Preface

The chaperonin field has captured the attention of numerous scientists in recent years. A rapidly increasing number of reviews and articles have tried to elucidate the mechanisms by which these multimeric complexes drive the folding of newly synthesized and denatured proteins.

An obvious common theme of chaperonin research first arose from the study of their structural features. All members of this class consist of multiple subunits that form cylindrical structures, which encage proteins in a cave-like environment where folding of proteins takes place according to the current view. Since the chaperonin structures are found even in very primitive organisms, the archaebacteriae, this "cave scheme" seems to be an evolutionarily successful feature that was conserved and that appears among evolutionarily distinct organisms.

Interestingly, almost all chaperonins have specific cofactors that are involved in the folding process. Even for the eukaryotic cylinder TRiC or CCT, a cofactor called prefoldin or GimC was recently discovered. Only for the archaeal chaperonins cofactors have not yet been discovered, although there seem to be GimC-like homologs in some archaeal species (unpublished observations by M. Leroux).

One key aspect of this volume is the purification of chaperonins along with their corresponding cofactors from different species, with examples ranging from archeae to higher eukaryotes. As there are of course many other species from which chaperonins could be purified, the focus here is to give a representative overview. Many proteins are purified today as recombinant proteins in *E. coli*, which makes the purification protocols easier and less time consuming than in previous years. The protocols are also not the sole protocols for chaperonin purification. Individual labs most likely utilize slightly different techniques, preferring one column over another, etc. However, the protocols described here have been used successfully by the authors and are described in a detailed way to be as reproducible as possible. The notes section especially is intended to reflect the experience of the experimenter with the procedure and should be an important source of troubleshouting for the user of this book.

Chaperonin activity assays for in vivo as well as in vitro work are the second main focus of this volume. Many assays are given for GroEL, as this is

one of the best charaterized and most investigated chaperonin. These assays can also be applied to mitochondrial Hsp60, for example. There are some protocols that describe assays specifically for the eukaryotic chaperonin TRiC. The reason for using more specific protocols for this chaperonin is that it has very specific substrates, such as tubulin and actin. But some of the protocols, for example the method for preparing labeled probes, can be also used for different purposes and might be helpful for numerous other protocols. The addition of purification protocols for the very recently discovered new cofactor of TRiC/CCT, called prefoldin in mammals and GimC in yeast, results from the very recent developments in the field.

I am grateful to series editor John Walker who offered me the opportunity to edit this book and to try something I have never done before. Without his helpful support and advice and without the patience and the encouragement of Tom Lanigan and his staff at Humana Press, I would not have had the opportunity to participate in this enjoyable project. Last but not least, I want to thank all the authors for their enthusiasm in writing the protocols. Their clear and careful descriptions of the protocols contribute to making chaperonin research a less mysterious field.

Christine Schneider

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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)***. Suprisingly, 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 (p*I* = 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-

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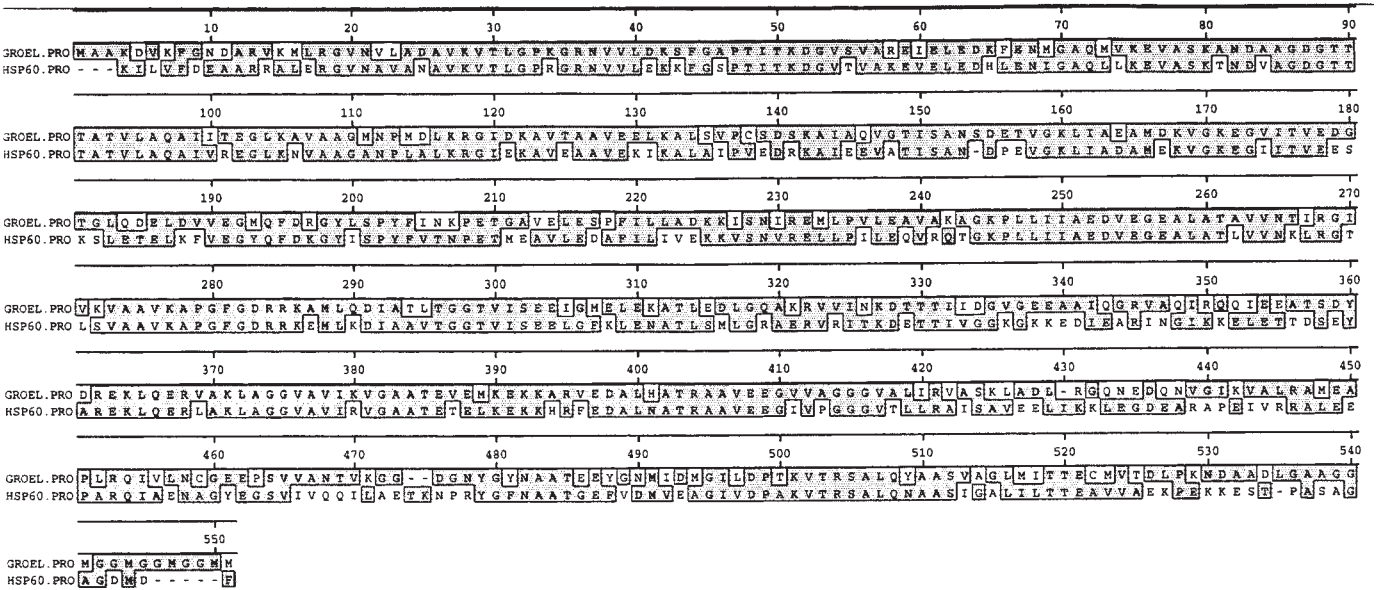

