two main types of experiment are described: the analysis of mutant forms of the *E. coli* chaperonin proteins, and the analysis of potential chaperonin proteins and/or homologs from other organisms. Both approaches are described here.

This first test involves the complementation of either *E. coli* mutants, which do not support the growth of bacteriophage λ and T4, or a temperature-sensitive (ts) chaperonin mutant strain. Growth at elevated temperature or growth of bacteriophages does not occur in such a mutant, but can be restored by expression of a functional chaperonin protein. This method has the virtue of simplicity, but has the drawback that chaperonin proteins are particularly important for growth of *E. coli* at high temperatures. Thus, chaperonin mutants or homologs with only partial function sometimes fail to support growth in this assay, since the conditions are too stringent (*see Note 6*). Moreover, we have observed some mutant forms of the GroEL protein that cannot be expressed in a strain carrying a *groELts* mutation at any temperature, presumably because a nonfunctional mixed complex is formed between the two mutant GroEL proteins present in the cell.

The second approach involves using P1 transduction to delete the *groEL* gene from the *E. coli* chromosome in a strain where the GroEL protein under investigation is already being expressed from a plasmid (*see Fig. 2*). Although longer, this procedure has the advantage that it can be carried out under normal growth conditions, where the cells do not require as much of the GroEL protein (*see Subheading 1*). Furthermore, such strains can then be used as a source of

purified mutant or heterologous GroEL protein, which is uncontaminated with wild-type *E. coli* GroEL.

3.2. Complementation of a Mutant Chaperonin Gene

In this protocol, the complementing plasmid is first introduced into a mutant chaperonin strain. The strain is then tested to determine whether it can either support bacteriophage morphogenesis or grow at elevated temperatures.

3.2.1. Preparation of Mutant *E. coli* Transformants

This is a quick and simple method for preparing competent *E. coli* cells. The level of competence will not be particularly high, but is enough for transformation with plasmid DNA. As stated above, the plasmid needs to have been characterized in advance and shown to express the desired chaperonin mutant or homolog by SDS PAGE analysis.

1. Streak the chaperonin mutant strain out on an LB agar plate containing the required antibiotic, and incubate the plate at 30 or 37°C overnight to give individual colonies.
2. Inoculate 5 mL of LB medium plus antibiotic in a 25-mL flask or MacCartney bottle with a single colony. Incubate the culture overnight at 30°C with shaking.
3. The following day, dilute the overnight culture 1:100 into 100 mL prewarmed LB medium in a 250-mL conical flask. Grow with shaking at 30°C while monitoring the optical density at 600 nm (OD₆₀₀). When the OD₆₀₀ reaches 0.4, place the cells on ice (*see Note 3*). To obtain good competence, all subsequent steps should be carried out cold, using prechilled equipment and buffers.
4. Harvest the cells by centrifugation for 5 min at 10,000g and 4°C.
5. Gently resuspend the pellets in 40 mL (per 100 mL culture) of an ice-cold 0.1 M CaCl₂ solution.
6. Harvest the cells again as described in **step 4** and resuspend the cell pellets in 2 mL (per 100 mL culture) of the ice-cold CaCl₂ solution.
7. Incubate on ice for 30 min.

The cells are now ready for transformation. Competence increases further with time up to a maximum of 24 h of incubation on ice, and declines thereafter. Competent cells can be stored long term by adding sterile glycerol to a final concentration of 15% (v/v). Transfer 100 mL aliquots to Eppendorf tubes, and freeze in liquid nitrogen. Store the competent cells at -80°C.

3.2.2. Transformation of the Mutant Strain

1. Mix an aliquot of about 1 µg of plasmid DNA (in a volume of 10 µL or less) with 100 µL of competent cells, and incubate on ice for 30 min.
2. Heat-shock the cells briefly by incubating the tubes in a water bath at 42°C for 90 s.
3. Return the cells to ice for a further 5 min.

4. Add 400 μL LB medium, and incubate the cells in a water bath at 30°C for 30–60 min (*see Note 4*).
5. Mix the cell suspension, and spread an aliquot on LB agar plates containing the appropriate antibiotics. We typically plate out 10 μL of the transformation mix, plus 50 μL sterile LB medium on one plate and 50 μL of the mixture on a second plate. A control should also be included of competent cells without added plasmid DNA to ensure that no growth occurs.
6. Incubate the inverted plates overnight at 30°C.
7. Colonies (transformants) will be visible after 18–24 h.
8. Select well-separated colonies, and restreak on fresh LB agar plates containing the required antibiotics. Incubated again at 30°C to obtain individual colonies. The plates can now be sealed with parafilm to prevent desiccation and stored at 4°C.

The competent cells will give 10^4 – 10^5 colonies/ μg of DNA, although this varies with the size and source of the plasmid and with individual batches of competent cells. Having produced mutant strains transformed with the plasmid, which expresses the protein of interest, these strains can now be analysed for their ability to support bacteriophage growth (**Subheading 3.2.3.**) and/or to grow at elevated temperature (**Subheading 3.2.4.**).

3.2.3. Ability to Support the Growth of Bacteriophages λ and T4

All the mutant chaperonin strains (*see Subheading 2., item 1*) are suitable to test bacteriophage λ since these strains were originally isolated for their failure to support λ growth. In contrast only the GroEL (E191G) mutant strain prevents the growth of bacteriophage T4 (*see Subheading 2., item 1*). The gene of the protein of interest needs to be present on a multicopy plasmid preferentially under the control of an inducible promoter. To test for chaperonin activity *in vivo*, a minimum of three transformed strains are needed, namely, the chaperonin mutant strain containing:

1. The vector.
2. A plasmid expressing the wild-type *E. coli* chaperonin.
3. A plasmid expressing the chaperonin protein of interest.

Selection for the plasmids and vector should be maintained with the appropriate antibiotic. If the genes are under the control of a regulatable promoter, e.g., the *ara*-promoter, the inducer (arabinose) should also be included (*see Note 5*).

Bacteriophages can be grown in either liquid medium or on agar plates. To obtain a high-titer stock solution, one should always start with a well-separated single plaque from a fresh plate. Although the following protocols are adequate, for more information, the reader is referred to refs. (30,31) for bacteriophage λ and T4, respectively.

3.2.3.1. PREPARATION OF SINGLE PLAQUES

1. Streak *E. coli* strain B or B178 (for bacteriophage T4 and λ , respectively) out on an LB agar plate, and grow the cells overnight at 37°C to give individual colonies.
2. Use an individual colony to inoculate 5 mL of LB medium. For strain B178 (bacteriophage λ) add maltose and MgSO₄ to the medium to a final concentration of 0.2% and 0.01 M, respectively. Bacteriophage λ virions are sensitive to EDTA and other chelators and it is thus essential that the Mg²⁺ concentration is sufficiently high (between 0.01 and 0.03 M).
3. Grow the cells at 37°C overnight in a glass tube with head-over-head turning on a rotor wheel or grow in 10 mL LB medium in a 50-mL flask on a shaking platform.
4. The overnight culture is used for growing the bacteriophage as follows: Transfer an aliquot of 0.3 mL of the overnight culture to a 5-mL sterile glass tube. For bacteriophage λ : add in addition an aliquot of 35 μ L of 20% (w/v) maltose and 35 μ L of 1 M MgSO₄.
5. Add 10²–10³ bacteriophages from a stock preparation, and leave at room temperature for 10 min (this allows adsorption of the bacteriophage).
6. Add 3–3.5 mL of melted LB soft agar (0.7%) of about 45°C.
7. Mix the solution gently on a Vortex or by rolling the tube between the hands. Avoid making bubbles.
8. Pour the solution immediately onto a freshly made LB plate at room temperature. Tilt the plate slightly to allow the liquid to cover the entire plate.
9. Leave the plate at room temperature for about 5 min with the lid ajar to allow the agar to solidify.
10. Incubate the plate upside down at 37°C. Single well-separated plaques will be visible after an overnight incubation when the plate is held up against a window or a desk lamp. See **Note 2** for suggestions on how to increase the plaque size.

3.2.3.2. GROWTH OF BACTERIOPHAGES IN LIQUID CULTURE

1. Inoculate 5 mL of LB medium with a single colony as described under **Subheading 3.2.3.1., steps 1 and 2**.
2. Grow the cells overnight at 37°C as described under **Subheading 3.2.3.1., step 3**.
3. Transfer an aliquot of 0.1 mL of the overnight culture to 8 mL of LB medium (in a 50-mL flask). For bacteriophage λ , the medium should contain MgSO₄ and maltose at a final concentration of 0.01 M and 0.2% (w/v), respectively.
4. Take a fresh, single plaque (prepared as described above under **Subheading 3.2.3.1.**) using a sterile capillary by stabbing through a well-separated plaque into the hard agar beneath. Use a capillary with a large enough diameter so that the entire plaque can be taken. Withdraw the capillary with the agar and material from the plaque. Expel the contents of the capillary into the LB medium.
5. Incubate the flask at 37°C on a shaking platform for about 6–8 hours until the cells have lysed. The culture should appear clear. The small white debris that is observed is the result of lysed cells. If after 8 h the cells have not lysed (not enough bacteriophages have been produced to infect and subsequently lyse all the *E. coli* cells), proceed to **step 6** anyway.

6. Add an aliquot (two drops) of chloroform to the solution, and incubate at 37°C for another 20 min to allow lysis of all the cells.
7. Clear the solution by centrifugation for 10 min at 4000g and 4°C.
8. Transfer the supernatant to a sterile screw-cap tube that is resistant to chloroform.
9. Add a few drops of chloroform, and store the bacteriophage solution at 4°C.

The clarified bacteriophage T4 lysate is stable for at least a year, but a bacteriophage λ lysate is stable for up to a few months. Both lysates can be used directly for testing of chaperonin activity *in vivo*. A titer of 2×10^9 – 2×10^{10} PFU/mL is routinely obtained.

3.2.3.3. PREPARATION OF SERIAL DILUTIONS

1. Transfer aliquots of 2 mL of TSG or λ -diluent for bacteriophage T4 and λ , respectively, to 5 mL sterile glass tubes that can be closed. In general, six to seven tubes are required depending on titer of the bacteriophage stock.
2. To the first tube, add 20 μ L of the original bacteriophage stock solution as prepared under **Subheading 3.2.3.2.** (use a P20 Pipetman). Mix the solution on a vortex.
3. To the second tube, add 200 μ L of the solution of the first tube (use a P200 Pipetman with a new yellow tip), and mix the solution on a vortex.
4. To the third tube, add 200 μ L of the solution of the second tube (use a P200 Pipetman with a new yellow tip), and mix on a vortex.

Continue until all the dilutions have been made. It is absolutely essential that a new micropipet tip be used for every dilution to prevent the unwanted carryover of bacteriophages. If this happens, the titer of the solution cannot be determined precisely. Bacteriophage dilutions are stable at 4°C for a few weeks.

3.2.3.4. THE SPOT TEST FOR DETERMINING THE NUMBER OF PFU/ML

The titer of a bacteriophage solution is defined as the number of viable bacteriophages per mL, and is determined in the so-called spot test. Exactly the same procedure is used to determine whether a given protein can complement a mutant chaperonin strain (**Subheading 3.2.3.5.**). The only difference is the nature of the indicator strain. For a spot test, the indicator strain can be any strain of *E. coli* that is known to allow growth of the bacteriophage in question. Ideally, it will be the parental strain of which the mutant strains are derivatives, since only then can differences in plaque number and/or appearance be contributed to the mutated gene.

1. Prepare a bacterial lawn from a fresh overnight culture of *E. coli* B or B178 as described in **Subheading 3.2.3.1.** but omit **step 5** (adding the bacteriophage).
2. Use a P10 Pipetman to apply 5- to 10- μ L aliquots of 10-fold serial bacteriophage dilutions onto the bacterial lawn. The highest dilution should give rise to indi-

vidual plaques. When many bacteriophage dilutions need to be tested, use a “grid” under the Petri-dish to ease placing the aliquots.

3. Leave the plate on the bench with the lid ajar until the drops have dried. This takes between 30 and 45 min, depending on the volume of the drops.
4. Put the lid on the plate, and incubate upside down (agar side up) at 37°C overnight.

Plaques can easily be seen after 18–20 h when the plate is held up against a window or a desk lamp. To calculate the titer, multiply the number of plaques in a spot by the total dilution. This will give the number of PFU/mL of the original solution.

3.2.3.5. TESTING FOR BACTERIOPHAGE GROWTH

Bacteriophage growth is tested by placing aliquots of serial bacteriophage dilutions on a bacterial lawn prepared from a freshly transformed mutant *E. coli* strain. To test for chaperonin activity, a minimum of three different transformed strains is needed, namely the chaperonin mutant strain containing:

1. A plasmid expressing the chaperonin protein of interest.
2. The vector only (a negative control).
3. A same plasmid expressing the wild-type *E. coli* chaperonin gene, which is mutated in the strain, either *groEL* or *groES*, from the same promotor as the protein, which is under test (a positive control).

1. Prepare a bacterial lawn from fresh overnight cultures of the three transformants as described in **Subheading 3.2.3.1.** but omit **step 5** (adding the bacteriophage). Selection for the plasmid and vector should be maintained with the appropriate antibiotic. If the genes are under the control of a regulatable promoter, e.g., the *ara* promoter, the inducer (arabinose) should also be included (*see Note 5*).
2. Prepare serial dilutions of the bacteriophages that will be tested as described under **Subheading 3.2.3.3.**
3. Use a P10 Pipetman to apply 5 to 10 μ L aliquots of 10-fold serial bacteriophage dilutions onto the bacterial lawn as described under **Subheading 3.2.3.4.**
4. Leave the plate on the bench with the lid ajar until the drops have dried. This takes between 30 and 45 min.
5. Put the lid on the plate, and incubate upside down (agar side up) at 37°C overnight.

Plaques can usually be seen after 18–20 h. Full complementation means that the number of plaques and their size are indistinguishable from the positive control. If partial complementation is observed (plaques are visible as “spots” with a high number of bacteriophages), repeat the test, but incubate the plates at a lower temperature (between 30–35°C). Be aware that strain SF103 supports bacteriophage at the 30°C. If the bacteriophage produces small plaques on a wild-type *E. coli* host, suppression of a transformed mutant strain may be difficult to observe (*see Note 2*).

3.2.4. Complementation of the Growth Defect at Elevated Temperature of Chaperonin Mutants

This test involves the complementation of a temperature-sensitive chaperonin mutant strain. Growth at elevated temperature does not occur in such a mutant, but can possibly be restored by expression of a functional chaperonin protein. The three *E. coli* mutant strains SF103, SV7, and SV8, described in **Subheading 2., step 1.** can all be used for this test. As with the plating of bacteriophages (**Subheading 3.2.3.5.**), a minimum of three transformed strains:

1. Vector alone
 2. Plasmid expressing the chaperonin gene of interest.
 3. Plasmid expressing the wild-type chaperonin) are required in this assay.
1. Start overnight cultures of the transformed strains as described above (**Subheading 3.2.1., step 1**).
 2. Measure the OD₆₀₀ of the overnight cultures (*see Note 3*).
 3. Dilute all the cultures in LB medium to the same starting OD of 0.1.
 4. Make a series of six further 10-fold serial dilutions of each culture in LB medium as described above in **Subheading 3.2.3.3.**
 5. Use a P10 Pipetman to apply 5 to 10 μ L aliquots of the dilutions onto the LB agar plate containing the selective antibiotic, plus any inducers that may be needed for expression of the protein of interest. With practice it is easy to spot at least six dilutions of six different cultures across an LB plate (i.e., as a 6 \times 6 grid) without the spots running into each other. Plates must be well dried beforehand. Allow the spots to air-dry with the lids of the plates partially off, before inverting the plates and incubating overnight. At a minimum, two plates should be set up, one incubated at 30°C (permissive temperature) and one at 42°C (restrictive temperature). However, other intermediate temperatures may also be tested. This is fully discussed in **Note 6** below.
 6. The following day, examine the plates, and determine growth or no growth for the different dilutions. Also note whether there are significant differences in colony morphology or size with the different transformants.

Complete complementation means that the growth of the strain containing the plasmid with the gene of interest is indistinguishable from the strain containing the plasmid expressing the host *E. coli* chaperonin. It is therefore important that the number of colonies at the highest dilutions is the same for the tester transformant grown at 30°C compared to the plate from 42°C. No complementation means no growth, as with the vector alone. This kind of complementation test has been instrumental in establishing that the distantly related gene *31* from bacteriophage T4 is functionally related to GroES, since it can substitute for GroES during *E. coli* growth at elevated temperature (*see Fig. 2* and ref. *21*). The result is not always as clear as in **Fig. 2**, and even with

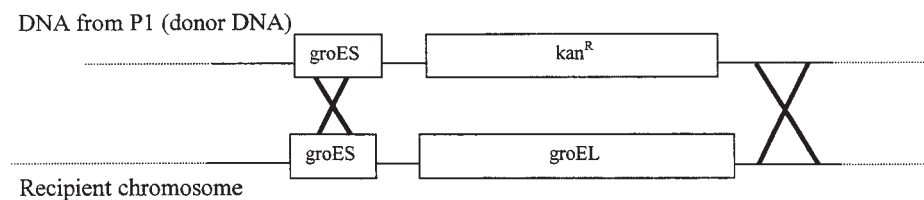


Fig. 2. Principle of the P1 transduction assay. A kanamycin resistant colony will only arise if a double crossover takes place between the incoming linear DNA from bacteriophage P1 and the chromosomal DNA (bold lines), if the recipient strain contains the gene for a GroEL mutant or homolog which is capable of function in *E. coli*. (Not to scale.)

the vector alone, faint growth may sometimes be observed at the highest cell concentration. Occasionally revertants or pseudorevertants may be seen, where normal or larger size colonies appear in an area that otherwise shows no growth (see Note 8).

3.3. Complementation of a *groEL* Deletion Strain

This test involves the use of bacteriophage P1 to delete the *groEL* gene from the *E. coli* chromosome in a strain where the GroEL protein under investigation is already being expressed from a plasmid (see Note 7). Construction of strains carrying a deletion of the chromosomal *groEL* gene is relatively straightforward with the use of bacteriophage P1. This bacteriophage packages DNA randomly when it grows lytically and will also package stretches of *E. coli* host chromosomal DNA. Thus, a P1 bacteriophage solution (or lysate) grown on any strain will contain a few phages that contain only *E. coli* chromosomal DNA. When such a lysate is now used to infect another strain, the *E. coli* DNA from the bacteriophage integrates into the chromosome of the recipient strain by homologous recombination via a double crossover event. It thereby effectively replaces the corresponding stretch of DNA on the recipient chromosome (see Fig. 2). Deletion of the chromosomal *groEL* gene can only give rise to colonies if the GroEL protein expressed from the plasmid is functional.

3.3.1. Making a P1 Transducing Lysate on Strain AI90/pBAD50

The strain AI90/pBAD50 was constructed as described in ref. (29). It contains a precise replacement of the *groEL* gene with the *nptII* gene from the transposon Tn5, which confers kanamycin resistance. The plasmid pBAD50 contains the *groEL* gene under control of the arabinose-inducible promoter. The strain will thus only grow on LB agar plates, which contain 0.2% (w/v) arabinose and 50 µg/mL kanamycin. A P1 lysate prepared from this strain can

be used to transduce potentially any other strain expressing a GroEL derivative to kanamycin resistance. If kanamycin-resistant transductants are obtained and it is confirmed that they have lost the chromosomal *groEL* gene, this demonstrates that the GroEL derivative in question is functional in *E. coli*. To prepare a P1-transducing lysate, any pre-existing solution of bacteriophage P1 can be used. Since the titer of the P1-lysate decreases on long-term storage, it is advisable to start by checking the titer of the stock solution on *E. coli* strain AI90/pBAD50 as described above under **Subheading 3.2.3.4.** (no maltose and MgSO_4 should be added). If the lysate stock has a reasonable titer (5×10^8 PFU/mL), the new lysate can be prepared.

1. Streak *E. coli* strain AI90/pBAD50 out on an LB agar plate containing 0.2% (w/v) arabinose and 50 $\mu\text{g}/\text{mL}$ kanamycin, and grow the cells overnight at 37°C to give individual colonies.
2. Use an individual colony to inoculate 5 mL of LB medium containing 0.2% (w/v) arabinose, 50 $\mu\text{g}/\text{mL}$ kanamycin, and grow the cells at 37°C overnight.
3. Make 10-fold serial dilutions of the bacteriophage P1 stock as described above in **Subheading 3.2.3.3.** in LB medium containing 0.0025 M CaCl_2 . A range from 10^{-1} to 10^{-4} should be sufficient.
4. Mix 100 μL of each of the dilutions with an aliquot of 1 mL of the overnight culture in a sterile glass tube.
5. Add 25 μL of 0.1 M CaCl_2 solution, and incubate the tubes at 37 °C for 30 min.
6. Add 3.5 mL of LB soft-agar (0.6%) containing 0.0025 M CaCl_2 to each tube, mix gently using a Vortex (avoid making bubbles), and pour the mixture onto a 1-h old LB plate containing 0.2% w/v arabinose, 50 $\mu\text{g}/\text{mL}$ kanamycin, and 0.0025 M CaCl_2 .
7. Incubate the plates overnight at 37°C.
8. Examine the plates the following day. On some plates, individual plaques should be clearly visible. On others, complete lysis will have occurred. Choose a plate where the plaques are all touching, but where lysis has not completely cleared the plate. Using a sterile glass spreader or metal spatula, scrape the soft agar from the surface of the plate, and transfer it to a Corex centrifuge tube. Wash the plate with 2 mL of LB medium containing 0.0025 M CaCl_2 , and add the liquid to the centrifuge tube. Vortex thoroughly.
9. Leave the tubes at room temperature for about 20 min. The bacteriophages will diffuse from the top agar into the solution.
10. Spin the mixture using a Sorval centrifuge for 15 min at 10,000g and 4°C.
11. Filter the supernatant through a 0.2- μm filter to remove unlysed bacterial cells and large debris. Transfer the supernatant to a sterile tube, and add few drops of chloroform to complete lysis of any residual cells. Determine the number of PFU of the lysate on strain AI90/pBAD50 as described under **Subheading 3.2.3.4.** A reasonable lysate should have at least 10^{10} PFU/mL. Store the P1 bacteriophage lysate at 4°C.

3.3.2. PI Transduction and Selection of Kanamycin-Resistant Colonies

This procedure involves mixing of the PI lysate with the recipient strain, allowing time for the bacteriophage to enter the cell, recombination to occur and for the phenotype to be expressed. The cells are then plated onto an LB agar plate containing kanamycin and arabinose. It is important that the multiplicity of infection (MOI) should be kept at about 1, i.e., equal numbers of PI bacteriophages and *E. coli* cells. In practice, it is easiest to set up several PI transductions each with a different bacteriophage concentrations and see empirically which one is successful. A minimum of three transductions should be set-up as described in **Subheading 3.2.3.5**.

1. Use a fresh transformant (**Subheading 3.2.3.1**) to inoculate 5 mL of LB medium containing the required antibiotics and inducer, and grow the cells at 37°C overnight. Some methods recommend including 0.0025 M CaCl₂ in the growth medium and growing the bacteria without shaking. We have not found that this makes any difference to the success of the transduction.
2. Transfer 1 mL of each overnight culture to an Eppendorf centrifuge tube, and harvest the cells by centrifugation for 2 min at 5000g and 4°C.
3. Resuspend the cells in 1 mL of salt solution containing MgSO₄ and CaCl₂ **item 15**.
4. Incubate at 37°C for 15 min with gentle shaking, e.g., on a rotor wheel.
5. Mix 100 µL of the cell suspension with 100 µL of the PI lysate obtained in **Subheading 3.3.1**. Use the PI lysate undiluted (at least 10¹⁰ PFU/mL) and also at 10X and a 100X dilution.
6. Incubate at 37°C without shaking for 20 min.
7. Harvest the cells by centrifugation for 30 s at 10,000g and room temperature.
8. Resuspend the cell pellet in 100 µL of LB medium, and incubate at 37°C for 60 min. Some protocols include the addition of sodium citrate at this point. However, citrate can apparently chelate kanamycin, so it is not used in this protocol.
9. Spread an aliquot of 100 µL of the cell suspension on an LB agar plate containing 10 µg/mL kanamycin (note that this is different from the normal 50 µg/mL), plus any inducers required for expression of the plasmid-borne genes. Also spread 100 µL of the 10X diluted cell suspension on a second LB agar plate. Controls: plate equal amounts (on individual LB agar plates containing 10 µg/mL kanamycin) of 100 µL of the recipient strain (transformant) alone and 100 µL of the PI lysate alone.
10. Incubate the plates at 37°C.
11. If the transduction has worked, colonies will begin to appear after 1–2 d incubation on the experimental plates. Colonies often come up at different times and with different sizes, unlike in a transformation (**Subheading 3.2.2**). Also, some growth may occur on the control plate with the recipient strain alone, since the initial levels of kanamycin used for selection are quite low. Importantly, no growth should occur on the plate with the PI lysate alone: if it does, either the

filtration step to remove unlysed cells failed to work properly or the lysate has subsequently become contaminated. If this happens, the experiment must be repeated after rectifying the problem.

12. After 2–3 d incubation, a large number of colonies should be visible on at least some of the plates. Pick a number of these (we usually pick 20 to 30) and streak them onto an LB agar plate containing at 50 $\mu\text{g/L}$ of kanamycin (plus any necessary inducer, for example, arabinose). Avoid picking only the larger colonies. Colonies should also be picked from the control plate with the recipient strain alone, if any have grown. These should not grow at 50 $\mu\text{g/L}$ kanamycin, and if they do, the experiment needs to be repeated.
13. Incubate the inverted plates overnight at 37°C. Colonies that grow on the higher amount of kanamycin are candidates for successful deletion of the *groEL* gene. Further tests are necessary to confirm this and are described below. If no colonies appear, either the transduction has been unsuccessful or the GroEL protein under investigation cannot support *E. coli* growth at 37°C. The success of the transduction can be gauged from the positive control with wild-type GroEL being expressed from a plasmid. Reasons for the protein failing to complement are many and are discussed further below (see **Note 8**).

3.3.3. Confirmation of the Correct Strain Construction

It is essential to confirm that any transductants are indeed the correct strain, and are not a consequence of either contamination of the PI lysate with unlysed AI90/pBAD50 cells or of a nonhomologous event leading to the integration of the “kanamycin resistance” gene into the chromosome without the loss of the *groEL* gene. Contamination can be ruled out by the absence of colonies on the plate of the PI lysate alone. The deletion of the *groEL* gene can be confirmed as follows.

3.3.3.1. REPRESSION OF THE INDUCIBLE PROMOTER ON THE PLASMID

If the complementing *groEL* gene on the plasmid is under the control of a tightly regulated promoter, such as the *ara* promoter, the construction of the correct strain can be established very simply by streaking candidate colonies onto an LB agar plate, which represses this promoter. In the case of the *ara* promoter, the plate should contain 0.5% glucose (29). No colonies will grow if the promoter is switched off and the chromosomal gene has been deleted. However, if the *groEL* gene has not been deleted, colonies will be visible even under repressing conditions. This experiment can only be performed if the promoter expressing the complementing *groEL* can be tightly regulated. **Fig. 3** shows an example of such a test.

3.3.3.2. LOSS OF THE SPECIFIC *GROES-GROEL* PCR FRAGMENT

Even if the results of the above test are favorable, we routinely confirm the presence of the correct deletion by using polymerase chain reaction (PCR) with a pair of primers. If the test cannot be done because the promoter cannot be

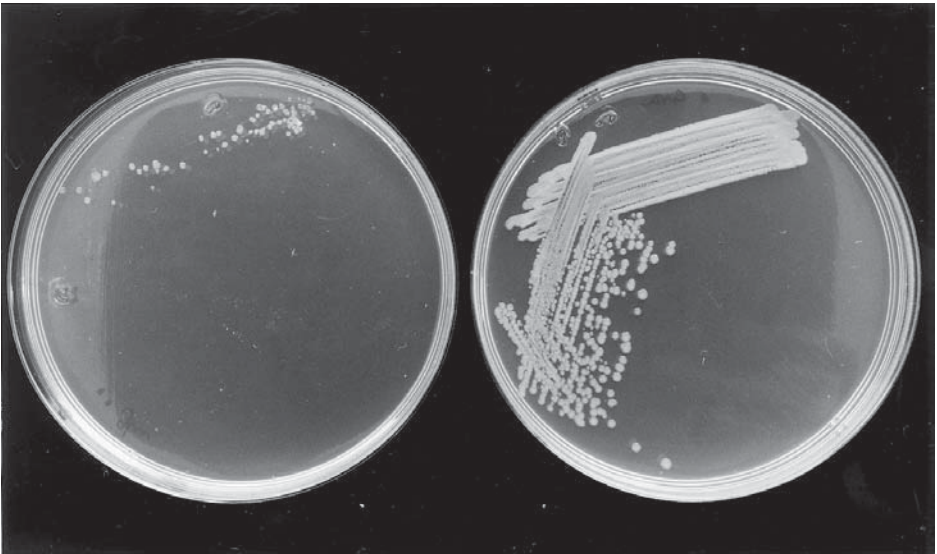


Fig. 3. Comparison of a *groEL* deletion by a plasmid-borne *groEL* gene. A loopful of an overnight culture of A190/pBAD50 has been struck onto LB plates containing either 0.5% glucose (left) or 0.2% arabinose (right). The plates were incubated at 37°C overnight. Growth is normal on the arabinose-containing plate (right), but only a few colonies are seen on the plate containing glucose (left). When the few colonies are analysed they are usually found to have arisen from mutations in the pBAD promoter preventing full repression.

fully repressed, confirmation using PCR is absolutely vital. The idea of this test is simple. Primers, which hybridize to the *groES* and the *groEL* genes, produce a signal from the chromosomal *groE* operon. The *groEL* signal is lost if the chromosomal *groEL* gene has been deleted. This can be tested using a simple PCR-based method as follows.

1. Use candidate transduced strains (**Subheading 3.3.2.**) to inoculate 5 mL of LB medium containing the inducer and grow the cells at 37°C overnight. Antibiotics to select for the plasmid will not be required, since if the plasmid is lost, the strain will die. Also grow cultures of strains A190/pBAD50 (positive control) and TG1 containing the plasmid with the mutant or homologous *groEL* gene to be tested (**Subheading 2, item 1**) (negative control).
2. For each strain, prepare a tube (PCR 0.5 mL) that contains the following reagents:
 - 12.5 μ L of a stock solution containing 0.001 M of each deoxyribonucleoside triphosphate (dNTP).
 - 5 μ L of the 10X PCR buffer (as supplied by the manufacturer; we use 10X PCR buffer without Mg from Gibco-BRL, Rockville, MD).
 - 2 μ L of a solution containing 0.025 M $MgCl_2$

5 μL of the *groES*-specific primer: 5'-GAAGTGTGATCATGTCC-3'.

5 μL of the *groEL*-specific primer: 5'-GATAGCCTGAGCCAGTAC-3'.

TE buffer to make the volume up to total of 48 μL .

3. Add an aliquot of 1 μL of *Taq* DNA polymerase enzyme solution of 5 U/ μL
4. Add an aliquot of 5 μL of the overnight cell culture of interest to each cocktail.
5. Invert each tube to mix the reagent, and centrifuge them very briefly for 1 or 2 s in an Eppendorf centrifuge at room temperature.
6. Carry out a 30-cycle PCR reaction with the following program:

93°C	1 min 45 s.
63°C	1 min 30 s.
72°C	2 min.
7. Load an aliquot of 10 μL of the PCR reaction onto a 6% polyacrylamide gel with appropriate size molecular weight markers to confirm the **absence** of the *groES-groEL* specific band from the strains of interest. It is essential that the positive and negative controls are on the same gel and give the expected results. The expected band size of 390 bp should be present in the TG1 control and absent in the AI90/pBAD50 control.

4. Notes

1. The choice of plasmid and promoter for expression of the heterologous or mutant chaperonin gene is critical. Complementation will obviously depend on the level of protein produced, but there are also more subtle factors involved. For example, we routinely see the best complementation of a *groEL^{ts}* mutant when the heterologous GroEL protein is itself expressed under the control of the *E. coli groE* heat-shock promoter. However, for complementation of the *groEL* deletion, we have almost always used the *ara* promoter system from the *E. coli* arabinose operon present in plasmid pBAD30 (32) and other pBAD-derived plasmids (33). We have also obtained deletion of the *groEL* gene in a strain containing the complementing *groEL* on a plasmid under the control of the *lac* or the *tet* promoter.
2. When bacteriophages are grown on plates, the plaque size of both λ and T4 can vary quite a bit. Using small plaques (about 0.1 mm in diameter) to start the liquid lysate (**Subheading 3.2.3.2.**) will result in a low yield. To increase the plaque size and thus the number of bacteriophages, three parameters may be varied.
 - a. Type of medium: Instead of using LB medium, the use of 2X YT for the growth of bacteriophage T4 sometimes result in larger plaque size. 2X YT medium contains 16 g Bacto-tryptone, 10 g yeast extract, and 5 g NaCl in 1 L of H₂O. Adjust pH to 7.0 with 5 N NaOH. Sterilize as described under **Subheading 2., item 2.**
 - b. Freshness of the bottom agar layer: For bacteriophage T4, it is essential that a freshly poured plate equilibrated at room temperature is used. The use of old (1–3 d) plates results often in very small plaques. Bacteriophage λ is less sensitive to the freshness of the plates, and even 1 to 3 d old plates will give satisfactory results that can be used for the preparation of liquid lysates.

- c. Diffusion rate in the soft agar: The diffusion rate is often dramatically increased when the soft agar concentration is reduced resulting in an increased plaque size. The relative dryness of the bottom agar affects the plaque size for similar reasons. Fresher plates are moister, dilute the “soft agar” more, and promote bacteriophage diffusion. When a concentration as low as 0.45% soft agar is used, do not incubate the plates upside down.
3. When measuring the OD₆₀₀ of dense cultures, it is important to remember that most spectrophotometers do not show a linear response at high optical densities. We typically dilute overnight cultures 10- to 20-fold into LB medium before measuring the OD. An interesting exercise to carry out with your spectrophotometer is to make a series of dilutions of a dense culture, measure the OD, and see the point at which linearity of response breaks down! An *E. coli* wild-type culture usually takes 90 min to 2 h to reach an OD₆₀₀ of around 0.4 when grown at 37. When preparing competent cells (**Subheading 3.2.1., step 1**), it is important that the OD does not go much beyond 0.4, since the cells will end up much less competent. Remember that the bacteria are growing exponentially, and so the OD₆₀₀ will change rapidly. Therefore, check the OD frequently to “catch” the cells at the correct OD.
4. The “time” needed after the transformation and before plating the cells is required for expression of the antibiotic marker from the plasmid. Ampicillin resistance only needs 30 min for expression, whereas kanamycin requires 60 min. Remember that you cannot use tetracycline resistance with this method, since SF103, like SV5, SV7, and SV8 cells, is already resistant to this antibiotic.
5. The required amount of inducer needed depends strongly on the type of promoter. Many promoters who technically require induction are actually sufficiently “leaky” to give enough expression for complementation. In addition, they are often present on high copy number plasmids. For example, in our experience, the presence or absence of isopropylthiogalactose (IPTG) in cultures where the *lac* promoter is being used has no effect on the complementation. Although for the *ara*-regulated promoter, arabinose must be present in both the overnight cultures and the LB agar plates. The requirement for inducer has to be determined empirically.
6. When determining the ability of a chaperonin protein to complement an *E. coli* mutant^{ts} strain, a number of temperatures can be tested. In our hands, the *groEL*^{ts} strains show no growth at all at 42°C and good growth at 37°C. However, if a particular plasmid fails to give complementation at 42°C, it is recommended to try intermediate temperatures and look for subtle differences. The presence of antibiotics and inducer will also have an effect on the growth characteristics of the mutant as well as the transformed mutant strain. The presence of arabinose often reduces the temperature at which the mutant cells can survive and thus complementation may well be observed at lower temperature (*see Fig. 3*). We have found that in order to observe complementation of certain chaperonin^{ts} mutants, 0.5°C was the difference between “life” and “death.” In addition partial complementation is sometimes seen where growth occurs in the first two or three spots of the serial dilution. This is why it is vital to have a negative (vector alone) and positive (vector plus wild-type *groEL*) control on every plate. It is also

important to realize that even with very careful calibration, most incubators show some variation in temperature at different places within the incubator. It is advisable to put a thermometer inside the incubator close to where the plates are placed, since the temperature displayed on the outside of the incubator is only an indication of what is going on inside! We have found that only in incubators, which are fan-assisted, a uniform and precise temperature is being maintained. If these are used, plates must be taped with parafilm to prevent drying out.

7. It is also important for the P1 transduction experiment that there is no *groES* gene present on the plasmid. If there is, recombination can take place between the plasmid and the chromosomal *groES* gene, which may lead to the formation of a functional operon on the chromosome under the control of the *groE* promoter, i.e., no longer subject to any inducer or repressor. Moreover, if a *groES* gene is present on the plasmid, the PCR test for successful deletion of the chromosomal *groEL* gene may not be informative. We have not looked in detail at the effects of expressing heterologous GroES proteins in *E. coli*, but this is obviously an area where interesting experiments can be done.
8. A negative or “intermediate” (partial but not complete complementation) results in the *in vivo* assays needs to be interpreted with care. In particular, a distinction must be made between two possibilities: either (a) protein is being expressed correctly, but is not functional in *E. coli*, or (b) some aspect of the expression is faulty. For example, the protein is being expressed at insufficient levels for complementation, the protein synthesis is not initiating at the correct ATG codon, or the protein folds or assembles in a nonactive conformation. If, however, it is clear that the expression is not the problem, the question still has to be asked why a given chaperonin mutant or homolog is not functional *in vivo*. In the case of mutant proteins, this is often because the mutant protein is defective in some aspect of chaperone function. For example, it is unable to release bound protein, or does so only very slowly. Examples of such mutants are described in refs. (34,35). In the case of a GroEL homolog from a different organism failing to function in *E. coli*, there are many possible reasons, but these have received less attention in the field to date. Examples of such reasons include (but are not limited to):
 - a. The protein may fail to interact correctly with the *E. coli* GroES.
 - b. The protein may have a different substrate specificity to *E. coli* GroEL and may fail to act on some essential *E. coli* proteins, which are GroEL-dependent for their folding.
 - c. The protein may have a distinct role in its normal host, which is not the same as the role of GroEL in *E. coli*.
 - d. The protein may end up in a incorrect location, e.g., in *E. coli* periplasm.

The experimental evaluation of these possibilities has the potential to add considerably to our knowledge of the role and mechanism of GroEL proteins in different organisms.

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Interaction of Nonnative Polypeptide Substrates with the *Escherichia coli* Chaperonin GroEL

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1. Introduction

A hallmark of the essential cellular factors known as molecular chaperones is their ability to bind tightly to a broad spectrum of nonnative polypeptides to facilitate protein folding in vivo and in vitro under nonideal or stressful conditions (1–3). A variety of methods have been used to assess substrate protein binding to the bacterial chaperonin GroEL qualitatively. One of the earliest and most popular approaches involves the inhibition of in vitro refolding of various model proteins, such as ribulose *bis*-phosphate carboxylase (4–6), citrate synthase (7,8), rhodanese (9,10), malate dehydrogenase (11–13), barnase (14), and glutamine synthetase (15–17). Additional, quantitative procedures have relied on the use of size-exclusion chromatography to separate free radio-labeled proteins from those bound to GroEL (8,9,18–20), and on the effect of nonnative proteins on the ATPase activity of GroEL (21). However, the most straightforward techniques to measure the affinity and stoichiometry of polypeptide interactions with the chaperonins accurately and precisely have involved a direct or indirect measurement of the change in the concentration of a substrate protein when it partitions to GroEL from solution. Some of these methods have relied on the heat exchanged on polypeptide binding to GroEL (22,23), on the distribution of bound and free substrates detected by ultracentrifugation (24–26), and on changes in surface plasmon resonance using BIAcore (27–29).

Many of the most convenient approaches for measuring substrate binding to GroEL, and often the most sensitive, involve the use of optical methods to detect changes in the spectral properties of substrates when bound to the

chaperonin. Optical methods, such as fluorescence spectroscopy, offer several important advantages among biophysical approaches in that they are reasonably sensitive and can be selective. Typically, fluorescence can readily detect micromolar to nanomolar concentrations of protein. Additionally, since wildtype GroEL is devoid of tryptophan (30), as is its cochaperonin GroES, they possess relatively little fluorescence when excited at 295 nm. On the other hand, tryptophan residues in many nonnative substrate proteins can be selectively excited and can change their emission properties when bound to GroEL. Consequently, fluorescence methods have found broad use in studies of GroEL–polypeptide interactions, and have provided information not only on the equilibrium binding of substrates (31,32), but also on the kinetics of polypeptide binding, dissociation, and folding (33–36). Furthermore, the biosynthetic incorporation of highly fluorescent tryptophan derivatives into protein substrates (37), or the derivatization of substrates with fluorescent chromophores (38), expands the possibilities of using fluorescence for measuring the energetics and kinetics of substrate protein binding to the GroEL chaperonin.

The fluorescence titration method described below can be used for equilibrium studies of chaperonin–substrate binding, and should be considered a prerequisite prior to kinetic analyses of substrate binding and dissociation. Two examples are used to illustrate the method and analysis. The first utilizes a single tryptophan-containing, nonnative variant of the serine protease subtilisin BPN'. This substrate, previously shown to possess significant affinity for GroEL under nondenaturing conditions (22,29), exhibits a considerable increase in fluorescence when bound to the chaperonin. The second example describes GroEL binding to an unfolded variant of the small, single domain nuclease from *Staphylococcus aureus*, an intensively studied model of protein folding and stability (39,40). The nonnative L103A variant of nuclease undergoes a significant decrease in the fluorescence of tryptophan 140 on binding to the chaperonin.

Generally, the simple binding of a model nonnative substrate to GroEL can be expressed by the following equilibrium:



The binding reaction can be studied by varying the concentration of the substrate, [S], GroEL, [EL], or both, and measuring the amount of the bound species, [S_B], to determine the equilibrium constant, K_d . This is achieved most conveniently with the fluorescence method by adding an increasing concentration of one of the reactants to a fixed concentration of the other, while measuring a change in the emission of one of the components on complex formation.

Within the limit that one of the reactant concentrations (e.g., GroEL) is well below the binding constant, the concentration of bound material, $[S_B]$, is negligibly small relative to the total concentration of added material, and can therefore be omitted in any analysis, i.e., $n[EL_T] \ll K_D$ and $[S_T] \gg [S_B]$, (where $[EL_T]$ and $[S_T]$ are the total concentrations of GroEL and substrate and $[S_T] = [S_B] + [S]$). Under these conditions, one can determine the equilibrium dissociation constant for the reaction from the fractional saturation using:

$$\frac{\Delta F}{F_{\max}} = \frac{[EL_B]}{n[S_T]} = \frac{[EL_T]}{K_d + [EL_T]} \quad \mathbf{A}$$

$$\frac{\Delta F}{F_{\max}} = \frac{[S_B]}{n[EL_T]} = \frac{[S_T]}{K_d + [S_T]} \quad \mathbf{B}$$

where ΔF is the change in fluorescence of the starting material, F_{\max} is the maximal change for the binding reaction, and n is the number of moles of added ligand that is needed to saturate a given concentration of the fixed reactant. **Equation 2a** is used when a given amount of substrate is titrated with increasing GroEL concentration, and **Eq. 2b** applies to the reciprocal experiment in which a fixed amount of GroEL is titrated with increasing substrate concentration. It can be seen from **Eq. 2** that the fraction of bound complex increases in proportion to the total ligand concentration. Thus, any method that detects bound substrate from the total, or the difference between the total and bound substrate, will allow estimation of the equilibrium constant.

Since **Eq. 2** is of limited use for estimating the affinities of substrates for GroEL because of their strong binding to the chaperonin, a different expression is usually needed to determine the equilibrium constant. Using the method described here, the concentrations of substrate and GroEL are significantly higher than the binding constant, and therefore, the total ligand concentrations are not accurate estimates of the free concentrations. Consequently, under this so-called tight binding limit, the equilibrium constant must be estimated from the fractional saturation using the quadratic expression:

$$\frac{\Delta F}{F_{\max}} = \frac{[EL_B]}{n[S_T]} = \frac{K_d + [EL_T] + n[S_T] - \sqrt{(K_d + [EL_T] + n[S_T])^2 - 4[EL_T]n[S_T]}}{2n[S_T]} \quad \mathbf{A}$$

$$\frac{\Delta F}{F_{\max}} = \frac{[S_B]}{n[EL_T]} = \frac{K_d + n[EL_T] + [S_T] - \sqrt{(K_d + n[EL_T] + [S_T])^2 - 4n[EL_T][S_T]}}{2n[EL_T]} \quad \mathbf{B}$$

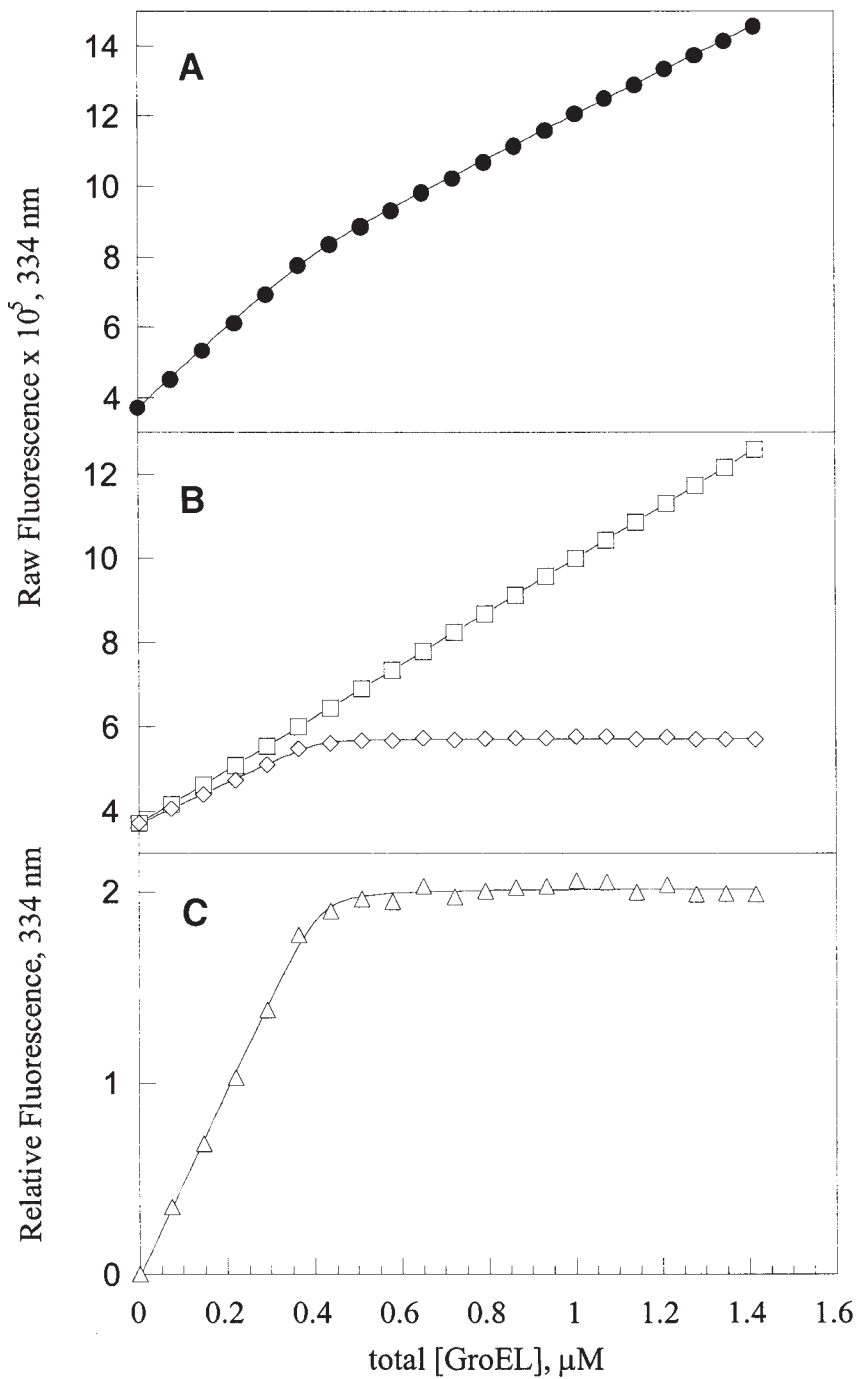
Assuming that any spectral changes are attributable to complex formation, one can analyze an isotherm for the addition of GroEL to a substrate protein using **Eq. 3a**, or for the addition of a substrate to GroEL using **Eq. 3b**. In these analyses, both $[EL_T]$ and $[S_T]$ would vary independently. However, as in the examples provided of titrating GroEL into substrate, the variation in $[S_T]$ is small enough so that one may set the total substrate concentration $[S_T]$ constant, and use $[EL_T]$ as the independent variable to determine F_{\max} , K_d , and n using **Eq. 3a**. One of the advantages of the titration method is that when the concentration of GroEL is in vast excess of the dissociation constant ($n[EL_T] \gg K_d \sim nM$), an accurate estimate for the stoichiometry (n) can be obtained simply by inspection of the intersection of the two asymptotes of the quadratic curve (see **Fig. 1**).

A final experimental concern is the significant scattering contribution of solutions containing milligram (micromolar) quantities of GroEL. Without appropriate filters in the fluorimeter to correct this condition, another parameter must be included to account for the linear increase in emission intensity on the addition of increasing concentrations of GroEL. Therefore, the reduced experimental data can be analyzed according to

$$\frac{\Delta F}{F_{\max}} = \frac{[EL_B]}{n[S_T]} = \frac{K_d + [EL_T] + n[S_T] - \sqrt{(K_d + [EL_T] + n[S_T])^2 - 4[EL_T]n[S_T]}}{2n[S_T]} + C[EL_T]$$

where C is a proportionality factor representing the increase in scattering from the addition of GroEL itself into buffer. An analogous equation is used for the titration of substrate into GroEL as described in **Eq. 3b**.

Fig. 1. Effect of increasing GroEL concentration on the fluorescence of a single tryptophan variant of subtilisin BPN'. Microliter aliquots of a stock GroEL solution (30–50 mg/mL) were added to a solution containing 1.0 μM subtilisin and the relative fluorescence increase was measured at 334 nm on excitation at 295 nm. **Panel A** illustrates the raw intensity change arising from the increase in subtilisin fluorescence and scattering from GroEL. **Panel B** shows the individual components of the fluorescence increase owing to the addition of GroEL (squares) and from binding of substrate (diamonds). **Panel C** shows a titration plot of the transformed data, corrected for the linear scattering contribution from GroEL. The transformed data can be analyzed in terms of the quadratic in **Eq. 3a**. Analysis of the data in panel A using **Eq. 4** yields a linear scattering component of 6.272×10^5 fluorescence U/ μM GroEL, a maximal change in fluorescence of 2.03×10^5 , a dissociation constant of 2.2 nM, and a value for n of 0.41 mol of GroEL needed to saturate 1 μM subtilisin. This latter value is equivalent to 2.4 sites for subtilisin per GroEL oligomer, or roughly one per toroid.



2. Materials

2.1. Apparatus

A steady-state fluorimeter is required for this measurement. Additionally, appropriate cuvettes (10 mm path length, Spectrosil Far UV quartz, Starna catalog number 21-Q-10) are needed for efficient and complete mixing of microliter aliquots of concentrated chaperonin stocks when added to the substrate. The results described below were acquired with a Spex FluoroMax 2000 spectrofluorimeter equipped with a stir motor to turn a magnetic flea for mixing and water jacketed cell for temperature control. Finally, a double-beam spectrophotometer is needed to measure accurate concentrations of the substrate and GroEL solutions, since they both tend to scatter light appreciably.

2.2. Chemicals

1. Assay buffer: 50 mM Tris-HCl, pH 7.8, 150 mM KCl, and 5 mM MgCl₂.
2. GroEL can be readily prepared by any of several protocols (15,41–43). However, for optical work such as that described here, the chaperonin should be free from tryptophan-containing contaminants that lead to high levels of fluorescence. Several procedures have been reported that yield GroEL free from fluorescent impurities, and all have been used successfully in this laboratory (15,41–43). Purified chaperonin can be stored at 30–50 mg/mL in assay buffer containing 20% glycerol at –80°C (see Note 1).
3. Nonnative subtilisin variants can be prepared as previously described (22), and stored at about 3–4 mg/mL at –80°C (Note 1).
4. Unfolded mutants of staphylococcal nuclease can be prepared according to Shortle and Meeker (44), and can be stored at 3–4 mg/mL at –80°C (Note 1).
5. Solutions of GroEL, nonnative subtilisin, and staphylococcal nuclease in assay buffer may be kept either at room temperature for 1 wk, or up to 1 mo at 4°C.

3. Method

1. The concentrations of GroEL, nonnative subtilisin, and nuclease should be determined from the absorbance at 280 nm obtained from corrected absorbance spectra. This is especially important for GroEL, at concentrations of 30–50 mg/mL, since it typically displays a substantial scattering contribution at 280 nm (see Note 3). The extinction coefficients used for wildtype GroEL ($M_r \sim 805,000$), subtilisin ($M_r \sim 27,000$), and nuclease ($M_r \sim 16,800$) are 0.213, 1.12, and 0.93 cm²/mg, respectively. The concentrations of amino acid variants of these proteins can be estimated from data at 280 nm compiled by Gill and von Hippel (45).
2. A fluorescence emission spectrum of assay buffer alone is recorded at the assay temperature from 310–450 nm to measure background fluorescence, which must be subtracted from all protein spectra.
3. The fluorimeter cell is filled with a 1- μ M solution of the nonnative substrate protein (either nuclease or subtilisin) and stirred gently until equilibrated at constant temperature (e.g., 25°C).

4. A fluorescence emission spectrum of the nonnative substrate protein should be obtained upon excitation of tryptophan residue(s) at 295 nm; the maximal fluorescence emission for nuclease and subtilisin is seen at 334 nm. For the substrates, scans were taken from 310 to 450 nm at a scan rate of 1 nm/min.
5. Microliter aliquots of a concentrated stock of GroEL (30–50 mg/mL) are added to the protein solution and stirred for 1 min before collecting a new emission spectrum to assess the fluorescence change (**Note 2**). For a 1- μM solution of substrate protein, aliquots of GroEL are added to increase the chaperonin concentration by approx 0.1 μM increments until a two- to threefold molar excess of chaperonin binding sites over the substrate is achieved. This enables a sufficient data set over the entire titration for accurate analysis. A plot of a typical titration of a nonnative variant of subtilisin with GroEL is shown in **Fig. 1**.
6. At the end of the entire titration, the solution is removed, and the cuvet is cleaned with detergent, rinsed with doubly deionized water and ethanol, and then dried.
7. The fluorescence spectrum for the GroEL-substrate complex is then compared to the substrate in the absence of the chaperonin to assess the wavelength at which the maximal change occurs on binding. The effect of dilution on $[\text{EL}_T]$, $[\text{S}_T]$, and the fluorescence at this wavelength is estimated, and then the fluorescence change is plotted as a function of the total GroEL concentration as seen in **Figs. 1** and **3**.
8. At this stage, the data can be analyzed directly with **Eq. 4**, if one includes a term for a nonzero intercept. This analysis yields F_{max} , the maximal fluorescence change, K_d , the dissociation constant, n , the number of moles of the GroEL oligomer needed to saturate the initial concentration of substrate, and C , the correction term for GroEL scattering. The results for subtilisin and nuclease can be seen in **Figs. 1A** and **3A**, respectively.
9. A separate experiment should be performed in which aliquots of GroEL are added to a cuvet containing only assay buffer to determine independently the scattering contribution from the chaperonin. These data, which are dependent on instrumental conditions such as excitation and emission slits and lamp intensity, can then be subtracted from the raw data to yield the fluorescence change owing solely to binding, as seen in **Figs. 1B** and **3B**.
10. The resulting data, presented as classical titration plots in **Figs. 1C** and **3C**, can be analyzed according to **Eq. 3a** to yield the maximal fluorescence change and the binding parameters.

4. Notes

1. It is essential to use highly purified preparations of chaperonin and substrate proteins for accurate estimates of the dissociation constants and binding stoichiometries. Additionally, although the components are stable for a week at room temperature and even longer at 4 °C, they should be assessed routinely for aggregation prior to use. Aggregation can be detected by extensive light scattering in absorbance scans that show a gradual increase in absorbance between 400 and 230 nm. Finally, although the subtilisin and nuclease variants employed in this study are nonnative in structure, they should be stored in small aliquots since repeated cycles of freezing and thawing result in their aggregation.

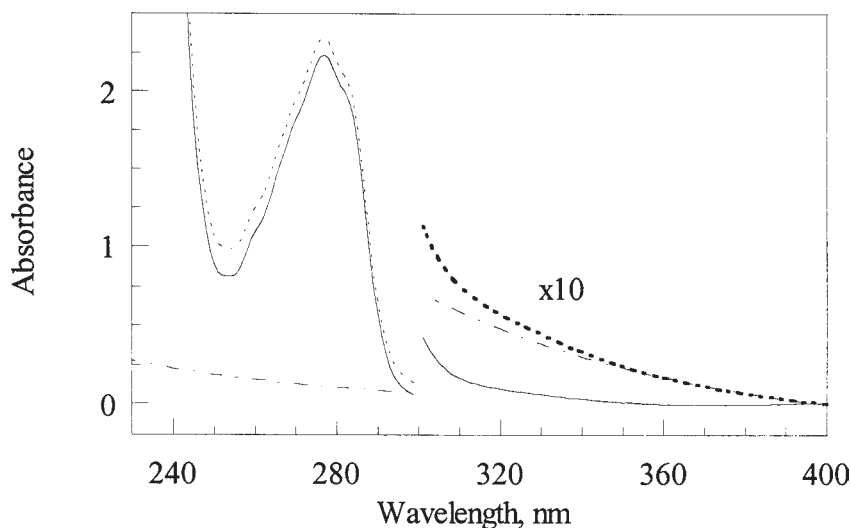
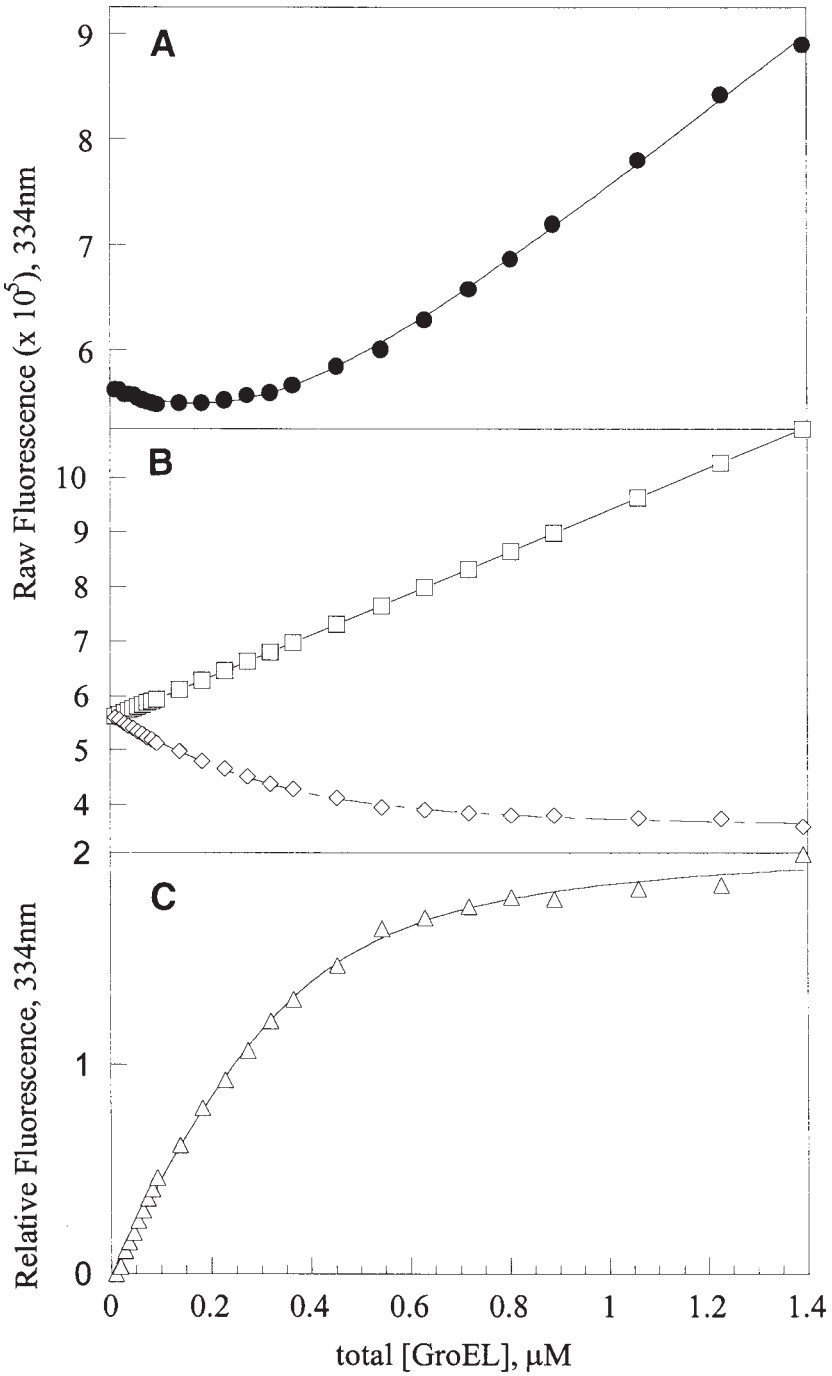


Fig. 2. Effect of light scattering on the estimation of GroEL concentration from absorbance spectra. The spectrum of a GroEL solution was scanned from 400 to 230 nm, and corrected for light scattering as described in **Note 3**. The dotted line represents the raw data, the dashed line represents the scattering contribution, and the solid line, the corrected spectrum. Use of the extinction coefficient for GroEL given in **Sub-heading 3**, with the raw spectrum yields an uncorrected concentration of 10.3 mg/mL, whereas the corrected spectrum yields a concentration of 9.8 mg/mL, or a difference of about 5% for this sample.

2. Since a concentrated stock of GroEL is added to the cuvet containing the substrate protein, the substrate is only slightly diluted, which need not be corrected in the analyses. However, the correction for substrate dilution is straightforward in several numerical analysis programs using column format data (e.g., KaleidaGraph, SigmaPlot, Excel) or by using nonlinear least-squares analysis with multiple independent variables (46).
3. The significant light scattering seen for concentrated GroEL solutions necessitates correction of absorption spectra to estimate protein concentrations accurately. The scattering causes an increase in absorbance, which is proportional to $1/\lambda^4$ (47). The correction is performed by first obtaining an absorption spectrum of GroEL from 400 to 230 nm. Then, the data from 400 to 350 nm are used to estimate the scattering contribution in the absorbing region by transforming them as absorbance vs $1/\lambda^4$ and fitting to a straight line. The fitted parameters are then used to estimate the scattering contribution to the absorbance, which is subtracted from the original data. An example that shows the results of this procedure, including the original spectrum, scattering contribution, and corrected spectrum for GroEL is shown in **Fig. 2**.



4. Although the binding stoichiometry can be easily determined by visual inspection for tight binding substrates, it is difficult to assess for weakly bound protein. For example, a clear break or inflection point that approximates n can be seen in the raw fluorescence data for nonnative subtilisin presented in **Fig. 1A**. However, the stoichiometry is more difficult to determine in **Fig. 3A** for nuclease. This is primarily owing to the decrease in fluorescence that occurs for nuclease when it binds to GroEL, which is superimposed on the increasing signal resulting from scattering. Thus, estimating n solely by visual inspection can be misleading, especially in cases where substrates bind relatively weakly to the chaperonin or when the overall fluorescence change is a composite of an increase and a decrease in the signal. An analysis of the nuclease data in terms of **Eq. 4** still applies, yielding a negative value for the maximal fluorescence change on binding, but a positive value for the scattering factor C owing to GroEL addition.
5. An important consideration in these experiments is that the substrate and chaperonin concentrations are reasonably large in comparison to the binding constant. Thus, there is a high level of sensitivity, and it is clear that **Eqs. 3** and **4** must be used in the analysis. Decreasing the concentrations of substrates significantly below $1 \mu\text{M}$ may lead to ambiguities, however, since the detection of changes in substrate fluorescence may be limiting. Also, for weak binding substrates that undergo a fluorescence decrease on binding to GroEL, it may be unclear initially if the use of **Eqs. 3** and **4** are valid. In principle, it may be acceptable to use **Eq. 2** in a study of chaperonin–substrate interaction at low concentrations if one employs modified substrates that contain highly fluorescent chromophores for increased sensitivity.

Fig. 3. (Previous page). Effect of increasing GroEL concentration on the fluorescence of the staphylococcal nuclease variant L103A. Microliter aliquots of a stock GroEL solution (30–50 mg/mL) were added to a solution containing $1.0 \mu\text{M}$ nuclease, and the relative fluorescence decrease was measured at 334 nm on excitation at 295 nm. **Panel A** illustrates the unusual raw intensity change arising from the fluorescence decrease for nuclease upon binding to the chaperonin and the increase due to scattering from GroEL. **Panel B** shows the linear scattering contribution of 3.83×10^5 relative fluorescence $U/\mu\text{M}$ from GroEL (squares), and the resulting titration plot (diamonds). **Panel C** shows the corrected titration plot of the transformed data, which can be analyzed in terms of the quadratic in **Eq. 3a**. Nonlinear least-squares analysis of the data in panel A using **Eq. 4** yields a maximal fluorescence change of -2.13×10^5 , a dissociation constant of 95 nM, a scattering contribution as shown in panel B, and a value for n of 0.32 mol of GroEL needed to saturate $1.0 \mu\text{M}$ nuclease. This is equivalent to about 3.1 mol of nuclease bound/mol of GroEL oligomer, or a little more than one per toroid.