Fig. 1. Sequence comparison of *E. coli* GroEL and Hsp60 from *Thermus thermophilus*. Identical residues are shaded.

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_2SO_4	0.5 mL
Mn SO_4	2.2 g
Zn SO_4	0.5 g
H_3BO_3	0.016 g
$CuSO_4$	0.025 g
Na_2MoO_4	0.025 g
$CoCl_2 \cdot 6H_2O$	0.046 g
Distilled H_2O	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
$CaSO_4 \cdot 2H_2O$	0.12 g
$MgSO_4 \cdot 7H_2O$	0.2 g
NaCl	0.016 g
KNO_3	0.21 g
$NaNO_3$	1.4 g
Na_2HPO_4	0.22 g
Distilled H_2O	to 1 L

c. 1% TYE: Prepare fresh for each preparation

Tryptone (Difco 0123)	10 g
Yeast extract	10 g
Distilled H_2O	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 m*M* Tris-HCl, pH 7.5, 1 m*M* ethylenediamine tetra-acetic acid (EDTA), 1 m*M* DTT, 50 m*M* NaCl.
2. Buffer B: 50 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, 1 m*M* DTT, 1 *M* NaCl.

3. Buffer C: 50 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, 1 m*M* DTT, 250 m*M* NaCl, 20% (v/v) ethanol.
4. Buffer D: 50 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, 1 m*M* DTT, 500 m*M* NaCl.
5. Buffer E: 50 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, 5 m*M* DTT, 250 m*M* NaCl.
6. Buffer F: 50 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, 5 m*M* DTT, 250 m*M* NaCl, 50% (v/v) glycerol.
7. Washing buffer: 50 m*M* 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 H_2O 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:H_2O 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% 50m*M* Tris-HCl, pH 7.5.
5X Native gel buffer: 1 mg bromophenol blue dissolved in10 mL 75%glycerol/25% 50m*M* 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% Commassie brilliant blue R250, 60% H_20 (v/v).
2. Destain I: 10% (v/v) acetic acid, 30% (v/v) methanol, 60% H_20 (v/v).
3. Destain II: 10% acetic acid, 10% glycerol, 60% H_20 (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. The100 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 µL 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 am 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 5000*g* 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 m*M* 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 17,000*g* 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 m*M* 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 m*M* 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.