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Prevention of Rhodanese Aggregation by the Chaperonin GroEL

Frank Weber and Manajit Hayer-Hartl

1. Introduction

A common feature of molecular chaperones is their ability to recognize hydrophobic surfaces of unfolded proteins to which they can bind, and thus, stabilize unfolded polypeptides at various levels of conformational compactness (1,2). Depending on the substrate, different chaperone systems are required to allow folding to the native state. Some proteins fold with high yields in a chaperone-unassisted reaction (3,4) whereas other proteins exhibit highly aggregation-sensitive structures. These proteins generally tend to aggregate and show higher yields of refolding in the presence of chaperones.

GroEL, a member of the Hsp60 class of molecular chaperones, cooperates in prokaryotic cells with the Hsp10 cofactor, GroES. Substrate protein is bound in a molten globule state in the central cavity of GroEL (for review, see 2,5,6). Although GroEL-mediated folding of substrate is dependent on nucleotides and GroES, the prevention of aggregation of denatured substrate is achieved by GroEL alone. Stabilization of unfolded structures and the inhibition of aggregation are general activities of molecular chaperones, and thus, aggregation assays test for a basic chaperone activity. The procedure described here for the chaperonin GroEL and the denatured substrate protein, rhodanese, is applicable for a wide variety of substrates and chaperones.

The formation of protein aggregates can be detected by scattering of a light beam passing through the sample. The scattering causes a decrease in light intensity detected by absorbance measurement in a spectrophotometer.

The inhibition of aggregation on dilution of an unfolded protein into buffer containing chaperones depends on the concentration of substrate and chaperone as well as their binding constant. The substrate binding affinities of chap-

erones as well as the effect of cofactors on substrate binding can be assessed in a semiquantitative manner (7,8).

2. Materials

1. Denaturation buffer: 6 M guanidinium hydrochloride, 5 mM dithiothreitol (DTT). (*see Note 1*).
2. Assay buffer: 20 mM MOPS-NaOH, pH 7.2, 100 mM NaCl (*see Note 2*).
3. 10 μ M GroEL in assay buffer (for purification, *see Chapter 3*).
4. 50 μ M rhodanese in assay buffer (*see Note 3*).
5. 50 μ M bovine serum albumin (BSA) in assay buffer.
6. Distilled deionized water.
7. Methanol.
8. Spectrophotometer.

3. Methods

3.1. Denaturation of Rhodanese

1. Lyophilize 20 μ L of a 50 μ M rhodanese solution in assay buffer (*see Note 2*).
2. Add 20 μ L of denaturation buffer, and incubate at 25°C in a thermomixer (1400 rpm) or vortexer for 1 h. A 50 μ M stock solution of denatured rhodanese in 6 M guanidinium-hydrochloride, 5 mM DTT, 20 mM MOPS-KOH, pH 7.2, and 100 mM NaCl is obtained (*see Note 1*).

3.2. Aggregation assay

The following protocol describes the detection of aggregation by absorbance measurement at 320 nm in a total volume of 500 μ L at 25°C.

1. Dilute 30 μ L of 10 μ M GroEL into 570 μ L assay buffer to give a final concentration of 0.5 μ M GroEL (*see Note 2 and 4*).
2. Transfer this solution into a cuvet. Change the wavelength of the spectrophotometer to 320 nm (*see Note 5*) and set the absorbance to zero.
3. Remove and save the 0.5 μ M GroEL solution. Clean the cuvet with water and methanol, and dry carefully.
4. Place 5 μ L of denatured rhodanese (50 μ M) on the bottom of the clean and dry cuvette.
5. Add 495 μ L of 0.5 μ M GroEL solution into the cuvet, pipet quickly up and down, and start the continuous measurement at 320 nm immediately (*see Note 6*).
6. Record the absorbance for 30 min or until the signal plateaus.

Rhodanese aggregation in buffer alone and buffer containing BSA (*see Note 7*) should always be included in a series of measurements (*see Fig. 1*). The aggregation prevention by GroEL may also serve as a positive control when the chaperone function of other putative chaperones is being tested.

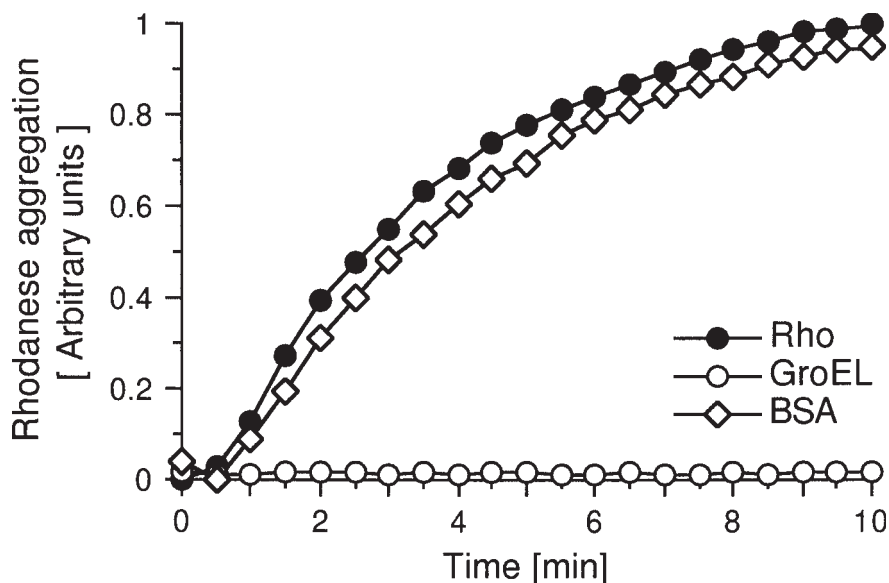


Fig. 1. Rhodanese aggregation monitored at 320 nm and 25°C for 0.25 μM rhodanese in buffer alone (filled circles) or buffer containing 0.25 μM GroEL (open circles) or 14 μM BSA (open diamonds).

These aggregation assays can be done with any substrate-protein which preferentially aggregates upon dilution from denaturant (9,10). If inhibition of aggregation is observed, it might be interesting to test whether the nonaggregated protein is refolded or maintained competent for refolding by other chaperones (7,8,10,11). Such additional experiments are useful to judge whether the aggregation is prevented or only retarded (10,11) (see Note 8).

4. Notes

1. Instead of 6 M guanidinium hydrochloride, rhodanese can be denatured in 8 M urea (12). To avoid carbamylation, urea solutions should be prepared fresh. Note that denaturation must occur in the presence of a reducing agent (DTT, β -mercaptoethanol). Otherwise, oxidation will prevent the reactivation of rhodanese.
2. Buffer conditions for aggregation assays are variable. In general, any buffer can be chosen, but note that the kinetics of aggregation are strongly effected by buffer conditions as well as temperature. Protein concentrations, buffer conditions, and temperature should be such that the aggregation is detected with appropriate kinetics. The detection of aggregation by light scattering is limited to aggregates above a certain size.
3. Bovine liver rhodanese, commercially available as lyophilized protein (Sigma, St. Louis, MO, type II, highly purified, #R1756), is dissolved in assay buffer and

centrifuged at 20,000g in a microcentrifuge tube for 10 min at 4°C to remove insoluble material. The supernatant is divided into aliquots and frozen at -80°C after the protein concentration has been determined spectroscopically using an $\epsilon_{280} = 60890 M^{-1} \text{ cm}^{-1}$ (**13**). Since this rhodanese is only ~60% pure, further purification by FPLC cation-exchange chromatography and gel filtration is recommended. The lyophilized protein purchased from Sigma is dissolved in buffer A (20 mM Na-acetate pH 5.0, 20 mM Na-thiosulfate) at a concentration of 10 mg/mL and 1 mL applied on a Mono-S HR 5/5 column (Pharmacia, Uppsala, Sweden) equilibrated in buffer A. The protein is eluted in buffer A with a 0–500 mM NaCl gradient. Fractions containing rhodanese are pooled and concentrated through centricon 10-filters (Amicon, Bedford, MA) to a final volume of 200 μL and applied on a Superdex 75 HR 10/30 column (Pharmacia), equilibrated in assay buffer. The protein is eluted in refolding buffer, and rhodanese-containing fractions are combined. Rhodanese can also be purified from an overexpression strain following published procedures (**14–17**).

4. Great care must be taken to ensure defined conditions. If proteins are stored as glycerol stocks or ammonium sulfate precipitates, the glycerol and ammonium sulfate may be removed by gel filtration, since they may effect the aggregation kinetics.
5. The detection wavelength may be set between 320 and 650 nm. However, shorter wavelengths are more sensitive for smaller aggregates (scattering $\propto \lambda^{-4}$).
6. Avoid air bubbles, since they create a very unstable background scatter, but a fast and thorough mixing is essential. It is important always to keep to the same start time of measurement after mixing.
7. Generally BSA is used as an unspecific control. However a protein of similar molecular weight might be chosen instead, since the corresponding concentration based on mg/mL may give different results to μM -based concentrations.
8. Small aggregates are not detected by light scattering. A putative chaperone might only slow down aggregation or stop aggregation after small oligomers are formed, which are prevented or slowed down to form bigger aggregates. In this situation, neither folding nor the stabilization of monomeric folding competent substrate-intermediates is achieved by the putative chaperone (**11**). However, for the interaction of, e.g., Hsp104, it has been shown that aggregation is stopped at the level of small aggregates, which can be refolded in concert with chaperones of the Hsp70 and Hsp40 families (**10**); *see also* (**8**).

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Refolding of Bovine Mitochondrial Rhodanese by Chaperonins GroEL and GroES

Frank Weber and Manajit Hayer-Hartl

1. Introduction

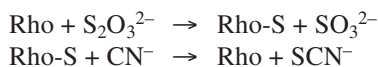
The *Escherichia coli* chaperonin GroEL and its cochaperone GroES facilitate protein folding in an ATP-dependent reaction cycle (for review, see **1,2**). GroEL is a cylindrical complex formed by two 7-mer-toroids, which are stacked back to back (**3,4**). Both toroids describe a central cavity in which denatured proteins are bound in a molten globule state. The bound substrate is sequestered inside the cavity after GroES, a dome-shaped seven-membered ring (**5**), closes the toroid in a *cis*-complex. Binding of GroES causes the release of substrate into the central cavity, which switches from a hydrophobic to a hydrophilic environment, allowing the enclosed substrate to acquire its native structure (**6–8**). This reaction is accompanied by ATP binding and hydrolysis in the substrate-bound ring. Subsequent ATP binding in the opposite ring causes the GroEL/GroES/substrate complex to dissociate and the native substrate is set free into solution (**9**). Proteins that do not reach their native state in one round of GroES binding and ATP hydrolysis rebind to GroEL on GroES release and undergo another folding cycle. Folding in a sequestered environment, effectively preventing aggregation throughout the folding pathway, facilitates high yields of chaperonin-assisted renaturation.

The function of GroEL/GroES (Hsp60/Hsp10) has been analyzed in vitro with model substrates, whose spontaneous refolding from the denatured state is insufficient owing to their tendency to aggregate. A physiologically relevant substrate is bovine rhodanese, a mitochondrial protein without a cleavable targeting sequence. It has been shown that folding of rhodanese on import into yeast mitochondria or expression in *E. coli* cytosol is dependent on Hsp60/

Hsp10 or GroEL/GroES respectively (**10,11**). The folding reaction requires adenine nucleotides (**12**). Renaturation at 25°C is faster in the presence of ATP than ADP, whereas at 37°C refolding with ADP is quite efficient (**13**).

The ability of GroEL-bound substrate to reach its native state inside the enclosed cavity of a GroEL/GroES complex (**3,6**) has been demonstrated for rhodanese (**7**). Sequestration of aggregation-sensitive folding intermediates in the GroEL/GroES folding cage is an essential characteristic of the chaperonin mechanism (**14**). Whether the specific environment of the GroEL/GroES cavity promotes the formation of productive folding intermediates remains to be investigated.

Bovine mitochondrial rhodanese is a monomeric protein (33 kDa) comprised of two domains with very similar fold, but low sequence homology (**15**). The catalytic site is formed by both domains with an essential residue, cystein 247, in the second domain (**16**). The domains are folded into a five-stranded parallel β -sheet surrounded by two helices on one side and three helices on the other (**15**). Rhodanese activity has been found in many tissues (**17,18**), and the name rhodanese (systematic name is thiosulfate:cyanide sulfurtransferase EC 2.8.1.1) had been coined by Lang in 1894 (**19,20**), because this enzyme catalyzes the formation of thiocyanide (“Rhodanid”) from thiosulfate and cyanide in the following two-step mechanism:



The sulfur-bound rhodanese (Rho-S) is more stable than free rhodanese (Rho).

After denaturation of rhodanese by guanidinium hydrochloride or urea, refolding can be monitored by following the recovery of rhodanese activity (**21**). Thiocyanide is easily detected colorimetrically by the formation of its red iron complex. The unassisted refolding of rhodanese as well as refolding in the presence of detergents or lipids has been studied (**22–26**). For most conditions, refolding of denatured rhodanese is inefficient owing to aggregation, whereas the presence of detergents as well as chaperonin-assisted refolding allows high yields of recovery (**12,27**).

2. Materials

2.1. Rhodanese Refolding Assay

1. Denaturation buffer: 6 M guanidinium hydrochloride, 5 mM dithiothreitol (DTT) (see **Note 1**).
2. Refolding buffer: 20 mM MOPS-NaOH, pH 7.2, 100 mM NaCl, 10 mM KCl, 5 mM Mg-acetate (see **Note 2**).
3. 1 M Na-thiosulfate (dissolved in refolding buffer, prepare fresh before use).

4. 0.1 M ATP.
5. 25 μ M GroEL in refolding buffer (for purification, *see* Chapter 3).
6. 25 μ M GroES in refolding buffer (for purification, *see* Chapter 4).
7. 25 μ M rhodanese in refolding buffer (*see* **Note 3**).
8. Distilled deionized water.
9. Heating block.
10. Vortexer.

2.2. Rhodanese Activity Assay

1. 1 M Potassium cyanide (KCN).
2. 1 M Na-thiosulfate (prepare fresh before use).
3. 0.8 M KH_2PO_4 .
4. 0.4 M trans-1,2-Diamino-cyclohexane-N,N,N',N'-tetraacetic acid (CDTA), pH 8.0.
5. 15% Formaldehyde (prepare fresh before use).
6. Ferric nitrate reagent (prepare this reagent in a fume hood): Slowly add 100 mL of 65% HNO_3 to a stirring solution of 50 g $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ in 600 mL distilled water. Adjust to 750 mL, and filter the solution. The solution is stable for months.
7. Distilled deionized water.
8. Timer.
9. Spectrophotometer.

3. Methods

3.1. Denaturation of Rhodanese

1. Lyophilize 20 μ L of a 25 μ M rhodanese stock solution in refolding buffer (*see* **Note 3**).
2. Add 20 μ L of denaturation buffer, and incubate at 25°C in a thermomixer (1400 rpm) or vortexer for 1 h. A 25 μ M stock solution of denatured rhodanese in 6 M guanidinium-hydrochloride, 5 mM DTT, 20 mM MOPS-NaOH, pH 7.2, 100 mM NaCl, 10 mM KCl, and 5 mM Mg-acetate is obtained (*see* **Note 1**).

3.2. Rhodanese Refolding Assay

1. Mix in a 1.5 mL microcentrifuge tube at 25°C:
 - 235 μ L of refolding buffer.
 - 5 μ L of 1 M Na-thiosulfate (freshly prepared).
 - 2.5 μ L of 25 μ M GroEL.
 - 5 μ L of 25 μ M GroES.giving final concentrations of 20 mM MOPS-NaOH, pH 7.2, 100 mM NaCl, 10 mM KCl, 5 mM Mg-acetate, 20 mM Na-thiosulfate containing 0.25 μ M GroEL and 0.5 μ M GroES (*see* **Notes 2** and **4**).
2. Add 247.5 μ L of this mixture to 2.5 μ L of denatured rhodanese (final 0.25 μ M) (*see* **Note 5**). Mix rapidly by vortexing or pipeting up and down, but avoid air bubbles (*see* **Note 6**).

3. To determine rhodanese activity at 0-min time-point, withdraw 10 μL for the rhodanese activity assay described in **Subheading 3.3**.
4. Add 2.4 μL ATP of a 0.1 *M* stock solution to the refolding mixture giving a final ATP concentration of 5 *mM* and start timer. The addition of ATP will start the chaperonin-assisted refolding reaction which is carried out at 25°C.
5. At various timepoints take 10 μL aliquots and transfer them immediately to the rhodanese activity assays (*see Subheading 3.3*). Record the refolding reaction for 60–80 min. The refolding reaction is usually complete after approx. 45 min with $T_{1/2} \sim 10$ min at 25°C.

A control reaction containing native rhodanese is treated the same way using a corresponding amount of native rhodanese in a reaction without GroEL and GroES.

To test for spontaneous refolding of denatured rhodanese in the absence of GroEL and GroES, the refolding reaction is started by dilution of denatured rhodanese into refolding buffer to which ATP has been added.

3.3. Rhodanese Activity Assay

The amount of native rhodanese accumulated during the time-course of refolding is detected by enzyme activity assay (*see Note 7*).

1. Each 10 μL aliquot of the refolding reaction, withdrawn at various timepoints, is immediately transferred to prepared microcentrifuge tubes containing the following assay mixture, preequilibrated at 25°C for 5 min:
 - 10 μL of 1 *M* KCN.
 - 10 μL of 0.8 *M* KH_2PO_4 .
 - 10 μL of 1 *M* Na-thiosulfate.
 - 5 μL of 0.40 *M* CDTA.
 - 155 μL distilled deionised water.A total volume of 200 μL is obtained containing final concentrations of 50 *mM* KCN, 50 *mM* Na-thiosulfate, 40 *mM* KH_2PO_4 , and 10 *mM* CDTA (*see Note 8*) and rhodanese protein from the refolding reaction, which catalyzes the formation of thiocyanide when the enzyme is native. The presence of CDTA stops the GroEL-mediated refolding of rhodanese by chelating magnesium ions and inhibiting the GroEL-ATPase (*see Note 2*).
2. Incubate for 3 min at 25°C.
3. Stop the activity measurement with 100 μL of 15% formaldehyde, and vortex briefly. After addition of formaldehyde samples can be stored until the time-course is complete.
4. At the end of the time-course, 300 μL of ferric nitrate reagent are added to each sample, incubated for 5 min, and centrifuged in an Eppendorf benchtop centrifuge at 14,000 rpm for 10 min at room temperature.
5. The red iron–thiocyanide complex in the supernatant is quantitated spectroscopically by absorbance reading at 460 nm (*see Note 9*). The color is stable for at

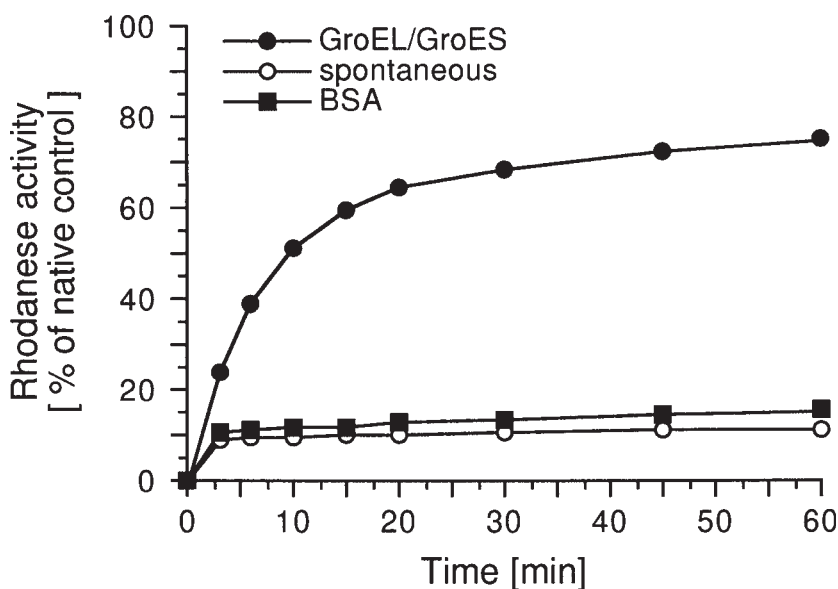


Fig. 1. Refolding of rhodanese by GroEL/GroES with control experiments for spontaneous refolding and spontaneous refolding in the presence of bovine serum albumin (BSA).

least 1 h. Subtract the absorbance reading of the 0-min time-point, which should be the same as a reagent blank.

For the 0-min time-point the formaldehyde is added to the activity mixture prior to addition of the 10 μ L sample from the refolding assay. This is particularly important for the control of spontaneous rhodanese refolding, since otherwise spontaneous refolding can occur during the activity measurement.

The control of spontaneous rhodanese refolding should always be included in a refolding assay with GroEL/GroES (see **Fig. 1**). Refolding yields are usually presented as percent of native control measured with a corresponding concentration of native rhodanese under the same conditions (see **Note 7**).

Since GroEL/GroES mediated refolding of rhodanese is very reproducible with high refolding yields, results are sometimes presented as percent of final yield of refolded rhodanese in the GroEL/GroES-assisted reaction. This is useful in particular if rhodanese is purified by overexpression under denaturing conditions from inclusion bodies and a native control is not available.

4. Notes

1. Instead of 6 M guanidinium hydrochloride, rhodanese can be denatured in 8 M urea (21). To avoid carbamylation, urea solutions should be prepared fresh. Note that

denaturation must occur in the presence of a reducing agent (DTT, β -mercaptoethanol). Otherwise, oxidation will prevent the reactivation of rhodanese.

2. Buffer conditions for refolding reactions are variable. 3-[N-Morpholino]propane-sulfonic acid (MOPS) or Tris buffer can be used in a pH range between 7.0 and 8.0. The addition of Na-thiosulfate stabilizes the native rhodanese by formation of Rho-S, which is more stable. Na-thiosulfate also prevents inactivation of native rhodanese by oxidation, and addition of further reducing agents, e.g., DTT or β -mercaptoethanol, is not necessary.

The presence of potassium and magnesium ions is essential for nucleotide binding and hydrolysis by GroEL (28,29). The recommended minimum concentrations are 5 mM KCl and 2 mM Mg-acetate or MgCl. Sodium chloride is added to give a total salt concentration, generally in the range of 50–200 mM.

It has been reported that the ratio of asymmetric GroEL:GroES complexes to symmetric GroEL:GroES₂ complexes changes with magnesium and ATP concentrations (30–35). When higher magnesium concentrations are used, the concentration of CDTA in the enzyme activity assay needs to be adjusted. CDTA is necessary to chelate magnesium ions, thus stopping the GroEL/GroES-mediated refolding reaction.

3. Bovine liver rhodanese, commercially available as lyophilized protein (Sigma, type II, highly purified, #R1756), is dissolved in refolding buffer and centrifuged at 20,000g in a microcentrifuge tube for 10 min at 4°C to remove insoluble material. The supernatant is divided into aliquots and frozen at –80°C after the protein concentration has been determined spectroscopically using an $\epsilon_{280} = 60890 \text{ M}^{-1} / \text{cm}$ (36). Since this rhodanese is only ~60% pure, further purification by FPLC cation-exchange chromatography and gel filtration is recommended. The lyophilized protein purchased from Sigma is dissolved in buffer A (50 mM Na-acetate, pH 5.0, 20 mM Na-thiosulfate) at a concentration of 10 mg/mL and 1 mL applied on a Mono-S HR 5/5 column (Pharmacia) equilibrated in buffer A. The protein is eluted in buffer A with a 0–500 mM NaCl gradient. Fractions containing rhodanese are pooled and concentrated through centricon 10-filters (Amicon) to a final volume of 200 μL and applied on a Superdex 75 HR 10/30 column (Pharmacia), equilibrated in refolding buffer. The protein is eluted in refolding buffer, and rhodanese-containing fractions are combined. Rhodanese can also be purified from an overexpression strain following published procedures (37–40).
4. The ratio of GroEL:GroES is usually 1:2 according to the expected physiological ratio of both chaperones in the cell. GroEL/GroES-mediated refolding of rhodanese is efficient at a rhodanese:GroEL ratio of 1:1, but higher concentrations of GroEL can be used.
5. The concentration of rhodanese in the refolding assay may be crucial for the interpretation of the results. Refolding assays described in the literature have rhodanese concentrations generally in the range of 0.1–1.5 μM . Rhodanese concentrations of at least 0.25 μM are recommended (14,25) to strengthen the requirement for sequestration of denatured rhodanese by the chaperonin over more unspecific aggregation inhibiting effects.

The GroEL/GroES-assisted refolding is 80–90% efficient compared to the native control. Spontaneous refolding of rhodanese under most conditions is only ~10%. However, at very low rhodanese concentrations (25) and buffer conditions that stabilize the native rhodanese, spontaneous refolding yields may increase to ~50%. Similar conditions may result in high yields of refolding owing to unspecific interactions with proteins like BSA or casein. If low rhodanese concentrations cannot be avoided, the use of siliconized tubes and the addition of reducing agents are suggested to avoid irreversible inactivation of rhodanese by surface adsorption and oxidation.

Further, it is advised to supplement the refolding data with aggregation assays performed under the same conditions used for rhodanese refolding. These assays are described in an accompanying chapter ?? of this volume. Aggregation should be clearly observed when rhodanese is diluted into buffer under refolding conditions in the absence of GroEL/GroES. The aggregation should be prevented in the presence of a stoichiometric amount of GroEL, but only minor effects on the aggregation behavior should be observed in the presence of corresponding amounts of BSA or casein.

6. Rapid mixing of rhodanese in buffer is achieved by adding denatured rhodanese into a fresh microcentrifuge tube followed by the addition of buffer and up-and-down pipeting of the solution. Alternatively, a drop of denatured rhodanese is put on the wall of a microcentrifuge tube, which contains the buffer, and fast mixing is achieved by vortexing.
7. Possible effects of carryover of denaturant as well as linearity of the enzyme assay with time and protein concentration should be controlled. If recovery of native rhodanese is low or inconsistent:
 - a. Add Na-thiosulfate (20–50 mM) to the refolding assay, since the formation of Rho-S has a stabilizing effect.
 - b. Add reducing agents (5 mM DTT or 200 mM β -mercaptoethanol) to the refolding buffer, since they prevent inactivation by oxidation (21).
 - c. Siliconized tubes may prevent loss of rhodanese by surface adsorption which may be a problem particularly at low rhodanese concentrations (*see also* Note 5).
 - d. If the native control loses activity during the time-course, the addition of BSA might stabilize rhodanese.
8. Rhodanese enzyme activity assays were described by Sörbo (41) and Westley (42). They may be performed at 25 or 37°C, at optimum pH 8.6 (0.8 M potassium-phosphate buffer can be used instead of 0.8 M KH_2PO_4). If KH_2PO_4 is used, the final pH of the assay mixture should not be below 6.0. The assay components should be mixed and aliquoted just before use. Na-thiosulfate solutions should always be prepared fresh.
9. If the measured rhodanese activity is too high or too low for the absorbance reading, adjustments can be made by varying the amount of refolding sample added to the activity mixture, the temperature at which the enzyme activity assay is performed (25 or 37°C) or the time the activity assay is stopped with formaldehyde.

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Assay of Malate Dehydrogenase

A Substrate for the E. coli Chaperonins GroEL and GroES

Manajit Hayer-Hartl

1. Introduction

Malate dehydrogenase (MDH) is found in all eukaryotic cells as two isozymes: mitochondrial (mMDH) and cytoplasmic (soluble, sMDH) (*1*). In contrast, prokaryotes contain only a single form of MDH. The crystal structures of MDH from *Escherichia coli* (*2,3*), porcine cytoplasm (*4*), porcine mitochondria (*5,6*), and *Thermus flavus* (*7*) have been solved and are essentially identical. Refer to ref. (*8*) for a comprehensive review on MDH.

Pig heart mitochondrial MDH has been extensively studied and has been the substrate used in several refolding studies with chaperones (*9–20*). It consists of two identical subunits of about 35,000 dalton (*21*) with two coenzyme binding sites per dimer (*22*). Each subunit functions independently in terms of catalysis with no evidence of cooperativity between catalytic sites (*23*). MDH catalyzes the interconversion of L-malate and oxaloacetate using β -nicotinamide adenine dinucleotide (β -NAD) as a coenzyme:



MDH activity is assayed photometrically by measuring the decrease in absorbance at 340 nm resulting from the oxidation of β -NADH (reduced nicotinamide adenine dinucleotide). The pH optimum for the reaction is 7.4–7.5. Phosphate, magnesium, zinc, and arsenate ions are activators of mMDH (*24*).

Here a method is described for the formation of a denatured state of mMDH that does not appear to aggregate rapidly (*18*) but constitutes a substrate for chaperonin-mediated refolding.

2. Materials

1. Siliconized microcentrifuge (sMC) tubes (*see Note 1*).
2. 2X buffer A: 40 mM 3-[N-morpholino]propane-sulfonic acid (MOPS)/KOH, pH 7.4, 200 mM KCl, 4 mM Mg(OAc)₂ (*see Note 2*).
3. 6 M guanidinium hydrochloride in buffer A.
4. 1 M dithiothreitol (DTT) in distilled deionized water.
5. Pig heart mitochondrial MDH (Sigma, St. Louis, MO) 410-13, suspension in 2.8 M [NH₄]₂SO₄.
6. Chaperonins: 25 μM GroEL and 100 μM GroES (*see Note 3*).
7. Bovine serum albumin (BSA) (Sigma, A-3059), 20 mg/mL in buffer A.
8. 0.2 M ATP (Sigma, A-5394), pH 7.5, with NaOH.
9. ATP-regenerating system (*see Note 4*):
 - 1 mg/mL pyruvate kinase (Roche Diagnostics GmbH) in distilled deionized water.
 - 0.5 M phosphoenolpyruvate (Calbiochem) in distilled deionized water.
10. 10 mM β-NAD reduced form (β-NADH) (Sigma, N 8129) (freshly prepared in buffer A) (*see Note 5*).
11. 25 mM oxaloacetate (freshly prepared in buffer A and stored in an ice bath during use) (*see Note 6*).
12. 0.45 M trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid (CDTA).
13. Disposable microcuvets.

3. Method

3.1. Preparation of Native mMDH and Denatured mMDH

One hundred microliters of 12 mg/mL mMDH suspension in 2.8 M (NH₄)₂SO₄ is centrifuged at 10,000g for 15 min at 4°C, the supernatant discarded, and the pellet resuspended in 50 μL buffer A. The protein concentration is determined spectroscopically using ε₂₈₀ as 7680 M⁻¹cm⁻¹. A concentration in the range of 450–500 μM is obtained. This solution is diluted to 50 μM native mMDH (N-MDH) in buffer A and 50 μM denatured mMDH (D-MDH) in buffer A containing 3 M guanidinium hydrochloride/5 mM DTT (*see Note 7*). To ensure complete denaturation, D-MDH should be incubated at 37°C for at least 30 min before use.

3.2. mMDH Refolding

For a 100 μL reaction, mix in 1.5 mL sMC tubes (*see Note 8*):

Reaction	1	2	3	4
2X buffer A	50	50	50	50 μL
Distilled deionized water	42	42	36	37.5 μL
25 μM GroEL (final 1 μM)	—	—	4	— μL
100 μM GroES (final 2 μM)	—	—	2	— μL
300 μM BSA (final 14 μM)	—	—	—	4.5 μL
1 mg/mL pyruvate kinase (final 20 mg/mL)	2	2	2	2 μL
0.5 M phosphoenolpyruvate (final 10 mM)	2	2	2	2 μL
0.2 M ATP (final 1 mM)	2	2	2	2 μL

Incubate at 37°C for at least 5 min. Transfer the respective 98 μL mix to sMC tubes containing:

50 μM N-MDH (final 1 μM)	2	—	—	— μL
50 μM D-MDH (final 1 μM)	—	2	2	2 μL

and simultaneously vortexing gently to mix thoroughly. At the zero time point, remove 5 μL for immediate assay (*see below*) and/or 10 μL into an sMC tube containing 2 μL 0.45 M CDTA (quenches the chaperonin reaction instantaneously; *see Note 9*) and incubate for 3–5 h (*see Note 10*). Continue to incubate the remainder of the reaction at 37°C for 30–60 min, and at various time-points, remove aliquots for immediate assay and/or transfer into CDTA.

3.3. Assay for mMDH Activity

- The assay is performed at 25°C in the spectrophotometer. Add 5 μL of refolding reaction (**Subheading 3.2.**) to a disposable microcuvet containing 446 μL of the assay mixture (*see Note 11*):
 - 400 μL buffer A.
 - 1 μL 0.45 M CDTA (final 1 mM).
 - 25 μL 20 mg/mL BSA (final ~1 mg/mL).
 - 10 μL 10 mM β -NADH (final 220 μM).
 - 10 μL 25 mM oxaloacetate (final 550 μM).
- Mix thoroughly by inverting cuvet sealed with parafilm, and monitor the decrease in absorbance at 340 nm over time (1–2 min). The slope of the line reflects the rate of β -NADH oxidation. The refolding yields are expressed as a percentage of N-MDH control activity. For the chaperonin-assisted folding of mMDH, the yield is generally between 40 and 60%.

4. Notes

- Siliconized MC tubes are used to prevent unspecific binding of MDH to tube walls and, hence, loss of material.
- Several different buffers have been used in the literature, ranging from pH 7.2 to 7.8 (**12,16,25,26**).
- The concentration of the chaperonins used may vary.
- Two most common ATP-regenerating systems used in the literature are the creatine kinase/phosphocreatine or pyruvate kinase/phosphoenolpyruvate combination.
 - Stocks for creatine kinase/phosphocreatine ATP regenerating system: 10 mg/mL creatine kinase (Roche Diagnostics GmbH) in 20 mM HEPES-KOH, pH 7.5, 1 M phosphocreatine (Roche Diagnostics GmbH) in 20 mM HEPES-KOH, pH 7.5, made into 10–20 μL aliquots, frozen, and stored at -80°C . Final concentrations in the assay are: 50 μg creatine kinase/mL of refolding reaction and 8 mM phosphocreatine.
 - Stocks for pyruvate kinase/phosphoenolpyruvate ATP-regenerating system: 1 mg/mL pyruvate kinase (Boehringer Mannheim) in double-distilled water,

0.5 M phosphoenolpyruvate (Calbiochem) in double-distilled water, made into 10–20 μL aliquots, frozen, and stored at -80°C . Final concentrations in the assay are: 20–30 μg pyruvate kinase/mL of refolding reaction and 5–10 mM phosphoenolpyruvate.

5. β -NADH should be dissolved in buffer (not in water) as it is decomposes in acid pH.
6. Oxaloacetate is unstable. It spontaneously decarboxylates to pyruvate (up to 10% decomposition within 30 min at 25°C) (27). It is recommended to use a fresh solution within 10 min. Oxaloacetate may also be generated *in situ* with glutamate-oxaloacetate transaminase (GOT) (28).
7. N-MDH and D-MDH are prepared fresh daily.
8. These are the four basic reactions to perform in any investigation to test the refolding of mMDH. Reaction 1 is the folded 100% native control. Reaction 2 is the fraction D-MDH that refolds spontaneously. Reactions 1 and 2 should not change with time. Reaction 3 is the fraction of D-MDH that refolds in the chaperonin-assisted reaction. In reaction 4 BSA (or casein) is used to test the effect of an unrelated protein on the folding of mMDH.
9. CDTA chelates the magnesium that is necessary for the ATP binding and hydrolysis of the GroEL-GroES-mediated refolding reaction .
10. The active form of MDH is the dimer. The refolding/assembly reaction is described as follows (16,20,25,29):



At low monomer concentrations of mMDH ($<0.2 \mu\text{M}$), the rate-limiting step is the formation of dimers, and it has been shown that under these conditions, it may take 3–5 h to regain activity. With concentrations of MDH $\geq 0.5 \mu\text{M}$, activities measured immediately after chaperonin-assisted refolding are virtually identical to those measured after 3–5 h of incubation.

11. A blank is read with assay mixture without addition of β -NADH. The first four components of the assay mixture are in the cuvet, and just before addition of the refolding reaction, the oxaloacetate is added (*see also Note 6*).

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Assay of Chaperonin-Assisted Refolding of Citrate Synthase

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1. Introduction

Chaperonins assist protein refolding by mechanisms that are poorly understood. In conditions under which a given protein exhibits chaperonin-dependent folding, the protein aggregates irreversibly in the absence of chaperonins. The *Escherichia coli* Hsp60, GroEL, displays a hydrophobic surface that binds folding intermediates. ATP-dependent binding of the *E. coli* Hsp10, GroES, promotes a conformational change in GroEL and simultaneously encloses the folding protein. The primary role of a chaperonin is to prevent aggregation and possibly to unravel misfolded protein-folding intermediates (1–3). Pig heart mitochondrial citrate synthase (CS) is a dimer of 50-kDa subunits (4,5). It has been shown that GroEL and GroES suppress aggregation of CS up dilution from denaturing solutions (6).

We and others have shown that chaperonin-dependent CS refolding absolutely depends on both GroEL and GroES components (7,8). For example, although GroEL can bind unfolded CS and suppress its aggregation, essentially none of the CS refolds in the absence of ATP or GroES. It has been shown that a single CS molecule may undergo several cycles of GroEL binding and GroES-dependent release before achieving the native state (9). Thus, CS serves as a useful substrate for assay of chaperonin-folding assistance.

2. Materials

1. Pig heart CS 10 mg/mL ammonium sulfate slurry (see **Note 1**) (Boehringer Mannheim Corp., Mannheim, Germany).
2. Denaturing buffer: 6 M guanidine hydrochloride (GdnHCl), 50 mM Tris-HCl, 125 mM NaCl, pH 7.5.

3. 4 mM oxaloacetic acid (OAA) (Boehringer Mannheim, Mannheim, Germany).
4. 1.6 mM acetyl-coenzyme A (acetyl-CoA) (Boehringer).
5. 50 mM dithiothreitol (DTT) (Boehringer).
6. Hsp60 prepared as described by Viitanen et al. (*see Note 2*) (*10*).
7. *E. coli* GroEL and GroES prepared as described (*11*).
8. Human cpn10 prepared as described (ref. *12* and the accompanying article).
9. 1 M potassium phosphate buffer, pH 7.4.
10. 20 mM potassium phosphate buffer, pH 7.4.
11. 0.5 M ethylene diamine tetracetic acid-NaOH (EDTA), pH 8.0.
12. 100 mM ATP (disodium salt) (Sigma, St. Louis, MO).
13. 1 M MgCl₂ (Fisher Biotech).
14. Centricon concentrators, 30,000-Dalton cutoff (Amicon, Bedford, MA).

3. Methods

3.1. Preparation of Protein

Aliquots of the ammonium sulfate slurry (typically 400 μ L) of CS, GroEL, Hsp60, and GroES are centrifuged in 1.5-mL tubes at 16,000g for 10 min (*see Note 3*). The supernatants are discarded, and the pellets are resuspended with 400 μ L 20 mM potassium phosphate buffer, pH 7.4. Each solution is transferred to a Centricon concentrator and brought to a volume of 1.5 mL with 20 mM potassium phosphate buffer, pH 7.4, and centrifuged at 5000g for 30 min at 4°C (this step was repeated three times) (*see Note 4*). Samples are collected using the manufacture's protocol and brought to 400 μ L with 20 mM potassium phosphate buffer, pH 7.4. Concentrations are determined by UV absorption at 280 nm of stock solutions diluted at least 100-fold in denaturing buffer using extinction coefficients determined by amino acid analysis (*see Note 5*). An aliquot of the CS solution is diluted to a final concentration of 67.8 mM.

3.2. Denaturation of Citrate Synthase

CS is denatured for 30 min at room temperature by combining 20 μ L of 67.8 μ M CS with 23 mg solid GdnHCl, 2.4 μ L 50 mM DTT, and 1.6 μ L 50 mM EDTA. The final concentrations are 33.9 μ M CS, 6 M GdnHCl, 3 mM DTT, and 2 mM EDTA in a volume of 40 μ L. An additional mock denaturation mixture is prepared by substituting 20 μ L potassium phosphate buffer, pH 7.4, CS.

3.3. Renaturation of Citrate Synthase

While vortexing, 2.4 μ L of denatured CS are added to a solution containing 0.02 M potassium phosphate buffer, pH 7.4, 10 mM MgCl₂, 1 mM OAA, and 4.2 μ M of GroEL or Hsp60 and 8.4 μ M of GroES or Hsp10, resulting in a dilution of the denaturation mix by 166-fold (*see Note 6*). ATP is added to a final concentration of 2 mM to initiate chaperonin-dependent refolding, and

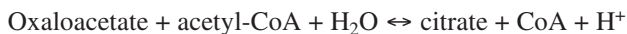
the reaction is incubated at 37°C for 1 h (*see Note 7*). The final CS concentration in the renaturation reaction is 0.2 μM (monomer). The CS/chaperonin ratio is 1:21, and the cochaperonin/chaperonin ratio is 2:1 (monomer) (*see Note 8*). The percent recovered CS activity is normalized to the activity (100%) of native CS subjected to mock renaturation (refolding mixture containing 2.4 μL mock denaturation mix).

3.4. Assay Mixture

In a quartz cuvet, 100 μL 4 mM OAA, 100 μL 1.6 mM acetyl-CoA, 750 μL 20 mM potassium phosphate buffer, pH 7.4 (all pre-equilibrated to room temperature), and 50 μL refolding mixture are combined, quickly mixed, and the absorbance is monitored at 233 nm with the spectrophotometer in time-base mode for 0–90 seconds (*see Note 9*).

3.5. Enzyme Assay

CS catalyzes the condensation of acetyl-CoA and OAA in the following reaction (4):



CS activity is measured by the decrease of absorption at 233 nm with the disappearance of acetyl-CoA (13). The initial slope is determined from a 10-s segment of the data that was judged to be linear by inspection of the graph.

The percentage of CS recovered after renaturation is calculated by dividing the initial slope by the slope of the mock-renatured CS (Fig. 1). The yield of CS renatured in the absence of chaperonins (spontaneous refolding) decreases with temperature (Taher et al., unpublished results), possibly owing to stronger hydrophobic interactions. Spontaneous refolding of CS is typically 20–30% at 27°C and 5–10% at 37°C. In the example described, the assay was carried out at 37°C to minimize the “background” level of spontaneous refolding (here 10% Fig. 1). A typical standard deviation in yield for three or more identical renaturation reactions is 5% (data not shown).

In Fig. 1, Hsp10 can substitute for GroES with GroEL, but the reverse is not true for Hsp60. Eukaryotic Hsp60s appear to be more specific for the counterpart Hsp10 than GroEL (10,14,15).

4. Notes

1. CS is provided as an ammonium sulfate slurry and must be prepared fresh daily. We have observed that newer lots of CS perform better than older lots. We typically order only as much CS as will be used within 2 mo time.
2. Hsp60 was supplied at a concentration of 355 μM in buffer.
3. 400 μL of CS slurry typically yield enough protein for 16 refolding reactions (~300 μM of CS).

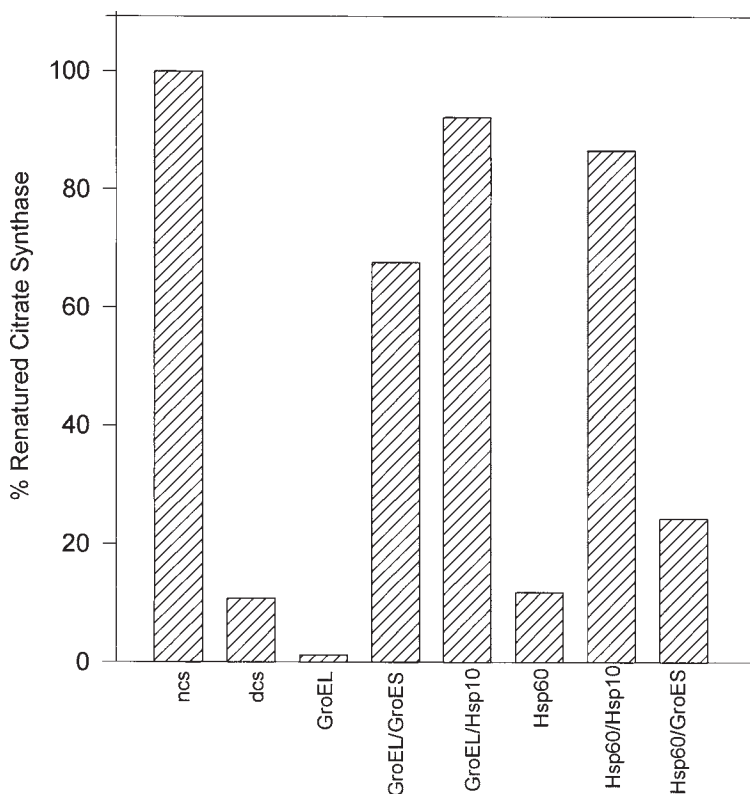


Fig. 1. Yield of CS after renaturation with various chaperonin combinations. Native CS (0.2 mM), mock-denatured CS (0.2 μ M); denatured CS (0.2 μ M), CS renatured in the absence of chaperonins (spontaneous refolding); GroEL, CS renatured in the presence of GroEL (4.2 μ M); GroEL/GroES, CS renatured with the *E. coli* chaperonins (4.2:8.4 μ M); GroEL/Hsp10, CS renatured with *E. coli* GroEL (4.2 μ M) and human Hsp10 (8.4 μ M); Hsp60, CS renatured in the presence of human Hsp60 (4.2 μ M); Hsp60/Hsp10, CS renatured with the human chaperonins (4.2:8.4 μ M); Hsp60/GroES, CS renatured with human Hsp60 (4.2 μ M) and *E. coli* GroES (8.4 μ M).

- This buffer exchange removes residual $(\text{NH}_4)_2\text{SO}_4$. The GroEL ATPase activity is dependent on K^+ (16). 0.1 M Tris-HCl (pH 8.0) can be substituted for K-PO_4 , and K^+ may be included in the desired reactions.
- The extinction coefficients is determined by Beer's Law: $A = \epsilon cl$; where A is absorbance at a specified wavelength, c = concentration in mol/L, and l = path length in cm. ϵ has the units $\text{M}^{-1}\text{cm}^{-1}$. Once the extinction coefficient is known, the concentration from this lot of protein can be determined by absorbance measurement and using the above equation. For this chapter, ϵ_{280} is CS = 8.9×10^4 ; GroEL = 13,606; Hsp60 = 6135; GroES = 2500; and Hsp10 = 9111.

6. The final volume once all the components are added is 400 μL . We find that use of volumes $<400 \mu\text{L}$ degrades reproducibility, presumably because of inconsistent mixing. Reactions were prepared 5 min apart to allow an incubation time of exactly 1 h prior to assay. After dilution of CS into the renaturation mix, the GdnHCl concentration is 36 mM. GdnHCl inhibits chaperonin-assisted refolding of RuBisCo at concentrations of 30 mM or more (17). Although similar levels of urea do not inhibit chaperonin-assisted refolding of RuBisCO (17), we find that the yield of CS is much greater after denaturation in GdnHCl as compared to denaturation in urea (7).
7. In the absence of ATP, CS complexes with Hsp60 are stable for hours. In the presence of ATP, the CS rapidly dissociates from Hsp60 without refolding, and misfolds or aggregates.
8. Previous studies have shown that chaperonin-assisted protein folding is most efficient in conditions that favor formation of the symmetric GroEL₁₄(GroES₇)₂ complex, as opposed to the asymmetric GroEL₁₄GroES₇ complex (18). We have found that GroES/GroEL ratios in excess of 1:1 are inhibitory when CS is added after GroES and ATP, presumably because symmetric complexes block CS binding to GroEL; therefore, ATP is added last. We have not observed inhibition by the cochaperonin with eukaryotic Hsp60/Hsp10.
9. The spectrophotometer should be zeroed at 233 nm with 100 μL 4 mM OAA and 900 μL 20 mM potassium phosphate buffer, pH 7.4.

Acknowledgments

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Purification of Yeast Mitochondrial Hsp60

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1. Introduction

Hsp60 from yeast is a close homolog of bacterial GroEL and located in the matrix of mitochondria. Yeast mitochondrial hsp60 is encoded by an essential gene and its expression is induced two- to threefold upon heat shock (1). Like its bacterial counterpart, hsp60 consists of 14 identical subunits of about 60 kDa that form a double-ring structure in yeast. The homolog from mammalian mitochondria has been found to be stable and functional as a 7 mer (2). The ATPase rate of yeast hsp60 based on protomer at 25°C is 2.5 min⁻¹. ATP hydrolysis is reduced to approximately half of this value by the cochaperonin hsp10 that binds to hsp60 in the presence of adenosine nucleotide. Hsp60 mediates the refolding; of mammalian mitochondrial malate dehydrogenase in vitro (3).

The mitochondrial system has been particularly useful for studying protein folding reactions under conditions that resemble most closely the in vivo situation. Isolated mitochondria are physiologically active and allow for the import of mitochondrially targeted proteins. During import, proteins unfold and thus have to refold in the matrix space. It is now clear that hsp60 mediates this refolding reaction for a subset of mitochondrially localized proteins either alone or in concert with its partner protein hsp10. Some of these in vivo substrates have been recently identified (4,5). One of the major topics in the chaperonin field remains the characterization of chaperonin-mediated folding of in vivo substrates. We describe here a large-scale purification procedure of hsp60 from *S. cerevisiae* expressed in the cytosol of the yeast *Pichia pastoris*. Hsp60 produced in this heterologous system is active in refolding of substrate proteins in vitro and assembles into a 14-mer double-ring. This finding is noteworthy, since inside the matrix of mitochondria, hsp60 requires pre-existing hsp60 to fold and assemble correctly (6).

2. Materials

2.1. Growth and Induction of Mature hsp60

1. *P. pastoris* strain YJP1, expressing mature yeast hsp60 (7).
2. 10X yeast nitrogen base solution (134 g/L), 500X biotin solution (200 mg/L), 5% (v/v) methanol in distilled water sterilized by filtration, 10% glycerol (v/v) in distilled water, autoclaved. Use these stocks to prepare the following media.
3. Glycerol plates: 13.4 g/L yeast nitrogen base, 0.4 mg/L biotin, 1% (v/v) glycerol, 1.5% (w/v) agar.
4. Minimal glycerol medium (MGM): 13.4 g/L yeast nitrogen base, 0.4 mg/L biotin, 1% (v/v) glycerol.
5. Minimal methanol medium (MM): 13.4 g/L yeast nitrogen base, 0.4 mg/L biotin, 0.5% (v/v) methanol.
6. Extraction buffer: 1.85 M NaOH, 7.4% (v/v) β -mercaptoethanol, 1 mM PMSF. Make fresh.

2.2. Preparation of *P. pastoris* Cytosol

1. Tris-dithiothreitol (DTT) buffer: 0.1 M Tris-SO₄, pH 9.4, 10 mM DTT. Prepare fresh just before use.
2. Sorbitol buffer: 1.2 M sorbitol (Baker Inc. Phillipsburg, NJ), 20 mM potassium phosphate, pH 7.4.
3. Breaking buffer: 0.6 M sorbitol, 20 mM potassium phosphate, pH 7.4, 1 mM PMSF, protease inhibitor mix: leupeptin (1.25 μ g/mL), antipain (0.75 μ g/mL), chymostatin (0.25 μ g/mL), elastinal (0.25 μ g/mL), pepstatin (5 μ g/mL), phenylmethylsulfonyl fluoride (1 mM), and EDTA (1 mM) (see **Note 1**).
4. Zymolyase (ICN Biochemicals, Costa Mesa, CA).
5. Dounce homogenizer (Bellco Glass, Vineland NJ).

2.3. Purification of hsp60 from *P. parstoris* Cytosol

1. 50 mM Hepes-NaOH, pH 7.4. \pm 2 M NaCl, filtered 0.2 μ m.
2. 10X stock of 200 mM KCl, 200 mM MgCl₂, and 10 mM MgATP. Keep at -20°C.
3. FPLC equipment at 4°C.
4. TMAE superformance anion-exchange column (150 \times 10 mm; Merck, Darmstadt, Mannheim, Germany).
5. Mono Q (HR10/10; Pharmacia, Uppsala, Sweden).
6. Centricon 100 microconcentrator (Amicon, Bedford, MN).
7. Superose 6 (HR10/30, Pharmacia).

3. Methods

3.1. Growth and Induction of Mature hsp60

1. Grow *P. pastoris* strain MP1 on a glycerol plate at 30°C for 2–3 d.
2. Prepare a preculture by inoculating 100 mL MGM with a single colony of YJP1, and incubate at 30°C on a shaker for 3 d.

3. Use the preculture to inoculate 10 L of MGM. Use five 10-L flasks containing each 2 L of MGM. Incubate on a shaker for 3 d at 30°C. The cells will have reached stationary phase by then.
4. Collect cells by centrifugation in a Sorvall rotor HG-4L at room temperature (5000g, 10 min).
5. Induce hsp60 expression by resuspending the cell pellet in 10 L of MM and incubating the culture for 3 d at 30 degrees on the shaker (*see Note 2*).
6. Replenish methanol 2 d after start of induction to 0.5% (v/v) in order to compensate for evaporative losses.
7. Monitor the expression of hsp60 by immunoblotting of total cellular protein extracts (**8**).
8. For that purpose, remove 1 mL of culture immediately after transfer to MM and then after 1, 2, and 3 d.
9. Spin the cells down in a 2.2-mL Eppendorf tube, and add 0.15 mL of extraction buffer to the pellet.
10. Mix immediately by inverting the tube three to four times.
11. Leave on ice for 10 min.
12. Add 0.15 mL of 50% trichloroacetic acid and mix by inverting.
13. Leave on ice for another 10 min.
14. Spin down cell debris, and precipitate protein for 2 min in a microfuge.
15. Remove supernatant, and wash the pellet with 1.5 mL ice-cold acetone.
16. Let the pellet dry, and resuspend it in 400 μ L SDS sample buffer. Heat to 95°C for 5 min.
17. Remove undissolved material by a microfuge spin.
18. Load 40 μ L of the samples on a 10% SDS-PAGE, and probe for hsp60 by immunoblotting.
19. Harvest cells by centrifugation after maximal induction of hsp60 is achieved (HG-4L rotor, 5000g, 10 min). Usually that is the case after 2–3 d. Typical yields range from 10 to 14 g wet cell paste/L of MM. Store the cell paste at –80°C until used (*see Note 3*).

3.2. Preparation of *P. pastoris* Cytosol

1. Resuspend the cell pellet from a 10 L culture in 100 mL Tris-DTT buffer.
2. Incubate at 30°C for 15 min, shaking slightly.
3. Spin cells down for 5 min at 4°C at 5000g.
4. Resuspend the cells in 80 mL sorbitol buffer, incubate for 5 min at 30°C, and recollect the cells by spinning at 4°C.
5. Resuspend the pellet in 80 mL 1.2 M sorbitol buffer containing 100 mg/mL zymolyase.
6. Incubate for 30 min in a shaking water bath at 30°C to generate spheroplasts.
7. Collect the spheroplasts by centrifugation 5000g. Resuspend the pellet twice in 1.2 M sorbitol buffer, and recollect the spheroplasts at 5000g to remove residual zymolyase.
8. Resuspend the cell pellets in 100 mL breaking buffer and break the cell wall using a Dounce homogenizer (Belco Glass, Vineland, NJ). Dounce 20 times with a tight-fitting pestle on ice.

9. Separate cell debris and intact organelles from the cytosol by centrifugation in an SS34 rotor at 3000g for 10 min.
10. Save the supernatant, and keep on ice.
11. Repeat **steps 8–10** and combine the supernatants.
12. Clarify the supernatant by ultracentrifugation at 125,000g for 30 min at 2°C (*see Note 4*).

3.3. Purification of hsp60 from *P. pastoris* Cytosol

1. Equilibrate the TMAE column with 50 mM HEPES-NaOH, pH 7.4.
2. Load half of the clarified extract (70–80 mL) at a rate of 1 mL/min onto the column.
3. Apply the following gradient in 50 mM HEPES-NaOH, pH 7.4:

0–140 mL	no salt.
140–260 mL	0.2 M NaCl.
260–310	linear increase to 0.5 M NaCl.
310–360	0.5 M NaCl.
4. Collect 2-mL fractions. Expect the hsp60 containing fractions to be between 0.45 and 0.5 mM NaCl. Detect hsp60 by immunoblotting using antibodies against hsp60.
5. Pool the fractions that contain hsp60 (approx. 10–15 mL, termed TMAE pool). Supplement the TMAE pool with 20 mM KCl, 20 mM MgCl₂, and 1 mM MgATP (final concentration), and incubate for 1 h on ice. This incubation will dissociate chaperonin–substrate complexes formed in the *P. pastoris* cytosol or during the purification procedure.
6. Equilibrate a MonoQ column with 50 mM HEPES-NaOH, pH 7.4, 200 mM NaCl.
7. Dilute the TMAE pool (approx. 14 mL) twofold with 50 mM HEPES-NaOH, pH 7.4, and load at a rate of 1 mL/min onto the MonoQ column.
8. Elute hsp60 with a 50-mL gradient from 200 to 800 mM NaCl in 50 mM HEPES-NaOH, pH 7.4. Identify the hsp60-containing fractions by immunoblotting.
9. Pool the peak fractions containing hsp60. Expect them to be between 600 and 700 mM NaCl.
10. Concentrate the collected material (termed MonoQ pool) to 0.5–1 mL with a Centricon 100 in the presence of 20 mM KCl, 20 mM MgCl₂, and 1 mM MgATP (final concentration).
11. Equilibrate a Superose 6 column with 50 mM HEPES-NaOH, pH 7.4, 50 mM NaCl.
12. Subject the concentrated MonoQ pool onto the gel-filtration column.
13. Collect fractions of 0.5 mL at a flow rate of 0.1 mL/min, and analyze by SDS-PAGE and Coomassie blue staining and immunoblotting.
14. Pool the hsp60-containing fractions, adjust to 10% glycerol, and freeze in liquid nitrogen (*see Note 5*).
15. The frozen hsp60 is stable at –80°C for at least 6 months as judged from ATPase assays (**3**).

4. Notes

1. Growth media are essentially prepared according to standard procedures using products from Difco Laboratories (Detroit, MI). A protease inhibitor cocktail is

used throughout the purification procedure and is essential to recover hsp60. EDTA is omitted during the incubations with MgATP.

2. The expression of hsp60 in YJP1 is under the control of the AOX1 (alcohol oxidase 1) promoter. Hsp60 expression is induced by shifting the cells bearing YJP1 from glycerol- to methanol-containing medium. The *Pichia* host strain is deleted in the AOX1 locus and, therefore, has lost the major alcohol oxidase 1 activity. As a result, the cells exhibit poor growth on methanol medium.
3. *P. pastoris* cells can be stored frozen. It is also possible to store the clarified cytosolic extract at -80°C .
4. To avoid contamination of the cytosolically expressed *S. cerevisiae* hsp60 with the homolog from *P. pastoris* mitochondria, we make use of the different intracellular locations of the two proteins. It is therefore important to prepare the cytosol by a procedure that removes intact mitochondria from the cytosol (9).
5. A normal yield obtained with this purification protocol is about 20%. Expect about 15–20 mg of purified hsp60 from a 10-L culture.

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Preparation of Recombinant Human Hsp10

N. Kalaya Steede, Jesse J. Guidry, and Samuel J. Landry

1. Introduction

This chapter describes the cloning, expression, and purification of human Hsp10, also known as chaperonin-10, cpn10. The function of Hsp10 is to bind Hsp60 (also known as chaperonin-60) cpn60, in the presence of ATP, thereby promoting a conformational change in Hsp60 and enclosing the protein substrate within the complex (1). ATP hydrolysis by Hsp60 destabilizes the Hsp60/Hsp10 complex, allowing it to dissociate and release the substrate protein. Crystal structures of the *Escherichia coli* (2), *Mycobacterium leprae* (3), and human Hsp10s (Hunt, et al., unpublished results) have been determined, and the architecture of the *E. coli* Hsp60/Hsp10 complex has been described by cryo-electron microscopy (4) and X-ray crystallography (5). Subunits of both Hsp10 and Hsp60 are arranged in sevenfold symmetric rings. Hsp10 is composed of 7 10-kDa subunits, and Hsp60 is composed of fourteen 60 kDa subunits.

In order to obtain high-level expression of Hsp10, we inserted the complementary DNA (cDNA) into the multiple cloning site of plasmid vector pET24d (Novagen, Madison, WI) downstream from the T7 promoter, and an *E. coli* strain bearing the T7 RNA polymerase gene (γ DE3 lysogen) was transformed with the resulting plasmid. Expression of the T7 polymerase is under control of the *lac* promoter, and thus inducible by addition of isopropyl-1-thio- β -D-galactopyranoside (IPTG). It is possible to minimize basal expression by including another plasmid, pLysS, which encodes T7 lysozyme, a natural inhibitor of T7 RNA polymerase. This is especially important if the gene of interest is toxic.

2. Materials

2.1. Reagents and Buffers

1. All solutions are prepared in deionized water.
2. SuperScript™ preamplification system (Gibco-BRL, Gaithersburg, MD).

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- GeneAmp PCR Core Kit (Perkin Elmer, Norwalk, CT).
- Phenol: chloroform:isoamyl alcohol (25:24:1) v/v.
- 1% Agarose gel prepared with SeaPlaque GTG agarose (FMC) in 0.04 mM Tris-acetate/0.001 M EDTA electrophoresis buffer (pH 8.0).
- Competent *E. coli* strain BL21(DE3)/pLysS (Novagen).
- LB medium: 10 g Tryptone, 5 g Yeast Extract, and 10 g NaCl per liter of deionized water (pH 7.0).
- Stock antibiotics: kanamycin (Sigma, St. Louis, MO) 30 mg/mL (50 mM) in deionized water and chloramphenicol (Sigma) 34 mg/mL (105 mM) in ethanol IPTG (Promega, Madison, WI): 1 M stock.
- Protease inhibitor stock solutions: 500 mM ethylene glycol-*bis*(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), pH 8.5, 1 mM *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E64), 200 mM phenylmethanesulfonyl fluoride (PMSF), and 1 mM aprotinin (Sigma).
- Lysis buffer: 100 mM Tris (hydroxymethyl) aminomethane-HCl (Tris-HCl) (pH 7.5) (Fisher Biotech), 5 mM MgSO₄ (Sigma), 1 mM dithiothreitol (DTT) (Boehringer), and 30 μ g/mL deoxyribonuclease I (Boehringer).
- Ammonium sulfate and saturated ammonium sulfate.
- 100 mM 2-*bis*[2-Hydroxyethyl]amino-2-[hydroxymethyl]-1,3-propanediol-HCl, pH 6.0 (*bis*-Tris-HCl) (Fisher Biotech).
- Tris-saline: 50 mM Tris-HCl, 125 mM NaCl, pH 7.5.
- 50 mM Tris-HCl, 125 mM NaCl, 6 M GdnHCl, pH 7.5.
- Loading dye: 0.1% bromophenol blue, 0.1% xylene cynaol FF, 25% w/v sucrose in water, and 40 mM EDTA (pH 8.0).
- TAE: 0.04 M Tris-acetate (pH 8.0) and 0.001 M EDTA.
- Li/MgAc: 2.5 M LiAc and 0.5 M MgAc.
- TE: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0
- QIAprep-spin Plasmid Kit available from QIAGEN Inc, Valencia, CA.

2.2. Diethylaminoethyl (DEAE) Cellulose Column

- DEAE-cellulose column (Whatman) with a bed volume of 650 mL.
- Wash buffer: 50 mM Tris-HCl, pH 7.7, and 1 mM EDTA.
- Strip buffer: 50 mM Tris-HCl, pH 7.7, 1 mM EDTA, and 2 M NaCl.

2.3. S-Sepharose Column

- SP Sepharose HP (Pharmacia) bed volume of 58 mL.
- Low-salt buffer: 20 mM morpholineethanesulfonic acid-NaOH, (MES-NaOH) pH 6.2.
- High-salt buffer: 20 mM MES-NaOH, pH 6.2, and 1 M NaCl.

3. Methods

3.1. Cloning of Hsp10

Total RNA was isolated from human umbilical vein epithelial cells (HUVEC) by the guanidinium thiocyanate method (6) and reverse-transcribed using the Gibco-BRL preamplification system. This system generates the first

strand of cDNA from total RNA with *E. coli* reverse-transcriptase primed with a degenerate hexamer (see **Note 1a**). Subsequently, *E. coli* RNase H is added to eliminate RNA from the cDNA preparation.

The Hsp10 cDNA coding sequence served as a template for polymerase chain reaction (PCR). PCR primer design (see **Note 1b**) was coordinated with the published sequence (7), and incorporated silent mutagenesis for restriction enzyme cleavage and cloning. The resulting amplification product contained *NcoI* and *SacI* sites at the 5' and 3' ends, respectively. PCR (see **Note 2**) was performed with the GeneAmp PCR Core reagent Kit (Perkin-Elmer).