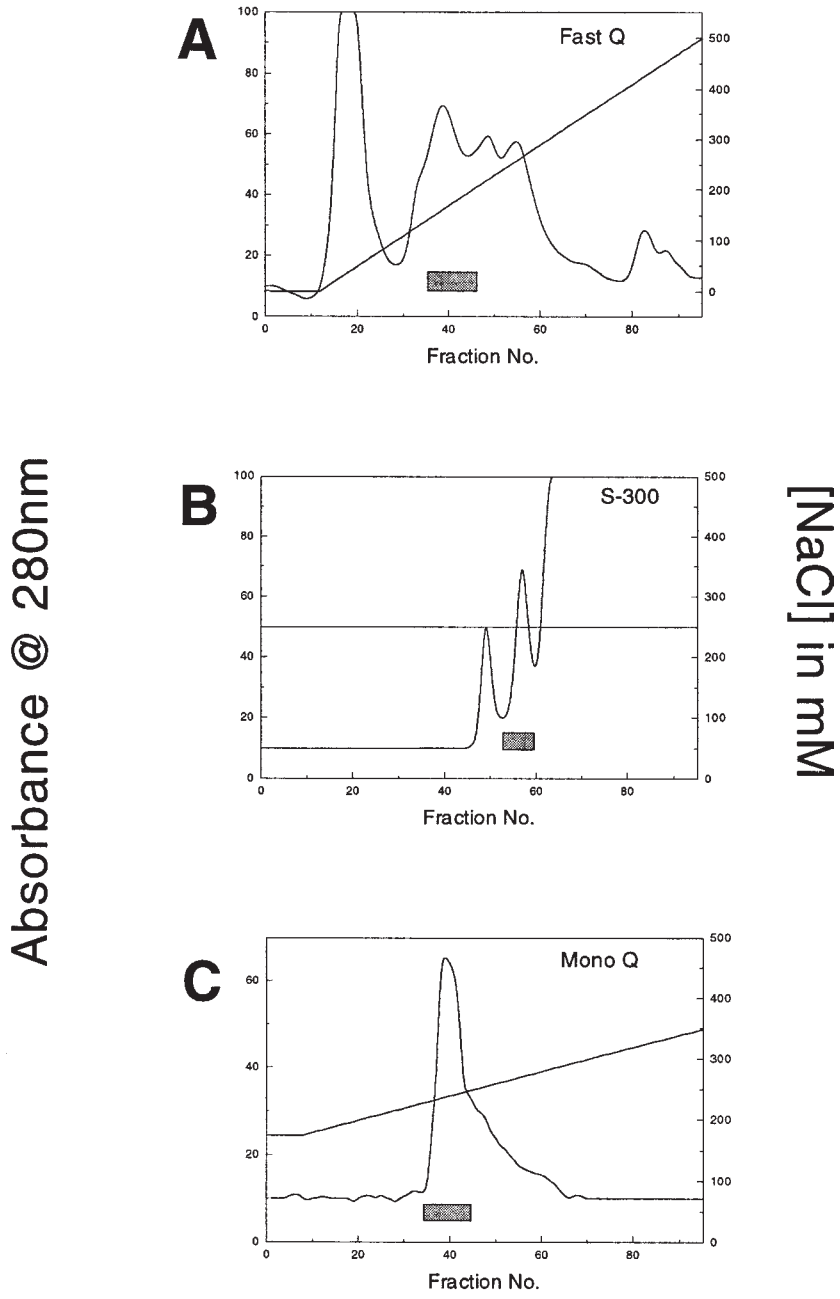

Fig. 2. Chromatographs from the purification of *T. thermophilus* Hsp60. **(A)** Fast Q anion-exchange elution profile; **(B)** S-300 gel-filtration elution profile; **(C)** MonoQ 10/10 elution profile. The elution position of Hsp60 is indicated by a shaded bar.

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 m*M* 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

*a*Estimated from gel electrophoresis.

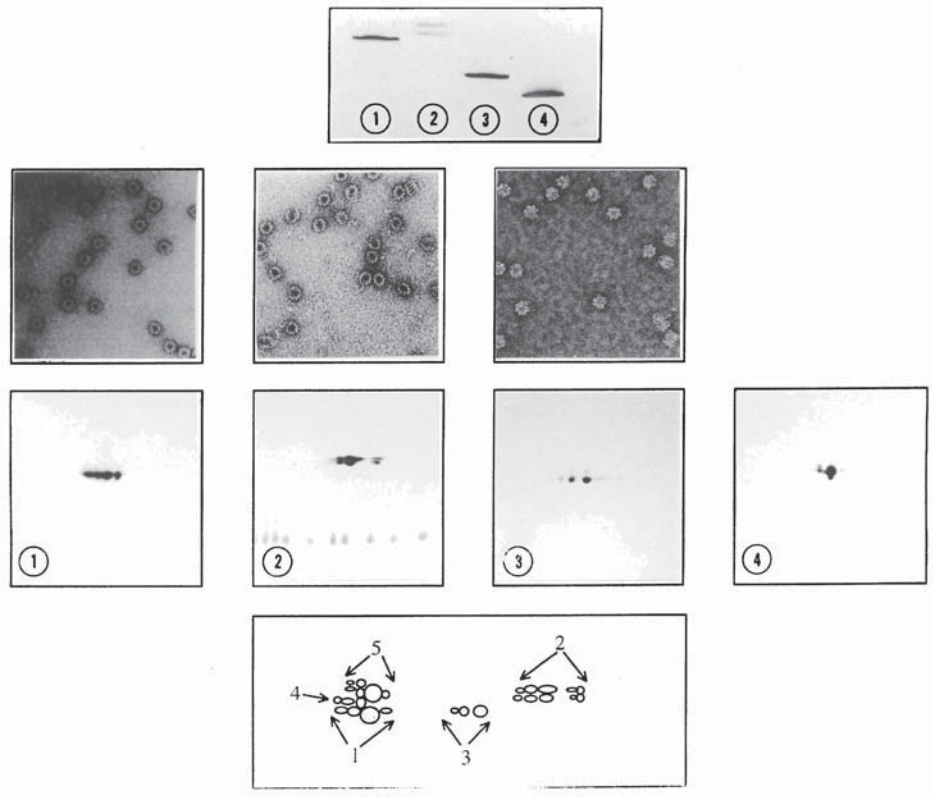

Fig. 3. Comparison of thermophilic chaperonin with GroEL. For a detailed discussion, *see* **Subheading 3.6.** **(A)** Native gel of purified chaperonins. **(B)** Electronmicrographs of thermophilic chaperonins. **(C)** 2D gel patterns of the different chaperonins. **(D)** Comigration studies. The numbering 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 M^{-1}/cm/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 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 m*M* NaCl. We know from anion-exchange chromatography that Hsp60 elutes between 200 and 250 m*M* 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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