After PCR, the product was screened for correct size and purified using a 2% SeaPlaque GTG agarose gel. After phenol/chloroform extraction and Li/Mg acetate-ethanol precipitation, the resulting pellet was washed, air-dried, and dissolved in TE buffer (8).

pET24d (Novagen) has been designed so that selection of kanamycin resistance identifies positive transformants. pET24d was selected for its ability to control expression levels tightly.

The pET24d and the Hsp10-coding PCR product was digested with *SacI* and *NcoI* (see **Note 1b**), the desired product was purified, and a ligation was performed (9). A portion of the product was used for transformation of competent *E. coli* DH5 α F' cells (8).

Several kanamycin-resistant transformants were screened for the presence of recombinant plasmids by restriction analysis of miniprep (QIAprep-spin Plasmid Kit). One of the positive clones was named pJG10-2.

3.2. Expression of Human Hsp10

Use 1 μ g of plasmid pJG10-2 to transform 100 μ L of competent *E. coli* BL21(DE3) containing the plasmid pLysS, which should be selectively maintained by expression of chloramphenicol resistance (Novagen). Plate 100 μ L of transformants on LB containing 30 μ g/mL kanamycin and 34 mg/mL chloramphenicol, and incubate at 37°C overnight. A 5-mL starter culture is prepared by inoculating a single colony into LB medium, which was allowed to shake at 37°C for ~9 h. Twenty microliters from this starter culture are used to inoculate 50 mL LB medium and allowed to shake overnight at 37°C. Eight microliters from the 50-mL culture are used to inoculate each of six 1-L cultures of LB containing the above antibiotics, and these are incubated with shaking at 37°C. When the cultures reach an A_{600} of 0.8 (~4 h), IPTG is added to a final concentration of 2 mM to induce expression of Hsp10, and incubation is continued for 2 h (**Fig. 1**, lane 2).

3.3. Harvesting and Purification of Hsp10

Subsequent steps are carried out at 4°C. Six liters of cells are harvested by centrifugation at 650 \times g for 20 min (Centrifuge: Sorvall RC-3C. Rotor: H-6000A).

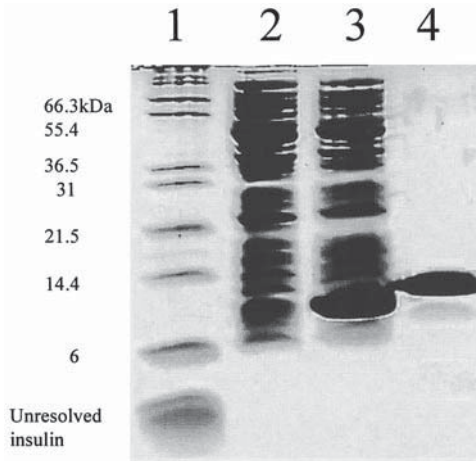


Fig. 1. Induction of Hsp10 expression analyzed by SDS-PAGE. Lane 1: mol-wt standards; lane 2: BL21(DE3)/pLysS cells transformed with pJG10-2 (uninduced); lane 3: BL21(DE3)/pLysS cells transformed with pJG10-2 (induced); lane 4: GroES standard.

The cell pellet is resuspended in 35 mL cold lysis buffer. Protease inhibitors are added to final concentrations as follows: 10 μ M aprotinin, 1.5 mM PMSF, and 10 mM EGTA. The cells are lysed by two passes through a French press at 1000 psi. The volume is increased threefold with cold lysis buffer, and cell debris is removed by centrifugation at 41,000g for 30 min. The supernatant is fractionated by ammonium sulfate precipitation. It is recommended that a series of ammonium sulfate concentrations be tested and analyzed by Tricine SDS-PAGE (**10**) using a 10% acrylamide gel to determine the optimum cut. In our hands, 2.2 M ammonium sulfate quantitatively precipitated Hsp10. While stirring gently, crystalline ammonium sulfate (338 g/L) (**11**) is slowly added and stirring is continued overnight at 4°C. The suspension is centrifuged at 41,000g for 30 min and stored at 4°C. The 0–2.2 M pellet is resuspended in 50 mM Tris-HCl, and 1 mM EDTA, pH 7.7. Another centrifugation step is required for 10 min to remove any nonresuspended material. The supernatant is applied to a DEAE-cellulose column equilibrated with 3 column volumes with wash buffer at a flow rate of 3.5 mL/min. The flowthrough is collected at this time (~650 mL), which contains Hsp10 (**Fig. 2**, lane 3). After loading, the column is washed with 650 mL of wash buffer (**Fig. 2**, lane 4). At this point, it is necessary to strip the DEAE-cellulose column with strip buffer to remove any remaining proteins that may be left on the column (**Fig. 2**, lane 5). The flowthrough, containing Hsp10, is diluted with an equal volume of 100 mM *bis*-Tris-HCl, pH 6.0 (*see Note 3*) and applied to an SP Sepharose HP (Pharmacia) column equilibrated with 20 mM MES, pH 6.2 at a flow rate of 2.5 mL/min, and washed with

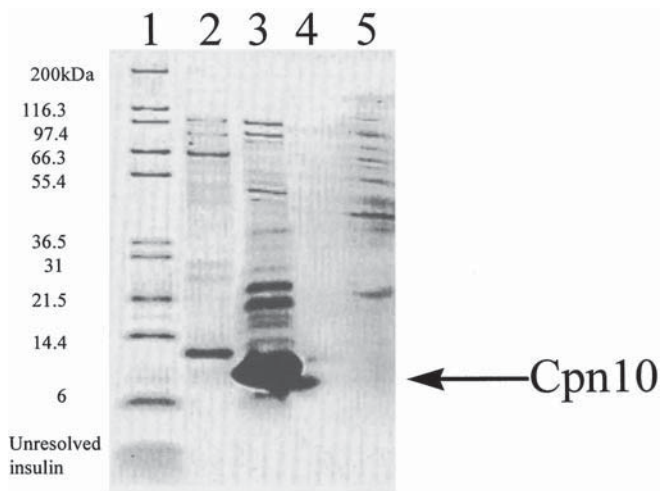


Fig. 2. Fractions from DEAE cellulose chromatography analyzed by 10% SDS-PAGE. Lane 1: mol-wt; lane 2: GroES marker; lane 3: flowthrough; lane 4: wash; lane 5: strip (see Note 7).

one column volume of low-salt buffer. Proteins are eluted using a linear gradient of 0–1.0 M NaCl in a gradient volume of 600 mL (total), collecting 8-mL fractions. Fractions containing Hsp10 are identified by Tricine SDS-PAGE (10) using a 10% acrylamide gel. Hsp10 elutes between 100 and 170 mM NaCl. After this step, Hsp10 is for the most part free of contaminants (Fig. 3). Fractions containing Hsp10 are pooled and concentrated by precipitating with 50% w/v of ammonium sulfate overnight at 4°C, and then resuspended in ~10 mL Tris-saline. The protein is aliquoted (~500 μ L) at a concentration of 20 mg/mL (see Note 4), precipitated by addition of 2X vol of saturated ammonium sulfate solution, and stored at 4°C (see Note 5). An extinction coefficient at 280 nm (ϵ_{280}) for each protein prep is calculated on the basis of quantitative amino acid analysis and the absorbance of a stock solution diluted by a factor of at least 100 in a solution of 50 mM Tris-HCl, 125 mM NaCl, and 6 M GdnHCl, pH 7.5. A typical value of ϵ_{280} is 7,900 (see Note 6).

4. Notes

1. Primer design:
 - a. The degenerate hexamer is designed to anneal with all possible sequence combinations of 6 bp (i.e., 5'-NNNNNN-3').
 - b. Cpn10 upper primer (*Nco*I site created): 5'-ACC ATG GCA GGA CAA GCG TTT AG -3'.
 - c. Cpn10 lower primer (*Sac*I site created): 5'-TCG AGC TCA GTC TAC GTA CTT TCC-3'.

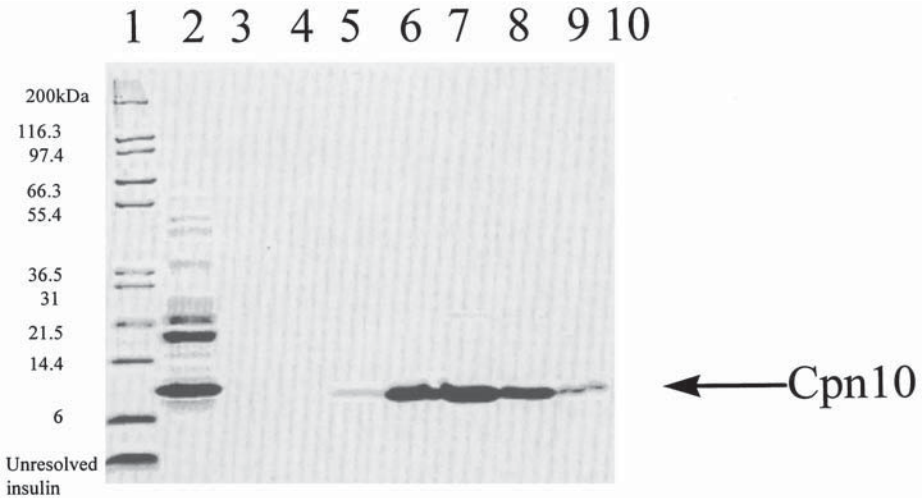


Fig. 3. Fractions from S-sepharose chromatography analyzed by SDS-PAGE. Lane 1: mol-wt; lane 2: flowthrough from DEAE cellulose; lanes 3–9: fractions eluted by 0–1 M NaCl gradient (see Note 7).

2. A 100- μ L PCR sample contains the following: 100 ng cDNA template, 100 ng of each nucleotide, 1X PCR buffer (supplied by Perkin Elmer), 2 mM each dAPs, dctp, dGTP, and TTP, 25 mM $MgCl_2$, 1 U *Taq* DNA polymerase (Perkin Elmer). The difference in volume is brought up with deionized water. A series of reactions were prepared using various concentrations of primers (i.e., 25, 50, 75, and 100 ng) and a constant concentration of template in order to increase the probability that one or more reaction contained an optimal primer:template ratio.
3. Diluting with this buffer decreases the pH of the sample. If the pH is too high, then Hsp10 will not bind to the column.
4. The concentration of protein will vary from each prep. It is recommended that at least 2 to 20 mg aliquots be made.
5. At this stage of purification, the protein is relatively pure and can be stored in an ammonium sulfate precipitate indefinitely.
6. The extinction coefficients is determined by Beer's Law: $A = \epsilon cl$; where A is absorbance at a specified wavelength, c = concentration in moles/L, and l = path length in cm. ϵ has the units M^{-1}/cm^{-1} . Once the extinction coefficient is known, the concentration from this amount of protein can be determined by absorbance measurement using the above equation.
7. This gel was selected as an ideal example and is not necessarily from the same preparation illustrated in the other figures.

Acknowledgments

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Purification of the Cytosolic Chaperonin TRiC from Bovine Testis

Raul G. Ferreyra and Judith Frydman

1. Introduction

The chaperonins are oligomeric ring-complexes composed of ~60 kDa subunits, which mediate the folding of polypeptide chains in an ATP-dependent reaction (**1**). Class I chaperonins are found in prokaryotes and organelles of prokaryotic origin (reviewed in refs. **2,3**). The most studied member of this class is the *Escherichia coli* chaperonin GroEL, which binds to and folds substrate polypeptides within its central cavity; this process requires the action of the ring-shaped oligomeric cofactor GroES (**4,5**). Archea and eukaryotic cells contain a related family termed Class II chaperonins (**6,7**). These functional homologs of GroEL are involved in the folding of a large subset of proteins in the eukaryotic cytosol, including actin, tubulin, and the G-protein transducin (**8–11**). The eukaryotic chaperonin, variously referred to as TCP-1 ring complex (TRiC), cytosolic chaperonin (c-cpn), or cpn-containing TCP-1 (CCT), is also ring-shaped, but consists of eight different, but homologous subunits ranging between 50 and 60 kDa (**12–14**). TRiC does not require a GroES homolog but has been shown to cooperate with a recently described hetero-oligomeric complex called GIM or prefoldin (**15,16**; also *see* chapters 20 and 21).

Based on the observation that TRiC is expressed to high levels in developing sperm cells, bovine testis was chosen as the starting material for isolation of the complex. In contrast to prokaryotic homologs, mammalian TRiC cannot be overexpressed, and thus, multiple purification steps are required (**12,14,17**). Our method for purification of TRiC from bovine testis consists of the following steps:

1. Binding of a cytosolic extract to DEAE-cellulose anion-exchange resin followed by a one-step elution with salt.

2. Concentration and further purification by ammonium sulfate precipitation
3. Gel-filtration chromatography on a Sephacryl S-300 column
4. Ion-exchange chromatography on a high-resolution MonoQ column
5. Affinity chromatography on a HiTrap heparin column
6. High-resolution Superose 6 gel-filtration chromatography.

This protocol yields high amounts of purified TRiC complex suitable for biochemical and biophysical studies.

Steps 3–6 are performed using a fast protein liquid chromatography (FPLC) system and commercial columns. However, all the steps can be easily adapted to other types of chromatographic systems and to conventional columns.

2. Materials (see Note 1)

1. Fresh bovine testis (~100 g, *see Note 2*).
2. Homogenization buffer (HB): 250 mM sucrose, 100 mM NaCl, 2 mM EDTA, 1 mM dithiotreitol (DTT), 50 mM HEPES-KOH, pH 7.4.
3. HB + 1 mM PMSF, 2 µg/mL leupeptin, 0.5 µg/mL aprotinin.
4. Column buffer (CB): 5% glycerol, 5 mM MgCl₂, 0.5 mM EDTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 20 mM HEPES-KOH, pH 7.4.
5. CB + 100 mM NaCl.
6. CB + 200 mM NaCl.
7. CB + 500 mM NaCl.
8. CB + 1 M NaCl.
9. 30% PEG 8000.
10. Ammonium sulfate (enzyme grade).
11. Protein determination reagent (Bradford or Bicinchonic Acid).
12. Chromatography columns:
 - a. DE52 Cellulose (Whatman).
 - b. Sephacryl S-300 26/60.
 - c. MonoQ HR 10/10.
 - d. HiTrap-Heparin (5 mL).
 - e. Superose 6 HR 10/30 (b–e from Pharmacia).

3. Methods

Perform homogenization on ice in the cold room. All buffers, tubes, and columns should be prechilled at 4°C. All subsequent manipulations and chromatographic steps are carried out in the cold room at 4–6°C. The purification schedule is as follows:

Day 1: **Subheadings 3.1–3.4.** The S-300 is run overnight (**Subheading 3.4.**) and assayed the next day.

Day 2: Perform and assay **Subheading 3.5.** **Subheading 3.6.** can be run overnight and assayed on day 3.

Day 3: Concentrate the sample from **Subheading 3.6.**, perform **Subheading 3.7.** and concentrate and aliquot the purified TRiC. With practice, **Subheading 3.6.**

can be finished on day 2, and the last column can be run overnight and assayed in the morning of day 3. It is advisable to do a protein determination to the pools after each column.

3.1. Preparation and Clarification of the Homogenate (See Note 3)

1. Wash ~70 g of tissue, cut in small (about 5-g) pieces, with 100 mL ice-cold HB (*see Note 4*).
2. Using a prechilled blender, mince washed tissue for no longer than 30 s in ~150–200 mL HB with protease inhibitors (PMSF, leupeptin, and aprotinin; *see Note 2*).
3. Homogenize the testes suspension using 15 strokes in a 40-mL Potter-Elvehjem tissue grinder (*see Note 5*).
4. Clarify by centrifugation at 16,000g for 30 min. This step also removes mitochondria and lysosomes.
5. Remove remaining insoluble material by ultracentrifugation at 180,000g for 1 h and filter the supernatant through two to three layers of tissue paper or cheesecloth (*see Note 6*).
6. Measure protein concentration to determine the amount of ion-exchange resin necessary for the next section (*see Note 7*).

3.2. Anion-Exchange Chromatography

1. Apply the clarified homogenate to the appropriate amount of ion-exchange column of DE52 cellulose (Whatman), pre-equilibrated in CB + 100 mM NaCl (*see Note 8*).
2. Wash the resin with 1.5–2 column volumes of CB + 100 mM NaCl (*see Note 9*).
3. Elute bound proteins with 1–1.5 column volumes of CB + 500 mM NaCl.
4. Collect all the protein eluting with CB + 500 mM NaCl. Protein concentration may be measured at this point, but it is not necessary for the next step.

3.3. Ammonium Sulfate Fractionation

1. To the DE52 eluate (from **Subheading 3.2., step 4**), add ammonium sulfate to 30% saturation (176 mg/mL) by slowly adding solid salt (*see Note 10*) and stirring gently on ice. After complete addition, continue stirring for 10 min more.
2. Spin out precipitated proteins by centrifugation at 16,000g for 20 min. Keep supernatant and discard the pellet.
3. To the supernatant of **step 2**, slowly add ammonium sulfate to 50% of saturation (127 mg/mL). After completing addition, stir gently on ice for 10 min.
4. Sediment the precipitated proteins by centrifugation at 16,000g for 30 min. Discard the supernatant. Briefly spin the bottles again to tighten the pellet and remove any remaining supernatant (*see Note 11*).
5. Gently dissolve pelleted proteins in a minimal volume of CB + 100 mM NaCl (~10 mL). Usually this solution is slightly turbid.
6. Clarify the sample by centrifugation at 20,000g for 20 min.
7. It is advisable to do a protein determination at this point.

3.4. Gel-Filtration Chromatography (see Note 12)

1. Equilibrate a Pharmacia HiPrep 26/60 Sephacryl S-300 column with 250 mL CB + 100 mM NaCl.
2. Load the clarified supernatant of **Subheading 3.3, step 6** using a 10-mL FPLC superloop (a maximum of 12 mL should be loaded).
3. Run column overnight at 0.2 mL/min, collecting 10-mL fractions (flow rate can be increased up to 1.5 mL/min).
4. Run 10 μ L of fractions on a 12% SDS-PAGE (see **Fig. 1**, lane 3). Keep fractions that elute between 100 and 140 mL, which should correspond to an apparent molecular mass of ~900 kDa, and pool them (see **Note 13**). If necessary, the fractions containing TRiC may be determined by either Western blot analysis or native gel (see **Note 14**).

3.5. High-Resolution Ion-Exchange Chromatography

1. Equilibrate a Pharmacia 10/10 MonoQ ion-exchange column by passage of 50 mL CB + 100 mM NaCl.
2. Run column on FPLC using a flow rate of 2 mL/min. Inject the pooled fractions from **Subheading 3.4.** by passage of 50 mL CB + 100 mM NaCl using a 50-mL FPLC superloop. Apply a 100-mL linear gradient in CB from 100 to 500 mM NaCl. Collect 2-mL fractions.
3. Analyze 10 μ L of fractions between 200 and 400 mM NaCl on 12% SDS-PAGE (see **Note 14**).
4. Keep and pool fractions that elute between 220 and 330 mM NaCl, typically about 20 mL containing 3–5 mg/mL of total protein.

3.6. Affinity Chromatography

1. Equilibrate a Pharmacia HiTrap Heparin 5-mL column with 20 mL CB + 200 mM NaCl (see **Note 15**).
2. Run column on FPLC using a flow rate of 2 mL/min. Inject the pooled fractions emerging from the MonoQ column using a 50-mL FPLC superloop. Apply a 100-mL linear gradient in CB from 200 mM to 1 M NaCl. Collect 3-mL fractions.
3. Analyze 10 μ L of fractions between 400 and 800 mM NaCl on 12% SDS-PAGE (see **Fig. 1**, lane 4).
4. Keep and pool fractions that elute between 0.57 and 0.68 M NaCl (see **Note 16**).
5. Concentrate the pooled fractions using a Centriprep-50 ultrafiltration unit (Amicon) until the final volume is 0.5 mL.

3.7. High-Resolution Gel-Filtration Chromatography

1. Load the TRiC preparation from 3.6.5 onto a Superose 6 HR 10/30 column (Pharmacia) equilibrated in CB + 100 mM NaCl and run at 0.2 mL/min. After 6 mL of eluted volume, start fraction collection, and collect 0.5-mL fractions. TRiC elutes as symmetrical peak at about 12 mL, corresponding in this column to an apparent molecular mass of about 900 kDa (see **Note 17**).

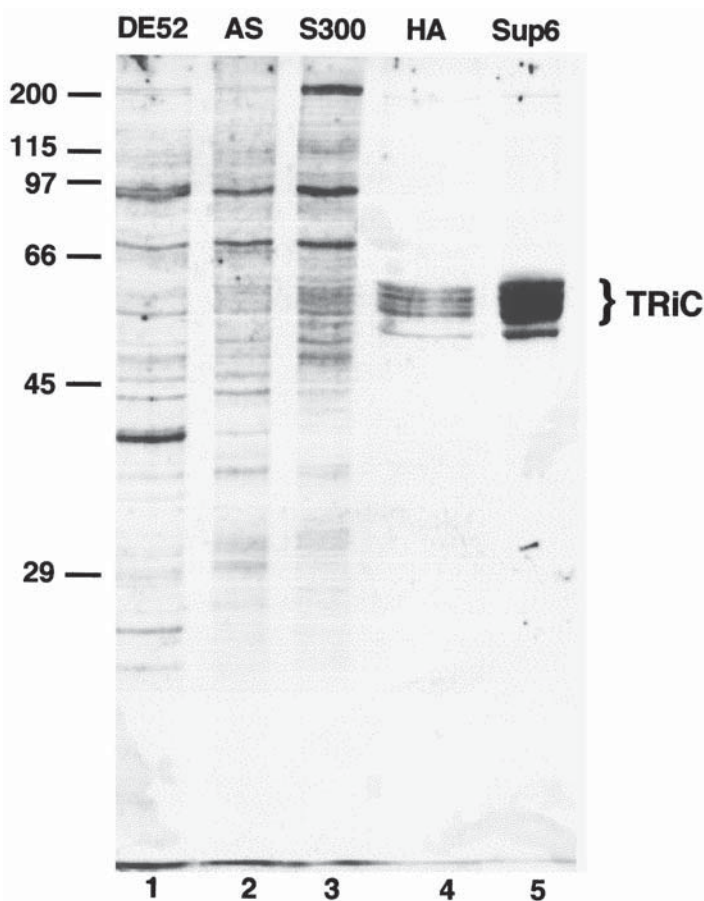


Fig. 1. 12% SDS-PAGE Analysis of TRiC Purification. Following the indicated purification steps, the TRiC-containing fractions were pooled and 5 μ g loaded on a 12% gel (except in lane 4, where 0.5 μ g were loaded). Lane 1: DE52 eluate; lane 2: 30–50% ammonium sulfate fraction; lane 3: Sephacryl S300 fractions; lane 4: HiTrap Heparin fraction; lane 5: Superose 6 purified TRiC.

2. Pool the TRiC fractions and supplement to a final concentration of 5% PEG8000 (see **Note 18**). If necessary, concentrate the pooled fractions using a Centricon-30 ultrafiltration unit to give a final protein concentration of 4–5 mg/mL (see **Note 19**), and clarify by centrifugation at 14,000g for 10 min to remove aggregates. Snap-freeze in small aliquots, and store under liquid nitrogen (see **Note 20**).
3. The purity of TRiC can be determined by 12% SDS-PAGE and Coomassie blue staining. See **Fig. 1** for samples of TRiC at different stages of the purification. Typically 3–5 mg of pure TRiC are obtained by this protocol.

4. Notes

1. Protease inhibitors and DTT should be added immediately before use to the buffers. The stock solutions are stored at -20°C : 100 mM PMSF in ethanol, and 10 mg/mL leupeptin and 2 mg/mL aprotinin (both in dimethylsulfoxide [DMSO]). CB can be prepared by dilution from a 10X stock solution.
2. Bovine testis should be obtained from freshly sacrificed animals (for storage, *see Subheading 3.1., step 1.* and *Note 4*). Other sources (including rabbit reticulocytes, yeast extracts, and so forth) may be used as starting material.
3. Homogenization is a critical step. Sucrose is added to prevent organelle disruption, primarily mitochondria that could contaminate the preparation with Hsp60. When using reticulocytes as a starting material, the lysate is prepared by simple hypotonic lysis with 2 vol of 20 mM HEPES-KOH pH 7.4, and 1 mM DTT. The purification is then continued from **Subheading 3.1., steps 5 and 6** onwards.
4. Testis are processed as follows: Remove blood and fat from tissue (250 g average/testis) and cut in small (about 5-g) pieces, washed with ice-cold HB (~200 mL/testis). The washed cut tissue pieces are snap-frozen in liquid nitrogen and stored at -80°C for several months.
5. The tissue suspension is divided into 40-mL aliquots, which are homogenized with 15 strokes each.
6. This step also sediments ribosomes and other particulate subcellular components. However, centrifugation and ultracentrifugation alone do not remove lipid compounds (phospholipids, lipoproteins, and so on), which float on the surface and stick to the bottle walls as a yellow layer; subsequent filtration of the ice-cold homogenate through tissue paper or cheesecloth removes most lipid contaminants.
7. Typically, the protein concentration of the clarified homogenate is 12–15 mg/mL.
8. DE-52 cellulose has a protein binding capacity of about 20–50 mg/mL packed resin. Approximately 50% of the proteins in the lysate bind to the resin under the conditions used here (~20 % in reticulocyte lysate). Usually, 100 mL of resin in a 5×10 cm column are appropriate for this step.
9. It is convenient to perform a quick protein assay on the wash and eluate from the column to determine when to proceed to the next step. This can be performed by adding 10 μL of column eluate to 0.5–1 mL Bradford assay reagent. The reagent will turn blue if the column output still contains protein.
10. A saturated solution (100%) of ammonium sulfate may be used in this step, avoiding the slow addition of the solid salt, but substantially increasing the volume of the sample.
11. Thorough removal of the ammonium sulfate solution will facilitate the solubilization of the pelleted proteins.
12. The following steps are performed on a Pharmacia FPLC system using commercial columns. They can, however, be easily adapted to other chromatography systems and/or conventional columns. In the latter case, the Superose 6 and S-300 gel filtration columns may be replaced by a number of excellent gel-filtration resins with a similar separation range: the MonoQ by one packed with Source Q (Pharmacia), and the Hi-Trap heparin by Heparin-agarose.

13. The S-300 column is calibrated using Blue Dextran (2 MDa), Thyroglobulin (680 kDa), Ferritin (440 kDa) and Aldolase (140 kDa). Collection was started after elution of the void volume (~90 mL). TRiC eluted in the fractions corresponding to an apparent molecular mass of ~900 kDa.
14. From this step onward, the typical TRiC pattern becomes recognizable on SDS gels. Column fractions may be assayed for the presence of TRiC by Western blot using TRiC-specific antibodies (available from a number of vendors, including Stressgen, Affinity Bioreagents, and Sigma). Alternatively, TRiC binding of denatured [³⁵S]-actin may be monitored using a native gel-based assay (see chapter 19).
15. If Heparin-agarose (or Heparin-acrylamide) is used instead of the Hi-Trap column, a 50 mL column should be used owing to the 10-fold lower binding capacity of these resins.
16. At this point, the TRiC preparation is already >90% pure. The last gel-filtration step is introduced to remove some low-molecular-weight impurities and the high salt from the previous step.
17. The Superose 6 column is calibrated using molecular-weight standards mentioned in **Note 13**, as well as ovalbumin (45 kDa) and ribonuclease A (13.7 kDa).
18. Glycerol may be used instead of PEG 8000. However, we find that PEG 8000 is a good stabilizing agent for TRiC.
19. Ideally, the concentration of TRiC should be determined using a standard of previously purified TRiC. We found that the bicinchonic acid protein determination assay (**18**) reflects more accurately the TRiC concentration than the Bradford assay reagent.
20. TRiC can be stored under liquid nitrogen (or -80°C) for up to 1 yr without loss of activity.

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Monitoring Actin Folding

Purification Protocols for Labeled Proteins and Binding to DNase I-Sepharose Beads

Vanitha Thulasiraman, Raul G. Ferreyra, and Judith Frydman

1. Introduction

Actin has been widely used as a model protein to study chaperone-mediated folding *in vitro* (1,2) and *in vivo* (3). In addition to being an essential and very abundant cytosolic protein, actin has the advantage of a very reliable assay, namely, that only the folded protein will bind to DNase I (1,4). Binding occurs with very high affinity ($>10^{-9} M$) (5,6), and the actin–DNase I complex is so tight that it can only be dissociated with mildly denaturing treatments (40% formamide or 3 M guanidinium hydrochloride). Consequently, the use of DNase I immobilized on Sepharose beads provides a very specific assay for the presence of folded actin. This assay has been used to measure the chaperone dependence, and the rate of actin folding in mammalian cells, yeast cells, rabbit reticulocyte lysate (RRL), and by purified chaperones (1,3,4). To assay TRiC-mediated actin folding requires the preparation of [^{35}S]-labeled purified actin. We include a protocol to purify [^{35}S]-labeled actin overexpressed in bacterial cells. This protocol can be used to purify any other labeled protein following overexpression in bacteria, including luciferase and tubulin. We also describe a protocol to purify [^{35}S]-labeled actin from mammalian cells. Notably, actin purified from mammalian cells is a much better substrate for TRiC-mediated folding than actin purified from bacteria.

2. Materials

2.1. DNase I-Sepharose Beads Preparation and Use

1. Formamide stored at $-20^{\circ}C$ in 1-mL aliquots.
2. Phosphorimager cassettes or X-ray film.

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3. DNase I binding buffer (DB): 10 mM Tris-HCl, pH 7.4, 1 mM CaCl₂, 10% glycerol, 1 mM dithiothreitol (DTT), 0.2 mM ATP, and 10% formamide (v/v). 2X DB stock without DTT, ATP, and formamide can be made and stored at 4°C for as long as 2 mo, and supplemented with ATP, DTT, and formamide before use.
4. Wash buffer (WB): DB with no ATP.
5. WB + 0.3 mM NaCl.
6. CN-Br-activated Sepharose 4B beads.
7. DNase I (Worthington, Lakewood, NJ).
8. 0.1 M NaHCO₃, 0.5 mM CaCl₂.
9. 0.2 M ethanolamine (pH 8.0), 0.5 mM CaCl₂.
10. 0.1 M NaAc (pH 4.5).
11. 0.1 M ethanolamine (pH 8.0).
12. 1X SDS sample buffer supplemented with 40 % formamide.

2.2. [³⁵S]-Labeled Actin Purification

1. Luria broth (LB).
2. Minimum Essential medium Eagle (MEM) modified without methionine, cysteine, and L-glutamine (Sigma, St. Louis, MO).
3. α-MEM (Gibco-BRL, Gaithersburg, MD) supplemented with 5% bovine calf serum (BCS) (Hyclone).
4. [³⁵S]-Methionine/cysteine express labeling mix (New England Nuclear, Boston).
5. Bacterial lysis buffer (BLB): 20 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 10% glycerol, 0.5% Triton X-100, 1 mM phenylmethylsulfonylfluoride (PMSF), 10 mM β-mercaptoethanol (or 2 mM DTT).
6. Mammalian cell lysis buffer (MLB): 10 mM Tris-HCl, pH 7.4, 1 mM CaCl₂, 1 mM DTT, 0.2 mM ATP, 0.5% Triton X-100, 1 mM PMSF.
7. 6 M GdmCl: 6 M guanidinium hydrochloride in 20 mM HEPES-KOH, pH 7.5, 5 mM DTT.
8. Ampicillin stock solution (4 mg/mL in water) stored at -20°C as 1-mL aliquots.
9. M9 salts.
10. 100 mM isopropylthiogalactoside (IPTG).
11. 20 mg/mL of rifampicin in dimethyl sulfoxide (DMSO).
12. 10 mg/mL of lysozyme in water.
13. Phosphate-buffered saline (PBS).

3. Methods

3.1. Preparation of DNase I-Sepharose Beads

1. Wash ~50 mL CN-Br-activated Sepharose 4B beads (~15 g) several times with 1 mM HCl (a total of ~2 l).
2. Dissolve DNase I (300 mg, Worthington) in 60 mL of 0.1 M NaHCO₃, 0.5 mM CaCl₂.
3. Add DNase I solution to the washed, packed beads, and rotate at 4°C for ~12 h (overnight).
4. Block unreacted groups in the resin by incubation with 0.2 M ethanolamine (pH 8.0), 0.5 mM CaCl₂ for 2 h at 4°C.

5. Wash the beads with two alternate cycles of 0.1 M NaAc (pH 4.5) and 0.1 M ethanolamine (pH 8.0).
6. Resuspend resin, and store in WB containing 0.02% NaN₃ at 4°C (*see Note 1*).

3.2. Purification of Labeled Actin from Bacteria

1. Using standard transformation protocols, transform BL21(DE3) cells with the pET plasmid (7) encoding chicken β -actin (4). Plate on LB plates containing ampicillin. Incubate overnight at 37°C.
2. Inoculate 5 mL of LB supplemented with ampicillin (0.1 mg/mL) with a freshly transformed colony, and grow with vigorous shaking at 37°C overnight (*see Note 2*).
3. On the next day, spin the cells (at 1,000g for ~5 min) and resuspend in 50 mL fresh LB-ampicillin.
4. Grow the cells by shaking for another 2–3 h or until an OD₅₉₀ of ~0.5 is obtained.
5. Spin the cells and resuspend in 50 mL of M9 salts with ampicillin, supplemented with 200 μ L of 100 mM IPTG, 0.5 mCi [³⁵S]-methionine/cysteine express labeling mix (New England Nuclear, Boston), and 5 ml of LB media (*see Note 3*).
6. Grow the cells for 30 min at 30°C (*see Note 3*).
7. After 30 min, add 500 μ L of rifampicin (20 mg/mL in DMSO), and grow the cells for 3–4 h.
8. Harvest the cells by centrifugation at 6000g for 15 min, and discard the media in the radioactive waste.
9. Resuspend the bacterial pellet in 1.5 mL of BLB by vortexing.
10. Add 100 μ L of freshly prepared lysozyme (10 mg/mL in H₂O) at room temperature. Within a few minutes, the cells should lyse, yielding a viscous mass.
11. Sonicate three to five times with 10-s bursts with a tip sonicator. At the end of the sonication, there will be a loss of viscosity (*see Note 4*).
12. Centrifuge the lysates at 20,000g for 15 min at 4°C to recover the insoluble inclusion bodies (*see Note 5*).
13. Wash the pellet thoroughly, at least twice with 1 mL BLB.
14. Discard the supernatant, and dissolve the pellet in 150 μ L of freshly prepared 6 M GdmCl.
15. Vortex vigorously and persistently until the pellet is no longer visible.
16. Centrifuge at 12,000g for 20 min at 4°C to clarify and take the supernatant.
17. Load the supernatant on a Sephadex G-50 column (medium grade, 1 x 3 cm) equilibrated in 6 M GdmCl. This step removes low-molecular-weight contaminants. Alternatively, Sephadex G-25 can be used (and should be used for proteins <30 kDa).
18. Collect drops from the column (4 drops/fraction), and monitor the radioactivity (by scintillation counting). Biochemical and radiochemical purity of the probe is determined by analysis on 10% SDS-PAGE followed by staining with Coomassie blue and autoradiography.
19. Pool the labeled fractions, freeze 10- μ L aliquots in liquid nitrogen, and store at -70°C (*see Notes 6 and 9*). This bacterially expressed actin (0.3–0.5 mC/ μ g of actin) is diluted 100-fold into the folding reaction. Usually 50–100 μ g of actin are obtained from 10 mL of culture.

3.3. Purification of Labeled Actin from Mammalian Cells

1. Grow CHO cells in five 150 mm dishes to 60–70% confluence. Any mammalian cells can be used in place of CHO cells.
2. Label the cells overnight with MEM modified medium supplemented with 0.2 mCi/mL of [³⁵S] labeling mix and 10% α -MEM with 5% BCS.
3. On the next day, remove the labeling media, and wash the plates twice with 5 mL of PBS. Scrape the cells in ~5 mL PBS/plate. Spin the cells at 1000g for 10 min at 4°C.
4. Lyse the cells with 3 mL of MLB, add formamide to 10% final, and incubate for 10 min on ice. All the steps after cell lysis of cells are performed at 4°C (*see Note 7*).
5. Spin the lysates for 20 min at 14,000g.
6. To the supernatant add 0.2–0.5 mL of a 1:1 DNase I-Sepharose slurry in DB, and incubate the mixture with gentle shaking for 1 h at 4°C (*see Note 8*).
7. Wash the DNase I-Sepharose beads once with two bed volumes of WB, once with WB supplemented with 0.3 M NaCl, and twice with WB.
8. Add 100 μ L of 6 M GdmCl to beads, and incubate for 1 h at 4°C with gentle mixing. Spin and collect the supernatant.
9. Repeat **step 8** once using 50 μ L of 6 M GdmCl.
10. Combine the supernatants of **steps 8** and **9**, divide into 5- μ L aliquots, and store at –70°C (*see Note 9*).
11. Biochemical and radiochemical purity of the probe is determined by running SDS-PAGE gel, staining with Coomassie blue and autoradiography. Usually, 3–5 μ g of actin (0.3–0.5 mC/ μ g) are obtained from one 150-mm plate.
12. Normally, mammalian actin is diluted 100-fold into the folding reactions.

3.4. DNase I-Sepharose Binding Assay

1. Translate actin in rabbit reticulocyte lysate in the presence of [³⁵S]-Methionine (*see Notes 2* and **10** and chapter 19).
2. Add 160 μ L DB to 2 μ L of the above sample. Spin for 10 min at 14,000g to remove aggregated protein (*see Note 11*).
3. Add 30 μ L of 1:1 DNaseI-Sepharose beads to the supernatant. Incubate at 4°C for an hour with gentle rotation.
4. At the end of incubation, wash the beads twice with WB, once with WB supplemented with 0.3 M NaCl, and twice with WB. The volume of each wash is 500 μ L.
5. Resuspend the beads in 1X SDS sample buffer supplemented with 40% formamide. Heat the sample at 80°C for 5 min before analysis of supernatants by 10% SDS-PAGE (*see Note 12*).
6. Stain and destain the gels with Coomassie blue. Dry and expose the gels to either X-ray film at –70°C or expose to a Phosphorimager cassette (**Note 13**). A typical example of an exposure is shown in **Fig 1**.

4. Notes

1. DNase I-Sepharose beads can be stored at 4°C for up to a year in 20% ethanol or buffer containing 0.02% sodium azide.

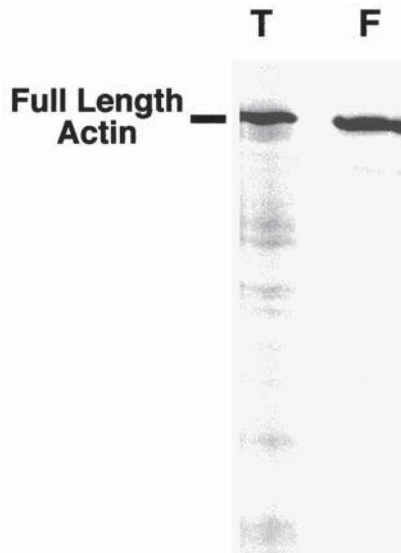


Fig. 1. Folded actin binds to DNase I-Sepharose beads. Actin was translated in nuclease-treated RRL (from Promega) for 10 min at 37°C in the presence of [³⁵S]-methionine. At the end of 10 min, 1 μ L was used for analyzing the total translation products (lane T), but 5 μ L were used to measure folded actin using binding to DNase I-Sepharose beads (lane F). Both the total and the DNase I bound actin analyzed using 10% SDS-PAGE, stained, destained, dried, and exposed to a Phosphorimager cassette overnight. Only folded full-length actin binds to the DNase I beads (lane F), but the unfolded and the incomplete actin polypeptide chains (*see* lower-molecular-weight species in lane T) do not bind to DNase I-Sepharose beads.

2. This protocol can be used to express a number of proteins in addition to actin. Actin and tubulin are recovered from insoluble inclusion bodies when expressed in bacteria. In contrast, other proteins like luciferase, fold correctly and are soluble when expressed. We describe the use of the inducible expression system based on T7-RNA Polymerase-driven promoters (7), but other systems can be adapted, using this protocol from **step 8** in **Subheading 3.2.** onward.
3. 10% LB is added to the M9 salts to ensure vigorous growth of cells during labeling. Obviously, for expression of the unlabeled protein, induction is performed in LB media with ampicillin, without radioactive methionine, and IPTG is used to induce protein expression. The temperature for optimal expression may vary among proteins. For instance, firefly luciferase should be induced at 25°C, since it is degraded at temperatures above 30°C.
4. Sonication is used to disrupt the bacterial DNA. For small volumes (< 200 μ L), sonication may be carried out in a sonicator bath. Alternatively, this step can be replaced by addition of 80 μ L of 1.0 M MgCl₂ and 50 μ L of DNase I (Worthington, Freehold, NJ; 0.5 mg/mL in water). Incubate for 1 min in ice. At

the end of incubation, there will be a loss in viscosity. However, commercial DNase I preparations may be contaminated with proteases.

5. As stated, not every protein accumulates in inclusion bodies. Some proteins can fold correctly and can be recuperated from the supernatant at this step. In this case, it is convenient to use an affinity purification step to purify the expressed protein further. The most commonly used affinity tags are poly-histidine (His₆ tag, purified using an Ni-column from Quiagen) or a glutathione-S-transferase fusion (purified using glutathione beads). The His₆ tag can also be used to purify denatured proteins using a buffer containing guanidinium hydrochloride or urea.
6. Denatured, labeled actin can be stored at -80°C for several months (in 5- μL aliquots), and should be discarded after one freeze-thaw cycle.
7. Cellular actin exists in two forms: as a monomer of globular G-actin and polymerized in actin filaments and patches (F-actin). Only G-actin binds to DNase I. In order to convert F-actin to G-actin quantitatively, the actin-containing samples (e.g., cell lysates, folding reactions, and so on) are diluted into a buffer containing 10% formamide, which induces actin depolymerization.
8. The binding step and the subsequent wash and elution steps can also be carried out using the DNase I-Sepharose resin packed into a small column (there are many commercially available columns; a disposable syringe plugged with glass wool can also be used). This method is preferable when using larger amounts of lysate, since it yields a more concentrated probe.
9. Actin purified from mammalian cells by the DNase I method can be stored at -70°C for at least 4 mo. It is far superior as a folding substrate than the actin purified from bacterial cells.
10. Alternatively, the sample may be labeled actin isolated from yeast or mammalian cells or labeled bacterial actin renatured by purified TRiC or rabbit reticulocyte lysate (*see* chapter 19).
11. If the volume of the sample to be assayed by the DNase I-Sepharose beads binding assay is larger, then 5X DB may be used instead of a larger volume of 1X DB. Importantly, the yield and rate of actin folding measured using the DNase I-Sepharose binding assay are very similar as those measured using the native gel assay.
12. The DNase I-Sepharose beads should not be loaded onto the gel since all the proteins are eluted by incubation with the sample buffer supplemented with 40% formaldehyde.
13. Exposure using Phosphorimager cassettes is preferred over exposure using X-ray films, owing to their increased sensitivity and broader linear range. Thus, they require a much shorter exposure time than X-ray film, and the quantitation of labeled bands is much easier in the Phosphorimager.

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Folding Assays

Assessing the Native Conformation of Proteins

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1. Introduction

To determine the efficiency and rate of chaperone-mediated folding and renaturation, it is fundamental to have a good assay for the native conformation of the substrate protein. In the case of enzymes, the ideal assay is clearly the enzymatic activity of the substrates, since most proteins are active only when correctly folded. As an example, we describe the folding of firefly luciferase, which has a very rapid and sensitive assay (1). Unfortunately, the assays available for some enzymes are not sensitive enough to detect the low concentrations of target protein used in chaperone-mediated folding assays (usually in the submicromolar range). In addition, the folding substrate may not have enzymatic activity. To overcome these problems, folding substrates can be radiolabeled (*see* chapter on actin folding for expression of labeled proteins in *Escherichia coli*) and the native state detected by either of two general methods: a protease sensitivity assay (2) or native gel electrophoresis (3,4).

The protease sensitivity assay is based on the fact that correctly folded proteins have a compact structure that is generally resistant to low concentrations of an unspecific protease, such as proteinase K (5). In contrast, unfolded or chaperone-bound proteins tend to be very susceptible to proteases (5,6). Since the substrate protein is radioactively labeled, its sensitivity to proteases can be assessed following SDS-PAGE and autoradiography. This assay can be successfully used to follow the TRiC-mediated folding of actin, tubulin, and luciferase (2,4,7). In addition, other proteases may also be used in this type of assay. Clearly, when trying a new folding substrate, the reaction conditions should be optimized, particularly regarding the protease sensitivity of the native protein.

The second kind of assay, native gel electrophoresis, relies on the characteristic migration of the folded substrate in a nondenaturing gel. This migration should be different from the migration of both the unfolded and the chaperone-bound substrate. In this case, the protein is also detected by autoradiography. Here we describe a native gel system used to study the folding of the TRiC substrates actin and tubulin (3,4,7). TRiC has a very characteristic migration in these gels, and the TRiC-substrate binary complex migrates at the same position as the empty chaperone complex. Native gels separate protein complexes by both charge and size. Consequently the migration of the native protein will depend on both the acrylamide–bis-acrylamide content of the gel and the pH of the buffer system used. Thus, a buffer whose pH is close to the isoelectric point of the target protein will result in little or no migration. There are many different buffer systems available for native gels, and it is always possible to find one that is appropriate to monitor the folding of a specific protein.

In addition to these general assays, a number of folding assays take advantage of specific properties unique to a correctly folded protein, such as binding to an immobilized ligand (e.g., DNase I in the case of actin [*see* Chapter 18] or methotrexate in the case of dihydrofolate reductase) or the ability to form filaments under certain conditions (e.g., tubulin). In this case, the labeled native protein will be separated from the unfolded protein through this property and analyzed by SDS-PAGE followed by autoradiography. It is possible to develop an assay of this type whenever the native protein binds with high affinity and specificity to a ligand that can be coupled to an affinity resin. Chapter 18 contains a description of the actin folding assay using DNase I-Sepharose beads.

2. Materials

2.1 Materials for TRiC-Mediated Folding

1. 2X Folding buffer (2X FB): 40 mM HEPES-KOH, pH 7.5, 200 mM NaCl, 10 mM MgCl₂, 10% glycerol, 10% PEG8000. This buffer can be stored at 4°C for up to a month and longer if stored in –20°C. 1X FB is made fresh from 2X FB supplemented with 1 mM dithiothreitol (DTT) (final) at the time of use.
2. 5X Stop buffer (5X SB): 100 mM HEPES-KOH, pH 7.5, 50 mM *trans*-1,2-cyclohexanediaminetetraacetate (CDTA), 1 mM CaCl₂.
3. 5X Native gel sample buffer (5X NB): 80% (v/v) glycerol, 80 mM MOPS-KOH, pH 7.2, 0.2% bromophenol blue. This buffer can be stored at 4°C for months.

2.2 Materials for Native Gel Analysis

2.2.1. Apparatus

Multiple-gel pouring stand, 10 × 10 cm glass plates, 1 mm thick spacers, 10-well combs (1 mm thick), and minilab electrophoresis apparatus were obtained from Idea Scientific. The gels were run using a Bio-Rad Power-Pac 300 power supply.

2.2.2. Buffers

1. 5X Running buffer (5X RB): 0.4 M MOPS-KOH, pH 7.2, and 5 mM MgCl₂ (stored at 4°C for up to 2 mo). For the analysis of tubulin folding, 0.4M MES-KOH pH 6.8 is used instead of 0.4 M MOPS-KOH, pH 7.2, in all buffers (*see Note 1*).
2. 1X RB: Top buffer is 1X RB supplemented with 0.095% cysteine, 0.1 mM ATP or 0.1 mM GTP, the latter two components being optional depending on whether the proteins of interest require the presence of these nucleotides to stabilize their native state. The lower chamber buffer is 1X RB.
3. 10% Ammonium persulfate (w/v).
4. TEMED.
5. Acrylamide-*bis*-acrylamide (30:0.5% v/v).

2.3. Materials for Protease Resistance Assay

1. Proteinase K, trypsin, chymotrypsin, V8, thermolysin, and subtilisin can be used. The stock solution (1 mg/mL in 10 mM Hepes-KOH, pH 7.4) can be stored at -20°C.
2. 0.1 M phenylmethylsulfonyl fluoride (PMSF) in isopropanol can be stored at -20°C for up to 4 mo. PMSF is not stable in aqueous solution for more than 3 h on ice. Consequently, the stock PMSF solution should be removed from -20 to 4°C just before use.

2.4. Material for Luciferase-Bioluminescence Assay

2.4.1. Buffers

1. Assay buffer (AB): 25 mM Tricine-HCl, pH 7.8, 8 mM MgSO₄, 0.1 mM EDTA, 33 μM DTT, 470 μM D-luciferin, 240 μM coenzyme A, and 0.5 mM ATP. Assay buffers are quick frozen in liquid nitrogen and stored at -70°C as 1.3-mL aliquots in amber-colored tubes. AB is light-sensitive and should be stored in dark.
2. Luciferase dilution buffer (LDB): 20 mM Tris-HCl, pH 7.4, 2 mM CDTA, and 1 mg/mL bovine serum albumin (BSA).

2.4.2. Apparatus

1. Lumac (3M) bioluminometer or Turner Luminometer (Promega).
2. Lumacuvet from Celsis (Monmouth Junction, NJ).

2.5. Materials for Tubulin-Polymerization/Depolymerization

1. 10 mg/mL tubulin (can be purchased from Cytoskeleton) are snap-frozen in liquid nitrogen and stored at -70°C as 10-μL aliquots.
2. Taxol (3 mM in DMSO) stored as 10-μL aliquots at -70°C.
3. 0.1 M GTP.
4. 10X PB contains 500 mM potassium phosphate buffer, pH 6.8, 20 mM EGTA, 1 mM EDTA, 20 mM β-mercaptoethanol, 5 mM MgCl₂.
5. 13.6 M Glycerol.
6. 50% Sucrose in 1X PB.
7. 12 mM CaCl₂ in 1X PB.

3. Methods

3.1. Methods for TRiC-Mediated Folding

As an example we describe the TRiC-mediated folding reaction of chemically denatured actin. This assay can be adapted to assay the folding of other denatured substrates or the activity of other chaperones.

1. Each reaction consists of 25 μL of 0.2 μM TRiC in 1X FB (*see Notes 2 and 4*).
2. Rapidly dilute into the reaction [^{35}S]-labeled, urea-unfolded actin (0.25 μL , 2–5 pmol, prepared as described in Chapter 18), mix immediately by gentle vortexing, and incubate for 10 min at 25°C (*see Note 3*).
3. Spin at 14,000g (full speed in a tabletop centrifuge) for 10 min at 4°C, to remove aggregates.
4. Add ATP to 1 mM final concentration to start the folding reaction, and incubate at 30°C. Typically, the reaction reaches a plateau after 45 min (*see Note 4*).
5. Terminate the reaction by adding 6 μL of 5X NB and loading on a native gel (or freezing in liquid nitrogen) (*see Note 5*). Alternatively, the reaction can be terminated by adding 6 μL of 5X SB (*see Note 6*).

3.2. Product Analysis on Native Gels

This native gel system is suitable for separation of native actin from unfolded actin. Native gels (4.5% final acrylamide concentration) are cast in a multiple-gel pouring stand, which can pour up to 14 native gels. Individual gels can be poured by sealing the bottom end with 1% agarose in water. Poured gels can be stored at 4°C wrapped in moist paper towels and Saran Wrap for up to 1 wk. The following protocol is for the simultaneous casting of 10 native gels, but can be adapted to any similar vertical gel apparatus or for pouring single gels. The buffer conditions described are suitable for the analysis of actin folding reactions; obviously, it may be necessary to change the pH of the gel and running buffer to accommodate proteins having different isoelectric points.

1. Mix 20 mL of 5X RB, 14 mL of acrylamide:*bis*-acrylamide (30:0.5% w/v), and 66 mL of water (*see Note 7*).
2. Start the polymerization reaction by adding 0.9 mL of 10% (w/v) ammonium persulfate and 90 μL of TEMED. Pour the mixture between the gel plates, and insert the combs quickly before the gel polymerizes.
3. Following polymerization (which takes ~30–45 min), remove the comb, wash the wells thoroughly with ddH₂O, and either store at 4°C or assemble the gel in the electrophoresis apparatus.
4. Prerun the gel at 100 V for 30 min at 4°C.
5. Load the samples in 1X NB (e.g., from **Subheading 3.1.**), and run the gel at 4°C for 3 h at 100 V.
6. Remove the gel from the apparatus, fix, stain with Coomassie blue, and destain (*see Note 8*).

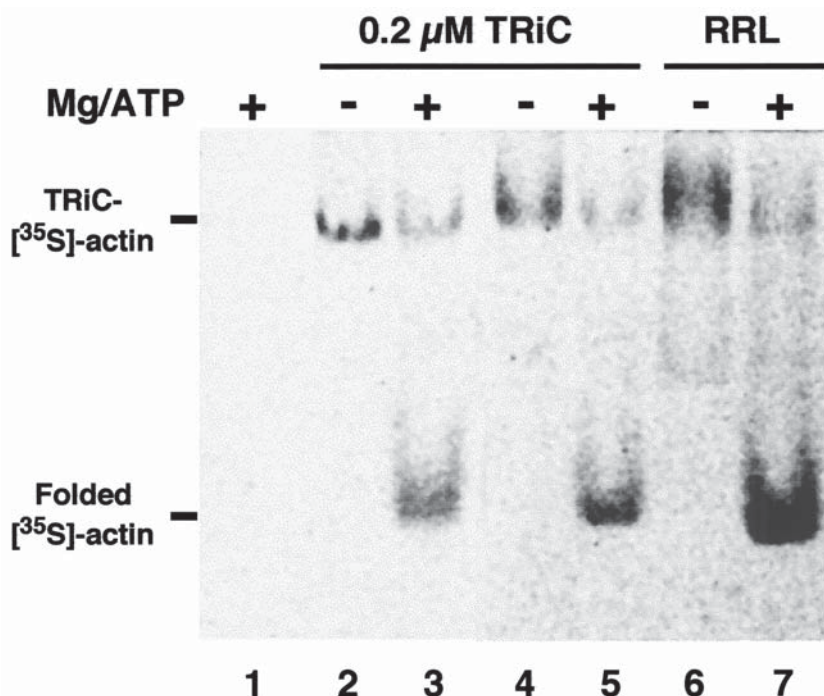


Fig. 1. Native gel analysis of TRiC-mediated folding. [³⁵S]-denatured actin, purified from mammalian cells, was diluted 100-fold into 0.2 μ M TRiC as described in **Subheading 3.1**. Aliquots of 25 μ L were then incubated for 40 min at 30°C in the absence (lanes 2, 4) or presence (lanes 3, 5) of 5 mM MgCl₂ and 1 mM ATP. The samples in lanes (4) and (5) were also supplemented with 10% RRL. As a negative control, [³⁵S]-denatured actin was diluted in buffer containing 5 mM MgCl₂ and 1 mM ATP (lane 1). As a positive control, [³⁵S]-denatured actin was diluted 100-fold into RRL (lanes 6, 7) in the absence (lane 6) or presence (lane 7) of ATP. The samples were loaded and analyzed by native gel electrophoresis as described in **Subheading 3**. The migration of native folded actin and the TRiC-[³⁵S]-actin binary complex are indicated.

7. Dry the gel on a gel drier, and expose to either X-ray film at -70°C or onto a Phosphorimager cassette (*see Notes 9 and 10*). **Figure 1** shows a typical result.

3.3. Protease Resistance Assay

1. The folding reaction containing TRiC-[³⁵S]-substrate (typically ~30 μ L, stopped with SB; *see Subheading 3.1*) is placed in ice and incubated with varying concentrations of the chosen protease. The protease concentration should be titrated to degrade the unfolded, but not the folded, substrate protein. For proteinase K, we have used concentrations ranging from 1 to 100 μ g/mL. Incubate on ice for 10 min (*see Note 11*).

2. The reaction is stopped by addition of 1 μL of 0.1 *M* PMSF to inhibit the proteases. Add 1X SDS sample buffer. Boil the sample for 5 min at 100°C.
3. Analyze the samples by 15% SDS-PAGE.
4. Stain and destain the gel with Coomassie blue.
5. Dry and expose the gels to either X-ray film at -70°C or to a Phosphorimager cassette.

3.4. Luciferase-Bioluminescent Assay

The efficiency of chaperone-mediated folding and renaturation of luciferase is commonly measured by the very sensitive and convenient bioluminescent assay (2,4,8).

1. Purified firefly luciferase (obtained from Sigma or expressed in bacteria) is denatured in 6 *M* guanidinium chloride or urea by incubating at room temperature for at least 30 min, but no more than 3 h at a concentration of 3.5 μM .
2. Binding to TRiC and the subsequent refolding reaction are performed as described in **Subheading 3.1**. Final concentration of luciferase in the refolding reaction is 35 nM. Alternatively, luciferase can be translated in RRL for 30–60 min at 30°C.
3. To 50 μL of the AB add 25 μL of the refolding reaction or 1 μL of the translation mix, vortex for 2 s at 22°C.
4. Immediately assay in the luminometer for 10 s (*see Note 12*).
5. If the amount of luciferase activity exceeds the detection range of the equipment, the sample may be diluted 2 to 10-fold with LDB and 2–5 μL of this diluted mix used for the assay.

3.5. Tubulin-Polymerization/Depolymerization

Only correctly folded tubulin will be assembled/polymerized into microtubules. There are two different methods of copolymerization: temperature-dependent copolymerization and taxol-dependent copolymerization (9) (*see Note 13*). Both methods are very similar and can be used indistinctly. Taxol-dependent polymerization is very fast and efficient, but yields very stable microtubules that are sometimes hard to depolymerize. Tubulin folding can also be assayed using native gel analysis.

3.5.1. Copolymerization by Taxol-Dependent Microtubule Assembly

1. To 20 μL sample, (e.g., [³⁵S]-labeled tubulin translated in rabbit reticulocyte lysate, or TRiC-bound tubulin expressed in bacteria [as described in chapter 18; *see Note 2*]), add 30 μL tubulin (10 mg/mL), 6 μL 10X PB, 0.6 μL GTP (0.1 *M*), 5.5 μL glycerol (13.6 *M*).
2. Incubate the mixture at 37°C for 20 min.
3. Place at room temperature, immediately add 1 μL of 3 mM taxol and mix.
4. Add 6 μL of 5 *M* NaCl, and incubate at 37°C for 20 min to remove microtubule-associated proteins (MAPS).

5. Layer the mix on 3 vol of 50% sucrose in PB. Spin in a TLA 120.1 Beckman rotor at 150,000g for 90 min at 23°C in a Beckman TL100 ultracentrifuge.
6. Resuspend the barely visible pellet at 4°C over 30 min in half the initial volume (30 µL) of 1X PB and 12 mM CaCl₂ to depolymerize tubulin. Taxol-stabilized microtubules are very stable, and the longer incubation ensures complete depolymerization.
7. Spin for 20 min at 80,000g at 4°C to remove denatured tubulin.
8. Initiate another round of polymerization in the supernatant by addition of 15 mM EGTA and 1 mM GTP.
9. Repeat **steps 2–8** again. This assembly/disassembly process is repeated for a total of three cycles to ensure that the pelleted material does not contain denatured tubulin.
10. Following the last round of polymerization, resuspend the pellet in 20–50 µL of 1X SDS sample buffer.
11. Analyze the samples by 10% SDS-PAGE (we use a 10 × 8 cm running gel). In a parallel lane, also load 20 µL of initial lysate to compare how much of the total tubulin was folded.
12. Stain and destain the gel with Coomassie blue. Dry the gel, and expose it to X-ray film or Phosphorimager cassettes.

3.5.2. Copolymerization

by Temperature Dependent Microtubule Assembly

1. To 20 µL sample (e.g., [³⁵S]-labeled tubulin translated in rabbit reticulocyte lysate, or TRiC-bound tubulin expressed in bacteria (as described in chapter 18; see **Note 2**), add 30 µL tubulin (10 mg/mL), 6 µL 10X PB, 0.6 µL GTP (0.1 M), 5.5 µL glycerol (13.6 M).
2. Incubate the mixture at 37°C for 40 min.
3. Layer the mix on 3 vol of 50% sucrose in PB. Spin at 150,000g in TLA 120.1 Beckman rotor for 90 min at 23°C in a Beckman TL100 ultracentrifuge.
4. Resuspend the pellet at 4°C over 30 min (to ensure complete depolymerization) in half the initial volume (30 µL) of 1X PB and 2.5 M glycerol to depolymerize tubulin.
5. Spin for 20 min at 80,000g at 4°C to remove denatured tubulin.
6. This assembly/disassembly process is repeated for a total of three cycles to remove aggregated tubulin and other associated proteins.
7. Following the last round of polymerization, resuspend the pellet in 20–50 µL of 1X SDS sample buffer.
8. Analyze the samples by 10% SDS-PAGE (we use a 10 × 8 cm running gel). In a parallel lane, also load 20 µL of initial lysate to compare how much of the total tubulin was folded.
9. Stain and destain the gel with Coomassie blue. Dry the gel, and expose it to X-ray film or Phosphorimager cassettes.

4. Notes

1. Tubulin is very sensitive to the buffer pH and denatures at pHs higher than 7.0. In contrast, actin denatures at pHs lower than 7.0.

2. Instead of using purified TRiC, actin, tubulin, luciferase, or any other chemically denatured protein can also be refolded by dilution into rabbit reticulocyte lysate (RRL) and incubation in the presence of ATP/Mg. RRL is a rich source of chaperones and can be used as a positive control of the specific activity of the purified chaperone. The protocol used in this case is briefly summarized:
 - a) To 25 μL of RRL (50% in 1X FB), add an ATP-regenerating system (10 mM creatine phosphate, 20 units/mL creatine phosphokinase).
 - b) To the above refolding mix, add 0.5 μL of [^{35}S]-labeled unfolded substrate protein and mix vigorously
 - c) Incubate at 30°C.
3. The denatured protein (in urea or guanidinium hydrochloride) should be added rapidly to the TRiC solution, and vortexed immediately but gently. This will prevent the formation of high local concentrations of denaturant, which may inactivate TRiC or the proteins in the lysate.
4. In the TRiC-mediated folding assay, one should always include “-ATP” control to be able to assess the folding reaction in the presence and absence of ATP. Thus, the minimal total volume for the folding assay will contain 75 μL (for three reactions). For the “minus ATP” control, 25 μL are removed before adding ATP in **step 4 (Subheading 3.1.)**. In addition, 25 μL are used to estimate the total actin present in these samples (*see Note 6*). For larger experiments (time-courses or when testing different conditions), the volume of the mix is increased accordingly.
5. To measure the rate of TRiC-mediated folding, 25- μL aliquots are removed from the master mix at different time-points after addition of ATP and added to 6 μL of 5X SB. The CDTA in SB will specifically chelate the Mg^{2+} required for TRiC function, whereas the Ca^{2+} will stabilize the folded actin (which is a Ca^{2+} /ATP binding protein).
6. The efficiency of folding is measured as the fraction of folded/total substrate protein present in the sample. Thus, it is necessary to have a way to estimate the amount of substrate in the starting folding reaction. Usually, a fraction of the total actin in each experiment is also included in the analysis by native gel electrophoresis.
7. In some cases, for example, in experiments involving generation of native actin (which binds ATP) or tubulin (which binds GTP), it is necessary to include ATP and/or GTP to a final concentration of 0.1 mM.
8. The 4% native gel is very fragile and sticky, and should be handled with extreme care, especially while trying to detach the gel from the glass plates for staining. The gel tends to stick to the glass plate, but using very clean plates when pouring the native gels alleviates this problem.
9. To dry the native gels, layer them onto Saran Wrap, and spread them properly. Then layer a wet Whatman 3MM paper on top of the gel. Flip the sandwich so that the Whatman paper is below the gel and the Saran Wrap above the gel.
10. Exposure using Phosphorimager cassettes is preferred over exposure using X-ray films owing to their increased sensitivity and broader linear range. Thus, they require a much shorter exposure time than X-ray film, and the quantitation of the digitized labeled bands is much easier in the Phosphorimager.

11. For the protease sensitivity assay, the protease concentration and the incubation time and temperature may be changed, depending on the sensitivity of the protein of interest. In general, proteinase K and thermolysin are favored because of their preference for hydrophobic amino acids, which are usually exposed in nonnative proteins and inaccessible in correctly folded proteins. In addition, TRiC-bound tubulin is less sensitive to proteinase K than the unfolded form (7).
12. Light intensity is very dependent on temperature. Optimal luciferase activity is achieved at approximately room temperature (20–25°C). It is important that the luciferase assay reagent be fully equilibrated to room temperature before beginning measurements. The light intensity of the reaction is nearly constant for about 20 s and then decays slowly, with a half-life of about 5 min. Thus, it is best to begin measurement within 2–3 s of mixing with the assay reagent.
13. Tubulin should not be frozen and thawed more than once, nor should it be exposed to pH values < 6.8 or > 7.0. Thus, the use of Tris-based buffers is not appropriate for the temperature-dependent polymerization and depolymerization assays, since the pH of Tris changes with temperature. In addition, tubulin is not stable at high NaCl concentrations (i.e., >100 mM) or in the absence of GTP. Once microtubules are stabilized, e.g., with taxol, they are less sensitive to buffer conditions.

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Purification of Prefoldin

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1. Introduction

Prefoldin is a recently discovered chaperone protein (*1,2*) that functions by directing unfolded target proteins to the cytosolic chaperonin, c-cpn (*3,4*). It thus serves to promote productive folding in the crowded molecular environment that prevails inside living cells, where there are many competing pathways for nonnative proteins. Prefoldin binds to c-cpn and transfers its target protein to the chaperonin (*1*). Like c-cpn, prefoldin is heteromeric: it is assembled from six different polypeptides in the size range 14–23 kDa. Each subunit is present in approximately stoichiometric amount. Prefoldin migrates with an apparent molecular mass of about 200 kDa on gel filtration, so each molecule contains either one or two each of the six subunit polypeptides. The prospect of engineering the simultaneous expression of six open reading frames in a prokaryotic host is a daunting one; in any event, it is uncertain whether the expressed subunits would assemble into functional prefoldin molecules in *Escherichia coli*. From a practical point of view, therefore, a biochemical study of prefoldin function requires its purification from a tissue source. Prefoldin is widely expressed; however, the protocol described here is for purification from bovine testis, because (1) testis tissue expresses relatively high levels of prefoldin and c-cpn, and (2) the tissue is easily obtainable from slaughterhouses and is relatively inexpensive. The purification procedure involves the preparation of a soluble protein extract, followed by ammonium sulfate fractionation and (minimally) four successive chromatographic dimensions.

2. Materials

1. Bovine testis tissue from freshly slaughtered animals (usually delivered on ice, and weighing in the range of 0.5–1 kg).

Table 1
Columns Used in the Purification of Prefoldin from Bovine Testis

Dimension	Matrix ^a	Size ^b	Buffers
1	Q-Sepharose HP	5/25	I/II
2	Phenyl Superose	2.6/12	III/IV
3	Hydroxylapatite	1/10	V/VI
4	MonoS	0.5/5	VII/VIII

^aThe columns and matrices (with the exception of hydroxylapatite, which is from American International Chemical, Inc., Natick, MA) are available from Pharmacia, Inc., Piscataway, NJ.

^bDiameter/height of column bed in cm.

2. Meat grinder and Waring-type blender.
3. Phenylmethylsulfonyl fluoride (PMSF): 0.1 M stock in ethanol; aprotinin, 1 mg/mL.
4. Cell debris remover (CDR), Whatman International Ltd., Maidstone, England, cat. no. 4025050.
5. Liquid nitrogen (several liters).
6. FPLC apparatus (or equivalent) equipped with the columns listed in **Table 1**.
7. Buffers for homogenization and for FPLC. All buffers contain 1mM dithiothreitol (DTT), plus the following: Homogenization buffer: 20 mM Tris-HCl, pH 7.2, 10 mM KCl, 5 mM MgCl₂, 1 mM EGTA. FPLC buffers (*see Table 1*):
 - I. 10 mM Na phosphate buffer, pH 7.2, 1 mM EGTA.
 - II. 400 mM Na phosphate buffer, pH 7.2, 1 mM EGTA.
 - III. 20 mM Tris-HCl, pH 7.2, 1.0 M (NH₄)₂SO₄, 1 mM MgCl₂, 1 mM EGTA.
 - IV. 20 mM Tris-HCl, pH 7.2, 1 mM MgCl₂, 1 mM EGTA.
 - V. 20 mM Na phosphate buffer, pH 6.8, 20 mM KCl, 1 mM MgCl₂, 0.1 mM CaCl₂.
 - VI. 0.5 M Na phosphate buffer, pH 6.8, 20 mM KCl, 1 mM MgCl₂, 0.1 mM CaCl₂.
 - VII. 20 mM 2-(N-morpholino) ethane sulfonic acid MES, pH 6.0, 20 mM NaCl, 2 mM MgCl₂, 1mM EGTA.
 - VIII. 20 mM MES, pH 6.0, 1.0 M NaCl, 2 mM MgCl₂, 1 mM EGTA.
8. Filters (0.4 μ) for extract preparation.
9. Sephadex G25 (medium) columns for buffer exchange.
10. Amicon ultrafiltration apparatus with YM30 membranes and centricon units (type 30) for protein concentration.
11. ³⁵S-labeled, urea-unfolded actin probe (2) for prefoldin assay (*see Chapters 18 and 22*).

3. Methods

3.1. Preparation of Tissue Extract

Unless otherwise stated, all procedures are done at 4°C.

1. Remove all skin and connective tissue, and weigh the testis tissue.
2. Cut the tissue into manageable chunks (with scissors), and insert these into the meat grinder.

3. Transfer the ground tissue to the Waring blender, and add ice-cold homogenization buffer (0.5l/0.5 kg tissue) containing 1 mM PMSF and 1 mg/mL aprotinin. Blend at full-speed for 2 min.
4. Transfer the creamy extract to 250-mL centrifuge bottles and centrifuge at 12,000g for 30 min.
5. Transfer the supernatants to ultracentrifuge tubes and centrifuge at 100,000g for 1 h.
6. Decant the supernatants, and stir for 5 min with 5 g of CDR (*see Note 1*). Filter the slurry on a Buchner funnel through a double layer of Whatman No. 1 filter paper.

3.2. Storage of Tissue Extracts

Preparation of the tissue extract (described above) takes several hours, and it is usually not convenient to proceed with protein purification on the same day. The extract can be conveniently stored frozen using the following protocol:

1. Fill a styrofoam container with several liters of liquid N₂.
2. Using a 25-mL pipet, allow the filtered tissue extract prepared as described above to fall into the liquid N₂. The rate of delivery should be such that the extract falls as individual drops rather than as a continuous stream; these drops form frozen “pearls” on entry into the liquid phase.
3. Transfer the frozen “pearls” to a convenient container, and store at –70°C.

3.3. Ammonium Sulfate Fractionation

1. Thaw the frozen extract prepared as described above in a flask at 37°C.
2. Add solid (crystalline) ammonium sulfate to 30% saturation (176 g/L). The addition should proceed slowly over a period of 20–30 min, with stirring, on ice. Continue stirring for a further 30 min after all the ammonium sulfate has been added.
3. Centrifuge at 10,000g for 10 min at 4°C.
4. To the supernatant, add solid ammonium sulfate to 55% saturation (162 g/L) as described in **step 2**, and centrifuge as described in **step 3**.

3.4. Anion Exchange Chromatography

1. Dissolve the pellet from **Subheading 3.3., step 4** in about 200 mL of buffer I by gentle swirling, and transfer to dialysis tubing.
2. Dialyze against 4 L of buffer I for 16 h with several changes.
3. Centrifuge the dialyzed extract at 10,000g to remove precipitated material.
4. Filter the supernatant through a 0.4 μ filter.
5. Load the filtrate (which should contain 4–5 g total protein) onto a pre-equilibrated 5 × 25 cm column of Q-Sepharose HP through the FPLC pump at a flow rate of 10 mL/min. Wash with buffer I until the absorbance (A₂₈₀) of material emerging from the column is <0.2.
6. Develop the column with a 1.5-L linear gradient (to 100%) using buffer II at a flow rate of 8–10 mL/min. Prefoldin emerges from the column in the range 0.34–0.39 M Na phosphate (*see Notes 2 and 3*).

7. Pool peak fractions containing prefoldin activity (typically about 150 mL, containing about 0.2–0.3 g total protein) and concentrate using an Amicon ultrafiltration cell fitted with a YM30 membrane to a final volume of about 20 mL.

3.5. Hydrophobic Interaction Chromatography

1. Exchange the concentrated pool from **Subheading 3.4., step 7** into buffer III by passage over a pre-equilibrated 50 mL column of Sephadex G25.
2. Load the protein-containing fractions from the G25 column onto a column of phenyl Superose HP pre-equilibrated in buffer III at a flow rate of 2 mL/min using a twice-filled 10-mL superloop.
3. Wash the column at 4 mL/min with buffer III until the absorbance at 280 nm is <0.2, and develop the column with a linear gradient (90 mL) to 100% buffer IV, collecting fractions of 4 mL.
4. Pool fractions containing peak prefoldin activity (typically 16 mL, containing about 20–40 mg total protein). Prefoldin emerges in the range 0.40–0.26 M ammonium sulfate (*see Notes 2 and 3*). Concentrate the pooled fractions using an Amicon ultrafiltration cell fitted with a YM30 membrane to a final volume of about 2 mL.

3.6. Hydroxylapatite Chromatography

1. Exchange the concentrated pool from **Subheading 3.5., step 4** into buffer V by passage over a pre-equilibrated 8-mL column of Sephadex G25.
2. Load the protein-containing fractions emerging from the G25 column onto a column of hydroxylapatite (**Table 1**) pre-equilibrated in buffer VI (*see Note 4*).
3. Wash the column at 2 mL/min with buffer VI until the absorbance at 280 nm is <0.1, and develop the column with a linear gradient (70 mL) to 100% buffer VII, collecting fractions of 2 mL.
4. Pool fractions containing peak prefoldin activity (typically 8 mL, containing about 1–2 mg total protein as measured in a Bradford assay). Prefoldin emerges in the range 50–100 mM Na phosphate (*see Notes 2 and 3*).
5. At this stage, the prefoldin should be recognizable by its characteristic band pattern on a 10% Tricine SDS-polyacrylamide gel (**Fig. 1**). Pool fractions containing peak prefoldin as judged by the Coomassie blue staining pattern, and concentrate on a Centricon 30 unit (Amicon) to a volume of about 1 mL.

3.7. Cation Exchange Chromatography

1. Exchange the concentrated pool from **Subheading 3.6., step 5** into buffer VIII by passage over a pre-equilibrated 8-mL column of Sephadex G25.
2. Load the protein-containing fractions emerging from the G25 column onto a MonoS column (**Table 1**) pre-equilibrated in buffer IX (*see Note 4*).
3. Wash the column at 1 mL/min with buffer IX until no further absorbance (at 280 nm) is detectable, and develop the column with a linear gradient (30 mL) to 50% buffer X, collecting fractions of 1 mL. Prefoldin emerges from this column as a clearly defined and symmetrical peak in the range 60–100 mM NaCl.

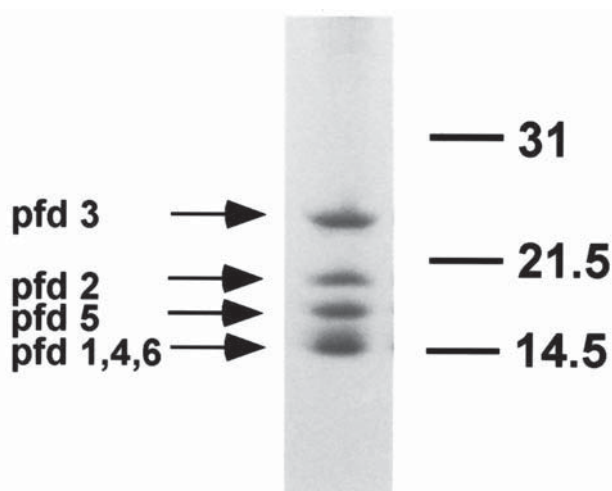


Fig. 1. Prefoldin analysis by tricine SDS-PAGE. Purified prefoldin consists of six subunits in the mol-wt range 14–23 kDa. (Note that not all of the subunits are perfectly resolved by SDS-PAGE [1]).

4. Check the purity of the prefoldin emerging from the MonoS column on a 10% Tricine SDS polyacrylamide gel. The purity should be greater than 90% (as judged by Coomassie blue staining) (see **Note 5**). The purified protein can be stored in aliquots either by flash-freezing in liquid N_2 , or by freezing following the addition of glycerol to 20% v/v. The yield of purified prefoldin obtained by the procedure described is typically in the range 150–250 μ g, starting from about 10 g of total soluble protein. This is sufficient for several hundred in vitro folding assays (1).

4. Notes

1. CDR is a cellulose-based very weak ion exchanger, which binds nucleic acids and some lipids (but not proteins) in crude cell extracts. Although not essential, the use of this material serves to reduce exposure of the initial anion-exchange column to unwanted materials; this avoids an inevitable reduction in the anion-exchange column's protein binding capacity, and reduces the frequency with which the column must be cleaned by treatment with strong base, acid, and so forth.
2. The buffer concentrations at which prefoldin emerges from each of the columns used in the purification described are only intended as a rough guide. Some variation occurs from one preparation to another, probably depending on such variables as small differences in temperature, protein concentrations, cleanliness of the chromatographic columns, and so forth. It is essential, therefore, to follow prefoldin activity with an assay throughout the purification procedure or at least until after the hydroxylapatite step, when prefoldin can be visually identified by SDS-PAGE. A reliable way to follow prefoldin activity is to use a functional

assay, i.e., binding of a labeled, unfolded actin probe and analysis of the resulting product on a nondenaturing gel (*see* Chapters 18, 19, and 22).

3. It is advisable not to discard any column fractions (including material that does not bind) until the location of prefoldin (determined either functionally or by inspection of SDS-PAGE—*see* **Note 1**) has been firmly established. Because the purification procedure involves at least four successive chromatographic dimensions, it is necessary to store fractions at least overnight between steps. Storage is also required pending the result of assays done to locate prefoldin activity precisely. Prefoldin seems to be a relatively resilient protein: we have found no appreciable loss of activity on overnight storage of column fractions at 4°C. The effect of more prolonged storage at 4°C is unknown. If more prolonged storage proves necessary, we have found that addition of glycerol (to 20%) and storage at -70°C is satisfactory. Note, however, that the presence of glycerol greatly slows the rate of concentration in subsequent ultrafiltration steps, and it may be necessary to remove glycerol by either dialysis or passage over Sephadex G25.
4. We have found that these dimensions may be run at room temperature without compromising the yield or activity of prefoldin.
5. A further dimension (i.e., gel filtration on a column of Superose 6) may be added to the purification procedure if required.

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Purification of GimC from *Saccharomyces cerevisiae*

Katja Siegers and Elmar Schiebel

1. Introduction

The recently identified hetero-oligomeric protein complex, named GimC, present in eukarya and archaea, is a critical cofactor of TRiC/CCT-assisted folding of actin and tubulins (1,2). Purification and characterization of this protein complex revealed that its subunits are encoded by six different genes, *GIM1* to *6*. In vitro studies with purified GimC showed that the protein complex interacts with the eukaryotic chaperonin TRiC/CCT and binds unfolded tubulin and actin, the major substrates of TRiC, but it does not bind to native actin.

Purification of low abundant protein complexes often requires several different purification steps, including the use of various column systems in order to yield highly enriched protein. Since this is in general a very time consuming and labor-intensive procedure, we use a different approach for the purification of GimC from yeast. This approach includes the construction of a yeast strain that expresses a fully functional protein A-tagged version of one of the subunits of GimC. The generation of the chromosomally integrated *GIM2-ProA* gene fusion follows a simple polymerase chain reaction (PCR) based strategy. This strategy is based on the use of a plasmid as PCR-template containing a module encoding the cleavage site of the TEV protease, protein A, and the heterologous *kanMX6* selection marker. This cassette is one of several modules that can be used to generate PCR products with flanking homologous sequences that allow their targeted integration into the genome of yeast cells (3–5). Once the yeast strain carrying the chromosomally integrated *ProA*-tagged version of *GIM2* has been generated, the functionality of the tagged gene can be tested easily, since the lack of function of GimC subunits results in distinct phenotypes, such as sensitivity toward the microtubule-destabilizing substance benomyl, cold sensitivity, and sensitivity toward high osmolarity

(I), which are common phenotypes for mutants with defects in the actin and tubulin cytoskeleton (6–8). Using the *GIM2-ProA* yeast strain, GimC can be purified by means of an affinity chromatography via the protein A tag. Since the protein A tag we use contains an adjacent cleavage site for the highly specific TEV protease, GimC can be liberated by treatment of the Sepharose-bound ProA-GimC with TEV protease. The cleavage between the protein A tag and GimC yields high amounts of functional GimC, whereas the protein A tag used for the affinity purification remains bound to the IgG Sepharose. After the treatment with TEV protease, GimC is subsequently subjected to a gel-filtration column to separate the purified protein complex from the added TEV protease.

2. Materials

2.1. Construction of the Tagged Yeast Strain

2.1.1. Generation of the PCR-Product for Tagging of *GIM2*

1. PCR primers:
 GIM2-S2: 5' TAGTGCCCTTTATTTTTCTTTGGATTTAAATTTACAGACA-CATTA atcgatgaattegagctcg
 GIM2-S3: 5' CAAGACTTGAAGCAGGCTCAAGAAGGGACTAAAAACCT-CAAGATA cgtacgctgcaggctgac
2. Template (plasmid containing *TEV-ProA-kanMX6* module): pYM9.
3. “Expand™ long-template PCR system” kit (Boehringer Mannheim, Gaithersburg, MD).
4. Vent™ Polymerase (Biolabs) and *Taq* Polymerase of any supplier.

2.1.2. Transformation of Yeast Cells

1. S288C derived yeast strain YPH499 (*MATa ura3-52 lys2-80Iamber ade2-10Iochre trpΔ63 hisΔ200 leu2Δ1*) (9).
2. Li-SORB (sterile): 100 mM lithium acetate, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA-NaOH, pH 8.0, 1 M sorbitol (special grade for molecular biology from Merck), pH 8.0 adjusted with diluted acetic acid. The solution has to be filter-sterilized and can be stored at room temperature for several months.
3. Li-PEG (sterile): 100 mM lithium acetate, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA-NaOH, pH 8.0, 40% PEG 3350 (Sigma). The solution has to be filter-sterilized and can be stored at 4°C for several months.
4. Carrier DNA: Shared salmon sperm DNA (10 mg/mL, Gibco BRL). The DNA was denatured at 100°C for 10 min and cooled on ice. The denatured carrier DNA can be stored at –20°C and thawed repeatedly.
5. Dimethyl sulfoxide (DMSO).
6. YPD medium: 1% Bacto-yeast extract, 2% Bacto-peptone, 2% glucose, 100 mg/mL adenine. Autoclave.
7. YPD agar plates: YPD medium plus 2% Bacto-agar (Difco).
8. YPD-G418 (geneticin) plates: add 200 mg/mL solid geneticin (Sigma, G-9515) to the autoclaved medium agar after it has cooled to a temperature below 60°C.

2.1.3. Control of Expression of GIM2-ProA

1. 1.85 M NaOH, 7.5% β -mercaptoethanol (prepare freshly before use).
2. 55% trichloroacetic acid (TCA) (w/v, stored in the dark).
3. HU buffer: 8 M urea, 5% SDS, 200 mM Tris-HCl, pH 6.8, 1 mM EDTA 0.02% bromphenol blue (as pH indicator), 5% β -mercaptoethanol. The buffer should be stored without β -mercaptoethanol at -20°C . Add β -mercaptoethanol just before use.
4. Blotting buffer: 25 mM Tris-HCl, 192 mM glycine, 0.25% SDS, 20% methanol.
5. Ponceau S (0.2% Ponceau S in 3% TCA).
6. TBS: 20 mM Tris-HCl, pH 7.6, 137 mM NaCl.
7. TBS-T: 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween-20.
8. Dried skimmed milk (99% fat-free).
9. Rabbit-antigoat-IgG/horseradish peroxidase (HRP) conjugates and goat-antirabbit-IgG/HRP conjugates (both from Jackson Immuno Research Laboratories).
10. ECLTM-Kit (Amersham).

2.1.4. Control of Functionality of Gim2p-ProA

1. Sterile YPD medium.
2. Sterile water.
3. YPD-BENOMYL plates: prepare YPD agar (*see Subheading 2.1.2., item 7*), autoclave, let it cool down to a temperature below 60°C , and add benomyl (DuPont) to a final concentration of 2.5 $\mu\text{g}/\text{mL}$ (use freshly prepared stock solution of 10 mg/mL benomyl dissolved in DMSO, and add the required amount of solution slowly, while stirring the YPD-agar).
4. YPD-SORBITOL plates: add sterile sorbitol solution (4.5 M) to a final concentration of 1.4 and 1.8 M to autoclaved YPD agar.

2.2. Purification of GimC

1. Buffer A: 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5% glycerol, and protease inhibitors: 6 $\mu\text{g}/\text{mL}$ antipain, 4.3 $\mu\text{g}/\text{mL}$ leupeptin, 4.5 $\mu\text{g}/\text{mL}$ aprotinin, 5 $\mu\text{g}/\text{mL}$ bovine trypsin inhibitor, 5 $\mu\text{g}/\text{mL}$ pepstatin, 6 $\mu\text{g}/\text{mL}$ chymostatin, 350 $\mu\text{g}/\text{mL}$ benzamidine-HCl, 1 mM PMSF.
2. Acid-washed glass beads (diameter 425–600 nm, Sigma).
3. IgG-Sepharose 6 Fast Flow (Pharmacia): 100 μL bed volume. 1 g of cells.
4. Buffer B: 50 mM Tris-HCl, pH 7.6, 150 M NaCl.
5. Buffer C: 0.5 M acetic acid, pH 3.4 (add 3 mL 100% acetic acid to 90 mL water, add solid ammonium acetate to adjust pH to 3.4, add water to a total volume of 100 mL).
6. Buffer D: 50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA.
7. TEV protease (Gibco-BRL).
8. Buffer E: 50 mM Tris-HCl, 300 mM NaCl.
9. Filtron Nanosep microspin concentrators.
10. Superose 12 PC 3.2/30 gel-filtration column.
11. Buffer F: 50 mM Tris-HCl, pH 8.0, 0.5 M EDTA, pH 8.0, 150 mM NaCl, degassed and filtered through a 0.22- μM filter.
12. Glycerol (87%).

3. Methods

3.1. Construction of the Tagged Yeast Strain

3.1.1. Generation of the PCR-Product for Tagging of *GIM2*

1. The PCR-based strategy used to generate the chromosomally integrated *GIM2-ProA* gene fusion is outlined in **Fig. 1** (see **Note 1**).
2. For the amplification of the *TEV-ProA-kanMX6* module, use standard PCR conditions and a mixture of thermostable DNA polymerases with and without 3'-5' exonuclease activity (e.g., 1 U *Taq* polymerase mixed with 0.4 U Vent DNA polymerase) to increase yield and fidelity of the reaction. Good results in terms of yield and reliability can be obtained with the "Expand™ long-template PCR system" Kit (Boehringer Mannheim) with the polymerase mixture.
3. Analyze a fraction (5 μ L) of the reaction by agarose-gel electrophoresis (expect a fragment of 2145 bp).
4. Precipitate the residual PCR-Product with ethanol, resuspend in 15 μ L of water, and use 5 μ L for the transformation of yeast cells.

3.1.2 Transformation of Yeast Cells

The transformation protocol is based on the LiOAC method published by Schiestl and Gietz (**10**).

1. Inoculate 100 mL YPD medium from a fresh preculture, and let the cells grow for at least 6–8 h or overnight to a density of 0.5–0.7 OD₆₀₀ at 30°C (this corresponds to 2–3·e⁷ cells/mL) (see **Note 2**).
2. Harvest cells by centrifugation (1000g, 5 min, room temperature).
3. Discard the supernatant and wash the cells once with 100 mL of sterile water and once with 25 mL sterile Li-SORB.
4. Resuspend the cells in a total volume of 540 μ L Li-SORB, and add 60 μ L denatured carrier DNA to the cell suspension.
5. Aliquot cells (50 μ L each) and store at –80°C or use cells directly for transformation (see **Note 3**).
6. Thaw an aliquot of the competent yeast cells, and add the PCR product to the cell suspension.
7. Mix well, and add 300 mL of sterile Li-PEG. Mix well again, and incubate at room temperature for approx. 30 min.
8. Add DMSO to a final concentration of approx. 10%, and incubate at 42°C for 15 min.
9. Sediment cells at 1000g for 2–3 min, remove supernatant completely and wash the cells with 0.5 mL sterile YPD medium.
10. Resuspend cells in 2 mL YPD medium, and incubate at 30°C for 2–3 h with shaking.
11. Collect cells by centrifugation (1000g, 2–3 min), resuspend pellet in 200 μ L of sterile YPD medium, and plate onto two YPD-G418 plates.
12. Incubate plates at 30°C for 2–3 d. Selection for the dominant resistance marker *kanMX6* on YPD/G418 plates usually results in a high background of microcolonies. Therefore replica plate onto fresh YPD/G418 plates and the transformants will appear after 2 d of further incubation at 30°C.

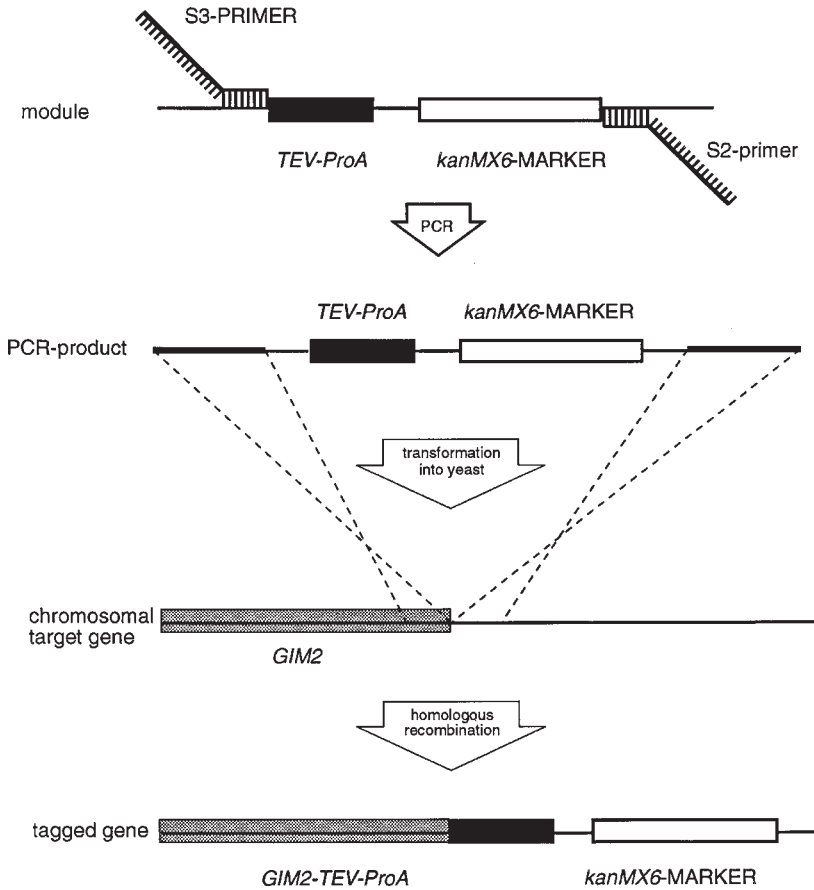


Fig. 1. Strategy used to generate the chromosomally integrated *GIM2-ProA* gene fusion. In a first step, the desired module is PCR-amplified using S2 and S3 primers. The DNA is then transformed into yeast cells. Both ends of the PCR product contain homologous sequences (that derive from the S2 and S3 primers) that target the DNA to be introduced at the desired chromosomal location via homologous recombination. Correct transformants now express an in-frame fusion of the target gene with the epitope tag. Terminators provided by the modules substitute for the original terminators.

- Pick a suitable number of clones (e.g., six transformants), and streak them out onto YPD-G418 plates to obtain single colonies.

3.1.3. Control of Expression of *GIM2-ProA*

In order to analyze the obtained transformants for the production of the Gim2p-TEV-ProA fusion protein, cells were lysed according to a protocol adapted from previously described methods (11,12).

1. Inoculate 5 mL of YPDmedium with a single colony of the obtained transformants and grow at 30°C to an OD₆₀₀ of approx. 1.0 (this corresponds to 4-5·e⁷cells/mL).
2. Collect cells by centrifugation, and resuspend in 1 mL of cold water. Alternatively, resuspend a toothpick full of cells from a fresh plate (from **Subheading 3.1.2., step 13**) in 1 mL of water (see **Note 4**).
3. Add 150 µL of 1.85 M NaOH, 7.5% β-mercaptoethanol to the cell suspension, mix well, and leave on ice for 15 min.
4. Add 150 µL of 55% TCA, mix well, and incubate for an additional 15 min on ice.
5. Spin for 10 min at 14,000g at 4°C and remove supernatant completely by aspiration.
6. Resuspend the pellet in 150 µL of HU-buffer, and incubate for 10 min at 65°C to denature the proteins.
7. If the capacity of the HU buffer is not high enough to neutralize remaining traces of TCA (indicated by the yellow color of the solution), add 1–3 mL of 2 M Tris base.
8. Spin for 5 min at 14,000g (room temperature), separate 10–15 µL of the sample by using a 12.5% SDS-PAGE (**13**), and transfer the separated proteins onto nitrocellulose by electroblotting. Incubate the gels in blotting buffer for 10 to 15 min before setting up the electrotransfer. The transfer is carried out at 2 mA/cm² for 90 min, and the efficiency of the transfer to the nitrocellulose is checked by reversible staining of the nitrocellulose membrane with Ponceau S (see **Note 5**).
9. Coat the nitrocellulose membrane in TBS/10% milk over night at 4°C or room temperature for 2 h.
10. Wash the blot once with TBS-T
11. The protein A tag of the Gim2p fusion protein can be detected using subsequent incubations for 1–2 h at room temperature with rabbit–antigoat–IgG/horseradish peroxidase (HRP) and goat–antirabbit–IgG/HRP conjugates (each diluted 1: 5000 in TBS-T). Wash the nitro-cellulose membrane three times with TBS-T after each incubation with antibody, and use the ECL Western blotting detection system according to the guidelines given by the manufacturer for protein detection. The Gim2p–ProA fusion protein has an apparent molecular mass of 60 kDa. No protein is detected in the control strain that does not carry the *GIM2–TEV-ProA* fusion (see **Note 6**).

3.1.4. Control of Functionality of *Gim2p-ProA*

Since a loss of function or even reduced functionality of the *GIM* gene products results in distinct phenotypes such as cold sensitivity, sensitivity to high osmolarity, and high sensitivity towards the microtubule-destabilizing substance benomyl (**1**), the functionality of the *Gim2p–TEV-ProA* fusion protein can be tested easily by comparing the growth of wild-type *GIM2* yeast cells, and the *GIM2–TEV-ProA* strain on YPD-BENOMYL and YPD-SORBITOL plates.

1. Grow cells of a wild-type *GIM2* strain and of the strain carrying *GIM2–TEV-ProA-kanMX6* instead of *GIM2* in 5 mL YPD medium by shaking at 30°C to an OD₆₀₀ of 1 (this should correspond to 4-5·e⁷cells/mL).

2. Collect cells from 1 mL of culture by centrifugation (1000g, 2–3 min), and wash in 1 mL of sterile water.
3. Resuspend cells in sterile water to 2×10^7 cells/mL, and make serial dilutions in sterile water ranging from 1:10 to 1:100,000.
4. Drop 5 μ L of each dilution onto YPD plates, YPD-BENOMYL plates, and YPD-SORBITOL plates, and incubate for 2–5 d at 30°C or 23°C, and compare growth of the *GIM2-TEV-PROA-kanMX6* strain with that of the wild-type yeast strain. You should not observe any noticeable differences in sensitivity toward benomyl or sorbitol, which indicates the functionality of the *Gim2p* fusion protein. If you observe reduced growth rates for the strain carrying the *GIM2-ProA* fusion, this results from a loss of function of the *GIM2* gene, which might be owing to a mutation acquired by PCR or during the transformation and recombination procedure.

3.2. Purification of *GimC*

1. Resuspend 20 g (this corresponds to approximately 4 L of culture grown in YPD medium to an OD_{600} of 1–2) of yeast cells (*GIM2-TEV-ProA* strain) in an equal volume of cold buffer A, and add an equal volume of cold acid-washed glass beads. Leave about 1–2 mm liquid above the glass beads (*see Note 7*).
2. Disintegrate the yeast cells by vortexing 6 times for 30 s with incubation on ice for 30 s in between (*see Note 8*).
3. Check the cell suspension under a microscope to make sure that more than 95% of the cells have been disrupted (broken cells appear dark in phase contrast)—if <95% of cells appear dark, continue vortexing two more times and check again.
4. Spin at 1000g for 10 min at 4°C to pellet unbroken cells and glass beads.
5. Collect supernatant, and wash glass beads twice with an equal volume of buffer A to recover as much cell lysate as possible.
6. Spin the combined supernatant for 1 h at 100,000g at 4°C.
7. In the meantime, prepare IgG-Sepharose: take 100 μ L bed volume/1 g cells. Wash Sepharose with 2–3 bed volumes each of:
 - a. Buffer C.
 - b. Buffer B
 - c. Buffer C.
 - d. Buffer B.Check pH of the last eluate with pH paper (should be neutral), and equilibrate with buffer A.
8. Take the supernatant from the 100,000g spin and incubate with (pre-treated) IgG-Sepharose with slight agitation overnight at 4°C.
9. Wash Sepharose beads with buffer A until you do not see any protein in the eluate.
10. Wash two more times with 5 bed volumes of buffer A.
11. Wash two times with 5 bed volumes of buffer D, and resuspend in 1 bed volume of buffer D.
12. Incubate the Sepharose beads with 5 U of TEV-protease for 2 h at 4°C with slight agitation.

13. Collect supernatant, and add an equal volume of buffer E to obtain a final concentration of 150 mM NaCl.
14. Concentrate three- to fivefold by using Filtron Nanosep microspin concentrators.
15. Spin for 10 min at 20,000g at 4°C and apply sample to a Superose 12 column.
16. Operate Superose 12 column at a flow rate of 20 $\mu\text{L}/\text{min}$ at 10°C using filtered and degassed buffer F as running buffer.
17. Collect 50- μL fractions, GimC elutes as a single peak at approx. 1.2 mL. Add glycerol to the respective fractions to a final concentration of 10%, freeze rapidly in liquid nitrogen, and store at -80°C .
18. This procedure should result in a yield of about 100–200 μg purified GimC (*see Note 9*).
19. The purified GimC can be used for in vitro actin or tubulin binding assays (*see chapters 19 and 22*).

4. Notes

1. The sequences that are required for homologous recombination have to be at least 45 bp in length. However, longer homologous sequences (up to 60 bp) increase the efficiency of the recombination significantly.
2. A refraction of 1 OD_{600} of cells grown in YPD medium to logarithmic growth phase corresponds to $4.3 \cdot 10^7$ cells/mL for strain YPH499, if measured with an LKB Ultrospec Biochrome II (Pharmacia) spectrophotometer. Note, the OD_{600} to cells/mL correlation has to be determined individually for each spectrophotometer because of the different path lengths. The samples have to be diluted to an OD_{600} of 0.05–0.5 for accurate measurements.
3. Do not freeze the competent yeast cells rapidly in liquid nitrogen. Place them at -80°C .
4. For the lysis of yeast cells by the TCA-based method, it is not absolutely necessary that the cells have been grown in liquid medium. Resuspending a toothpick full of yeast cells taken from a fresh plate usually gives satisfactory results.
5. For unknown reasons, TCA-treated proteins require a longer transfer time than nontreated proteins (about 1.5- to 2-fold the normal time) (*II*).
6. This sandwich procedure allows a highly sensitive detection of the protein A tag, but is not an absolute requirement, since using just one HRP-conjugated species of antibody is usually sufficient for detection of the protein A fusion proteins. If you just use one antibody for the detection, you should take into account that protein A has different affinities for IgGs from different species.
7. In order to achieve an efficient cell lysis, it is important not to add too many or too few glass beads to the resuspended yeast cells. The disintegration of the yeast cells works most efficiently when the glass beads move alongside the walls of the tube in circular movements during vortexing. Good results can be achieved by breaking the yeast cells in 50-mL FALCON tubes containing no more than 4–5 g yeast cells each.
8. Do not extend the vortexing intervals for longer than 30 s, because this will lead to the denaturation of proteins.
9. The yield of GimC from this procedure is critically dependent on the efficiency of the cleavage by the TEV protease.

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Analysis of Eukaryotic Molecular Chaperone Complexes Involved in Actin Folding

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1. Introduction

Actin, the most abundant protein of eukaryotes, plays critical roles in muscle contraction and in the movement of and within cells, including amoeboid locomotion, protoplasmic streaming, migration of cellular organelles and chromosomes, formation of cell evaginations, and stabilization of cell shape. The dynamics of actin filament assembly and disassembly, as well as the organization of the actin cytoskeleton into bundles and networks of filaments, are highly complex, being regulated by a large number of actin crosslinking, capping, and severing proteins (1,2). Interestingly, the biogenesis of actin also depends on at least two accessory proteins; the first to be described is the eukaryotic cytosolic chaperonin known as TCP-1 ring complex (TRiC) or chaperonin-containing TCP-1 (CTT), which is also involved in tubulin biogenesis (3–10). A second component involved in actin biogenesis has been recently identified independently by two groups. In a screen for *S. cerevisiae* mutants that are synthetically lethal with a mutant allele of γ -tubulin, Geissler et al. (11) identified five genes that encode proteins that assemble into a heterooligomeric complex named Gim-complex (GimC; Gim is an acronym for genes involved in microtubule biogenesis). Deletion of *GIM* genes causes both microtubule and actin cytoskeletal defects, a property also observed for genes encoding defective TRiC subunits (9,12–14). In addition, synthetic lethality is observed with certain combinations of *GIM* and *TRiC* gene mutations. Vainberg et al. (15) reported on the purification of the mammalian homolog of GimC from bovine testis based on its ability to form a stable binary complex with unfolded β -actin. The chaperone complex, named prefoldin (PFD) because of its ability

to transfer unfolded actin to the chaperonin TRiC for subsequent folding to the native state, consists of six proteins, PFD1–6. Although TRiC is apparently both necessary and sufficient for the ATP-dependent folding of actin *in vitro*, it is likely that *in vivo* both GimC/PFD and TRiC cooperate to ensure that actin reaches its native state following its synthesis. Additional components may be involved, for instance, an unidentified protein that for the purpose of this chapter, is referred to as IBP (intermediate mobility actin binding protein) because of its ability to form a complex with denatured actin that migrates on a native gel between the TRiC–actin and GimC–actin complexes. Here, methods for the detection and analysis of unfolded actin binding proteins are presented. In Chapters 17, 20, and 21, protocols for purifying TRiC from bovine testis, as well as GimC/PFD from *S. cerevisiae* and bovine testis are detailed.

2. Materials

2.1. β -Actin Overexpression Construct

This consists of high-level *Escherichia coli* expression plasmid encoding β -actin (see **Subheading 3.1.**).

2.2. [^{35}S]-Labeled Denatured β -Actin (D -*Actin)

1. Ampicillin: 100 mg/mL in water. Filter-sterilize and keep at 4°C for up to 1 mo, or freeze at –20°C.
2. Isopropyl-1-thio- β -D-galactopyranoside (IPTG), 100-mM stock made in water and filter-sterilized. Keep at –20°C in aliquots.
3. 5X M9 salts: Dissolve 30 g Na_2HPO_4 , 15 g KH_2PO_4 , 5 g NH_4Cl , and 2.5 g NaCl into water (final volume 1000 mL), autoclave, and store at room temperature.
4. Amino acid mix: To make a 60 mg/mL stock solution, add 0.12 g of all amino acids except methionine and cysteine (18 in total) to 200 mL water, and stir overnight or until all amino acids are dissolved. Filter-sterilize and store in 10-mL aliquots at –70°C.
5. Rifampicin: 20 mg/mL should be dissolved in methanol by vigorous vortexing. This stock solution is stable for 2 wk at 4°C. Rifampicin is light-sensitive; keep in the dark.
6. Labeling mix: To make 1 mL, mix together 0.684 mL sterile distilled water, 0.2 mL 5X M9 salts, 2 μL 1 M MgSO_4 , 0.1 mL amino acid mix, 10 μL rifampicin (20 mg/mL), 1 μL ampicillin (100 mg/mL), and 5 μL IPTG (100 mM).
7. [^{35}S]-labeled methionine and cysteine, e.g., Pro-Mix [^{35}S] cell-labeling mix (Amersham, Buckinghamshire, UK). Store in 50- μL aliquots at –70°C. It is preferable to use the labeling mix within about 3 mo, or approximately one half-life of ^{35}S .
8. Lysozyme, 5 mg/mL in 10 mM Tris-HCl, pH 7.5. Should be freshly prepared.
9. Deoxyribonuclease I (Sigma Type I, from bovine pancreas) (DNase I), made as a 10 mg/mL solution in 20 mM Tris-HCl, pH 7.5, 50 mM NaCl buffer. Store in small aliquots and freeze at –70°C.

10. *t*-Octylphenoxypolyethoxyethanol (Triton X-100) (Sigma, St. Louis, MO), 10% stock solution made in water.
11. MgCl_2 : 1 *M* stock solution prepared in water, autoclaved, and stored at room temperature.
12. 1 *M* tris(hydroxymethyl)aminomethane (Tris) (Sigma), adjusted to pH 7.5 with HCl, autoclaved and stored at room temperature.
13. 1 *M* dithiothreitol (DTT) (Sigma) prepared in distilled water, filtered, and stored in 1-mL aliquots at -70°C .
14. Denaturant buffer: To prepare a 10-mL solution, mix together 9.7 mL of a freshly prepared 8.25 *M* urea solution (deionized using mixed-bed resin TMD-8 from Sigma), with 200 μL of 1 *M* Tris-HCl, pH 7.5, and 100 μL of 1 *M* DTT. Filter-sterilize and freeze in small aliquots at -70°C (see **Note 1**).

2.3. Actin Refolding Assays in Rabbit Reticulocyte Lysate

1. Rabbit reticulocyte lysate was purchased in bulk quantities from Green Hectares, Oregon, WI, and aliquots (typically 50 μL and 1 mL) were flash-frozen in liquid nitrogen. Alternatively, the reticulocyte lysate from Promega is a reliable source of starting material.
2. Adenosine-5'-triphosphate (ATP) 100 mM concentrated stock is prepared by dissolving 0.605 mg ATP in 10 mL water and adjusting the pH to 7.0 with NaOH. The special quality ATP from Boehringer Mannheim, Germany, is best for this purpose.
3. 100 mM MgSO_4 , autoclaved and stored at room temperature.
4. 2-(*N*-morpholino)propanesulfonic acid (MOPS) (Sigma) adjusted to pH 7.0 with KOH. Prepare 20 mM, 0.4 *M*, and 1 *M* solutions, filter, and store at 4°C .
5. ATP/ATP-regenerating system: Mix 4 μL of 1 *M* phosphocreatine (PC, from Sigma) and 2.5 μL of 10 mg/mL creatine kinase (CK, from Sigma; both PC and CK are prepared in 20 mM MOPS-KOH, pH 7.0, and kept in aliquots at -70°C) with 5 μL of 100 mM ATP (pH 7.0), 5 μL of 100 mM MgSO_4 , and 3.5 μL distilled water. Use 2 μL of mix/50 μL refolding reaction to give final concentrations of 8 mM PC, 50 $\mu\text{g}/\text{mL}$ CK, and 1 mM Mg-ATP.

2.4. Native Gel Electrophoresis

1. 30% Polyacrylamide solution containing 0.8% *bis*-acrylamide. The ready-made solution (Protogel[®] from national diagnostics, Atlanta, GA) provides reproducibility and convenience.
2. Ammonium persulfate (Sigma), 10% stock prepared with distilled water. This can be stored at 4°C for at least 2 wk, or frozen in aliquots at -20°C for up to 6 mo.
3. *N*, *N*, *N*', *N*'-tetramethylethylenediamine (TEMED) from Sigma.
4. MOPS-KOH adjusted to pH 7.0. Prepare 1 and 0.4 *M* solutions, filter, and store at 4°C .
5. 5X native gel-loading buffer: Make a stock solution containing 50% glycerol (v/v) in water, and a sufficient amount of bromophenol blue to color the sample and use as a tracking dye during electrophoresis.

6. Native gel-running buffer: To make 1000 mL, mix together 80 mL of 1 M MOPS-KOH, pH 7.0, 1 mL of 1 M MgCl₂, and 919 mL of distilled water. This is stable at 4°C for many months.
7. 1 M ATP, adjusted to pH 7.0, for inclusion in native gel running buffer if necessary.
8. Gel-staining solution: 40% methanol, 10% acetic acid and 0.05% Coomassie brilliant blue R-250. Filter using Whatman® paper and keep at room temperature.
9. Gel-destaining solution: 40% methanol and 10% acetic acid kept at room temperature.

2.5. Analysis of Actin Refolding Reactions by Size-Exclusion Chromatography

1. For chromatography, a SMART™ System from Pharmacia provides excellent results. The Pharmacia Precision Column PC 3.2/30 (2.4 mL) prepacked with Superdex™ 200 has a broad separation range (approx. 10 kDa to over 1 MDa), which is ideal for the separation of relatively small proteins, such as actin (42 kDa), and large complexes, such as GimC-actin (~250 kDa) and TRiC-actin (~850 kDa). Alternatively, a Superose®6 PC 3.2/30 column would perform similarly well.
2. Gel-filtration buffer: 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, and 2 mM ATP. Filter-sterilize and degas before use.

2.6. Detection of Denatured Actin-Binding Activities from Purification Fractions

1. 0.5 M EGTA, autoclaved, and stored at room temperature.
2. 1 M KCl solution, autoclaved, and stored at room temperature.
3. 2X-concentrated hemoglobin buffer: Prepare a solution containing 0.4 mL 1 M MOPS-KOH, pH 7.0, 0.4 mL 1 M KCl, 40 µL 1 M MgCl₂, 20 µL 1 M DTT, 40 µL 0.5 M EGTA, and 9.54 mL distilled water. To the above solution, completely dissolve 100 mg of rabbit hemoglobin (Sigma), remove any particulate matter using a 0.2-µm filter, and store in aliquots at -70°C. The final composition of the solution in assays is 20 mM MOPS-KOH, pH 7.0, 20 mM KCl, 2 mM MgCl₂, 1 mM DTT, 1 mM EGTA, and 5 mg/mL hemoglobin. The 2X concentrated hemoglobin solution can be thawed and refrozen numerous times without any adverse effects.

3. Methods

3.1. Preparation of a β -Actin Overexpression Construct

The murine β -actin cDNA expressed from a high-copy T7 promoter-based prokaryotic expression vector (pRSET6a; **ref. 16**) yields an abundant amount of unfolded β -actin; other expression vectors, such as the pET vectors (**17**), can also be used, as can β -actins from other species owing to their high level of similarity. For example, chicken β -actin was used to purify the actin binding activity associated with the cytosolic chaperonin by Gao et al. (**3**). The *E. coli* chaperonin cannot fold actin (**18**), and therefore, the misfolded actin accumu-

lates into inclusion bodies, where it can be isolated in a nearly pure form. The murine β -actin cDNA (kind gift from J. Shephard) was subcloned into the *NdeI*-*Bam*HI-digested pRSET6a, where the last 3 bp of the *NdeI* site (CATATG) represent the initiating methionine for the coding region of actin. This construct, designated pRSET6a-mouse β -actin, is available on request.

3.2. Preparation of Labeled Denatured β -Actin (D-*Actin)

This section presents a description of how to prepare a highly radiolabeled denatured actin (D-*actin) that can be used (1) to detect denatured actin-binding activities in crude lysates or purified fractions, and (2) as a tool to examine how unfolded actin can be refolded by molecular chaperones, such as TRiC and GimC.

1. Transform the β -actin expression plasmid into *E. coli* BL21(DE3) cells (17), and incubate overnight on LB plates containing 100 μ g/mL ampicillin (for general molecular biology techniques, see ref. 19).
2. Inoculate 10 mL of LB medium containing 100 μ g/mL ampicillin using a small streak of colonies from freshly transformed cells, and grow with vigorous shaking at 37°C until the OD₆₀₀ reaches 0.7–1.
3. Induce the expression of the β -actin gene by adding IPTG to a final concentration of 0.5 mM, and continue incubation at 37°C for 30 min.
4. Centrifuge 1 mL of culture in Eppendorf tube at 5000g for 1 min, and carefully remove supernatant. Wash cells once with 1 mL of 1X M9 salts (see Subheading 2.2.).
5. Resuspend cells with 1 mL of labeling mix (see Subheading 2.2.), transfer to a round-bottom capped 10-mL culture tube (disposable), and grow cells at 37°C for 15 min.
6. Add 1 mCi of [³⁵S]-methionine/cysteine (~100 μ L of Pro-Mix), and continue incubating cells (with vigorous shaking) at 37°C for 1.5–2 h.
7. Transfer the culture to a 1.5-mL Eppendorf tube, centrifuge at 12,000g for 2 min at room temperature, and discard the supernatant into a container for liquid radioactive waste.
8. Resuspend the cells in 100 μ L of 10 mM Tris-HCl, pH 7.5, and perform 3 cycles of freeze thaw. Freeze on dry ice or in liquid nitrogen, and thaw at 37°C.
9. Add 1 μ L of lysozyme (5 mg/mL) and incubate for 5 min at room temperature.
10. At this point, the viscous cell lysate is clarified by adding 1 μ L of 1 M MgCl₂ and 1 μ g of DNase I, and incubating for 2–3 min at room temperature (check that there is a considerable decrease in viscosity).
11. Before pelleting the inclusion bodies containing insoluble β -actin, add 1 μ L of 10% Triton X-100 and mix. Centrifuge at 10 000g for 5 min at room temperature.
12. Wash the inclusion bodies once with 100 μ L of 20 mM Tris-HCl pH 7.5.
13. To solubilize the β -actin, add 100 μ L of denaturant buffer (see Subheading 2.2.) to the inclusion bodies, and vortex vigorously for 10 min at room temperature.
14. Centrifuge insoluble debris for 5 min at room temperature, and store 10- μ L aliquots of the supernatant into separate tubes at –70°C. The denatured β -actin probe (named D-*actin for short) is now ready for use (see Note 2 and ref. 20).

15. The radiochemical purity of the labeled β -actin can be verified by electrophoresing 1 mL on a 10% SDS-polyacrylamide gel (21), staining, and destaining the gel (see **Subheading 2.4.**), drying the gel on Whatman[®] filter paper, and exposing the gel to film (e.g., Kodax BioMax MR) for 5 min (see **Fig. 1A** for a typical preparation).

3.3. D-*Actin Refolding Reactions in Rabbit Reticulocyte Lysate

The labeled β -actin (D-*actin) can be used as a probe for denatured actin-binding proteins (TRiC, GimC, and IBP) in crude rabbit reticulocyte lysate or bovine testis lysates, or in fractions obtained during purification steps. Here, such actin binding activities can be detected by incubating the D-*actin into reticulocyte lysate and separating the complexes by native gel electrophoresis. All of the chaperone components required for efficient refolding of actin to its native state are present in reticulocyte lysate, so native actin is produced in the presence of ATP. Using this technique, native labeled actin and complexes between D-*actin and TRiC, GimC, and IBP can be resolved (for a typical refolding reaction analyzed on a native gel, see **Fig. 1B**).

1. Thaw aliquots of rabbit reticulocyte lysate (50 μ L) and denatured β -actin (D-*actin; 10 μ L) quickly at 37°C, and place immediately on ice.
2. Carefully pipet 0.25 μ L of D-*actin into 24.75 μ L of rabbit reticulocyte lysate containing 1 mM ATP, and mix by tapping the Eppendorf tube gently five to six times (see **Note 3**).
3. Incubate at 30°C for 5–10 min (see **Note 4**).
4. Add 6 μ L of 5X native gel loading (see **Subheading 2.4.**), mix thoroughly, and freeze reaction in liquid nitrogen if the sample is not used immediately; alternatively, a sample (5–10 μ L) of the reaction can be electrophoresed immediately on a nondenaturing gel (see **Subheading 3.4.** below). Actin refolding reactions can also be analyzed by size-exclusion chromatography (see **Subheading 3.5.**).
5. The rabbit reticulocyte lysate actin refolding reaction described above can be used as a lane of “denatured actin-binding protein standards”; it is good practice to include one such lane on all native gels that are run. With it, the relative positions of TRiC-actin, IBP-actin, GimC-actin, and native actin can be immediately ascertained. This is important, because there is some variation in the migration behavior of proteins on different batches of native gels.

3.4. Analysis of Denatured Actin-Binding Proteins on Native Gels

The separation of TRiC-*actin, GimC-*actin, IBP-*actin and native labeled actin produced from D-*actin refolding reactions (see **Subheading 3.3.**) is readily accomplished by nondenaturing gel electrophoresis. For this purpose, a 4.5% continuous native gel (i.e., no stacking gel) run in a Bio-Rad minigel apparatus is most convenient.

1. To prepare the 4.5% native gel solution, mix together 5 mL 0.4 M MOPS-KOH, pH 7.0, 3.75 mL 30% acrylamide–0.8% bis-acrylamide, 16.1 mL distilled water,

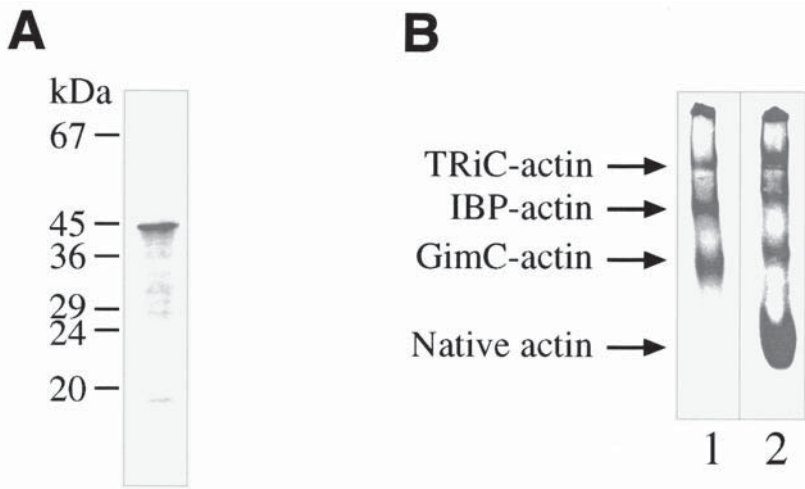


Fig. 1. Radiolabeled denatured β -actin (D-actin) and protein components in rabbit reticulocyte lysate that bind the unfolded actin. **(A)** Approximately 0.5 μ g of prepared labeled, denatured actin (D-actin) was separated on a 12.5% SDS-polyacrylamide gel. The gel was dried and exposed to film for 5 min. **(B)** D-actin was diluted 100-fold into rabbit reticulocyte lysate without (lane 1) or with (lane 2) 1 mM ATP, incubated for 5 min, and separated on a 4.5% nondenaturing polyacrylamide gel. The gel was dried and exposed to film for \sim 14 h. Note that the D-actin forms complexes with three different components: TRiC, IBP, and GimC (genes involved in microtubule biogenesis-complex; also called PFD). The fastest migrating species on the native gel corresponds to native actin, since it can be shifted with DNase I (not shown). The positions of the TRiC-actin and GimC-actin complexes on native gels correspond precisely with those of the same complexes formed with D-actin and purified TRiC and bovine GimC, respectively (not shown).

and 25 μ L 1 M $MgCl_2$. In the case where native actin is generated, 25 μ L of 1 M ATP should be added to the gel solution.

2. Stir in 125 μ L of 10% ammonium persulfate and 12.5 μ L TEMED to begin the polymerization reaction.
3. Pour the gel mixture between the plates in the casting chamber, and insert 10- or 15-slot combs (see **Note 5**). Allow at least 1 h for complete polymerization at room temperature. Fill the upper and lower chambers completely with native gel-running buffer (see **Subheading 2.4**). If required, add 0.2 mL of 1 M ATP/1000 mL running buffer in order to stabilize native actin.
4. Load the samples (usually 5–10 μ L of the actin refolding reactions done in rabbit reticulocyte lysate (see **Subheading 3.3**), or 10 μ L when assaying purification fractions for denatured actin-binding activities), and run the gel for about 1 h at 90 V constant, or until the tracking dye reaches the bottom of the gel (see **Note 6**).
5. Remove the gel from the apparatus, taking care not to tear the gel (4.5% gels are very soft), incubate gel in staining solution for 20 min, and remove some of the

stain with destaining solution (*see Subheading 2.4.*). Dry gels down using a gel dryer, and subject them to autoradiography, using a film sensitive to ^{35}S signals, such as Kodak BioMax MR. Alternatively, expose dried gel to a phosphorimager screen. Typically, strong signals from TRiC- α -actin, GimC- α -actin, IBP- α -actin and native α -actin appear after exposures of 4 h to overnight (about 18 h; *see Fig. 1B*).

3.5. Analysis of Denatured Actin-Binding Proteins by Size Exclusion Chromatography

The fractionation of TRiC- α -actin and GimC- α -actin complexes, and native labeled actin generated from a D- α -actin refolding reaction (*see Subheading 3.3.*) can be done by chromatography on an analytical sizing column, such as the Pharmacia SuperdexTM 200 PC3.2/30. Note that the IBP-actin complex observed by native gel electrophoresis is not recovered under the conditions described below (*see Fig. 2*).

1. To prepare the folding reaction, mix 0.25 μL of D- α -actin into 24.75 μL of rabbit reticulocyte supplemented with 1 μL of ATP/ATP-regenerating system (*see Subheading 2.3.*), and incubate for 5 min to 1 h at 30°C. Longer incubation times will yield less TRiC- α -actin and GimC- α -actin binary complexes, and more the native labeled actin (*see Note 4*).
2. Centrifuge at 12,000g at 4°C for 15 min to remove D- α -actin aggregates. Remove supernatant to a clean Eppendorf tube.
3. Load 15 μL on a SuperdexTM 200 PC 3.2/30 column maintained at room temperature and equilibrated in gel-filtration buffer. By using a program where the first 0.6 mL are discarded and a total of 30 fractions (50 μL) are collected at a flow rate of 40 $\mu\text{L}/\text{min}$, a profile such as that as shown in **Fig. 2** will be obtained (*see Note 7*).
4. Analyze the fractions on nondenaturing gels and detect the positions of the labeled actin by autoradiography; *see Subheading 3.4. above*.

3.6. Assay for Detecting Denatured Actin-Binding Activities During and After Purification from Rabbit Reticulocyte or Bovine Testis Lysates

During the purification of GimC from rabbit reticulocyte and bovine testis lysates, the total amount of protein present during denatured actin binding assays greatly affects the reliability of the assay. Only when a stabilizing agent is present at a sufficient concentration (e.g., hemoglobin at 5 mg/mL; *see ref. 15*) can the relative actin binding activity of GimC in different fractions be assessed reliably. A comprehensive assay procedure is presented below; the results of such an assay on fractions from MonoQ-purified bovine testis lysate are shown in **Fig. 3**. For refolding experiments using purified TRiC, GimC, and perhaps other components, the same assay conditions can be used (*see Note 8*).

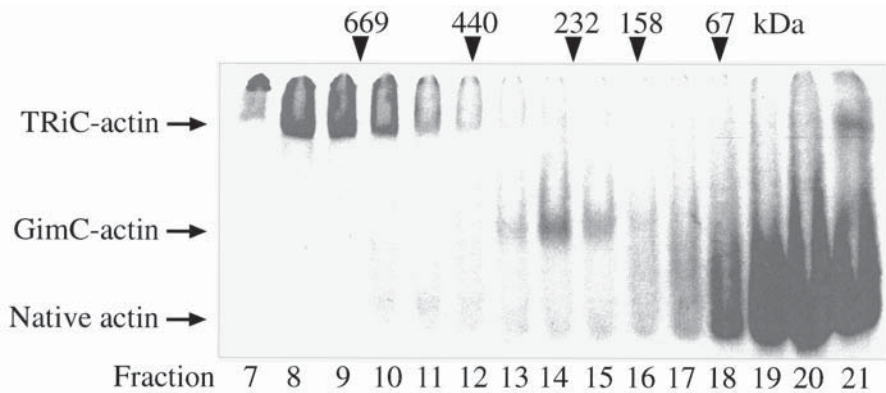


Fig. 2. Fractionation on a Superdex™ 200 1.2/2.6 column of a D-actin refolding reaction in rabbit reticulocyte lysate (15 μ L of the reaction were loaded). D-actin was incubated in rabbit reticulocyte lysate in the presence of 1 mM ATP and an ATP-regenerating system for 1 h at 30°C. The fractions collected were 50 μ L in size, and are numbered starting after 0.6 mL of flow through (approximate void volume of column). The position of migration of molecular-weight standards on the column are indicated above each fraction. The peak of the TRiC-actin (fractions 8–9) and GimC-actin complexes (fraction 14) correspond to molecular weights of approx. 850 and 250 kDa, respectively.

1. After each purification step, combine 20 μ L of each fraction to be assayed with 20 μ L of 2X concentrated hemoglobin buffer (*see Subheading 2.6.*), and place on ice.
2. When all fractions have been made 1X with concentrated hemoglobin buffer, add 0.4 μ L of D-actin (freshly thawed from -70°C) to each fraction, mix by gently tapping eppendorf tube five to six times, and incubate at 30°C for 5 min.
3. Add 10 μ L of 5X native gel-loading buffer (*see Subheading 2.4.*), and freeze fractions in liquid nitrogen; alternatively, keep on ice until they are ready to load on the native gel.
4. When all fractions have been assayed, thaw quickly and place on ice (if necessary). Analyze 10 μ L of each fraction on a 4.5% native gel as described in **Subheadings 2.4.** and **3.4.** (remember to include a lane containing a “denatured actin binding protein standards”; *see Subheading 3.3.* for an explanation).
5. The peak fractions containing denatured actin binding activities can be selected for further purification.

4. Notes

1. The solution should be deionized to remove by products of urea (isocyanates), which can carbamylate the unfolded actin, and therefore could interfere with binding or folding assays. Keeping the denaturing solution at -70°C reduces this problem, but it would be advisable to prepare a fresh solution every 6 mo.

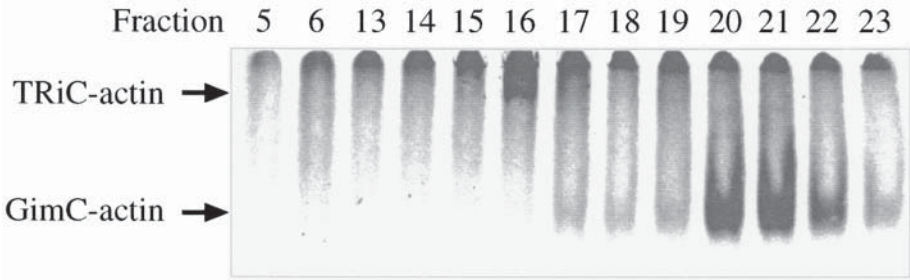


Fig. 3. Detection of denatured actin binding activities during purification of bovine testis lysate by anion-exchange (MonoQ) chromatography (for example, *see ref. 20*). An aliquot (20 μ L) of each fraction from the purification was mixed with 20 μ L 2X-concentrated hemoglobin buffer and 0.4 μ L D-*actin, incubated for 5 min at 30°C, and 10 μ L of each assay were analyzed on a 4.5% nondenaturing gel as described in **Subheading 3.4**. Top and bottom arrows point to TRiC-actin and GimC-actin complexes, respectively.

- It is not absolutely necessary to pass the labeled β -actin over a small Sephadex G-50 column as described by Gao et al. (3). This step can be avoided for routine actin binding assays, since omitting this step does not appear to make any difference in the behavior of the denatured actin. It is recommended that the probe not be used after a storage time of more than 2 wk at -70°C , because the formation of native β -actin from D-*actin by TRiC declines rapidly, presumably owing to radiochemical damage of the probe (20). However, formation of the TRiC- or GimC-*actin complexes is largely unaffected when the D-*actin is stored over a period of at least 1 mo at -70°C , even with a few thawings and re-freezings of aliquots.
- The D-*actin probe must be diluted at least 100-fold in order to reduce the concentration of urea from 8 M to a level that is generally tolerated by TRiC and GimC (e.g., 80 mM). Because the specific activity of the probe is extremely high, the D-*actin can be diluted \sim 150-fold when a large number of samples must be assayed. For greater accuracy, a larger reaction volume (e.g., 0.5 μ L of D-*actin into a total of 50 μ L) is advisable because of the inaccuracies in pipeting small volumes (0.25 μ L).
- Depending on the period of incubation, and the presence or absence of ATP and an ATP-regenerating system, there will be different amounts of the TRiC-*actin and GimC-*actin binary complexes. With short incubation times, more of the complexes will be present; longer incubation times will yield less of these binary complexes, and more of the native labeled actin, particularly in the presence of ATP. To generate native actin more efficiently, an ATP/ATP-regenerating system (*see Subheading 2.3.*) should be added.
- This protocol is adapted to use the Bio-Rad Mini-PROTEAN II system with plates separated by 1-mm spacers. There is enough gel mixture for four gels. For convenience, many gels can be poured at once and kept at 4°C wrapped up in water-

soaked tissue paper covered with plastic wrapping. The gels can be stored for up to 1 wk.

6. The gel can be run either at room temperature or at 4°C. The buffers from the upper and lower chambers can be mixed at intervals of 30 min to ensure a proper distribution of ions during electrophoresis. Since MOPS buffer is expensive, the buffers from the combined lower and upper chambers can be reused two to three times without adverse effects. Note that the hemoglobin in the rabbit reticulocyte lysate does not migrate into the native gel under these conditions; this does not indicate that the sample is not entering the gel.
7. To calibrate the sizing column, approx. 100 mg of each molecular-weight standard used (bovine serum albumin, 67 kDa; aldolase, 158 kDa; catalase, 232 kDa; ferritin, 440 kDa, and thyroglobulin, 669 kDa; all from Pharmacia) were run on the column using the same elution and fraction collection program.
8. The hemoglobin buffer (*see Subheading 2.6.*) used for the assays is essentially the same as the “folding buffer” described in Vainberg et al. (15). The latter buffer is used during the purification of bovine testis GimC (prefoldin) and for performing refolding assays using purified chaperone components. Addition of 0.5 mM ADP to denatured actin-binding assays is not necessary for detecting the GimC actin binding activity during purification. However, during refolding reactions or analyses of actin binding activities, nucleotides, such as ADP or ATP, can be added.

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