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Review

Purification of chaperonins

Elsie Quaite-Randall, Andrzej Joachimiak*

Argonne National Laboratory, 9700 S. Cass Ave, Argonne, IL 60439, USA

Abstract

The availability of protein samples of sufficient quality and in sufficient quantity is a driving force in biology and biotechnology. Protein samples that are free of critical contaminants are required for specific assays. Large amounts of highly homogeneous and reproducible material are needed for crystallography and nuclear magnetic resonance studies of protein structure. Protein-based therapeutic factors used in human medicine must not contain any contaminants that might interfere with treatment. The roles played by molecular chaperones in protein folding and in many cellular processes make these proteins very attractive candidates as biochemical reagents, and the class of chaperones called chaperonins is one of the most important candidates. Methods for successfully purifying chaperonins are needed to advance the field of chaperonin-mediated protein folding. This article outlines the strategies and methods used to obtain pure chaperonin samples from different biological sources. The objective is to help new researchers obtain better quality samples of chaperonins from many new organisms. © 1999 Published by Elsevier Science B.V.

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*Corresponding author. Tel.: +1-630-252-3926; fax: +1-630-252-5517.

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1. Introduction

1.1. Molecular chaperones and protein folding

The phenomenon of protein folding has been studied for several decades. Although the specific rules that govern protein folding remain elusive, we do know that some proteins can fold spontaneously. The pioneering work of Anfinsen, the Nobel Prize laureate, showed that ribonuclease activity could be regained upon its dilution from a denaturant [1]. This observation gave rise to the hypothesis that a protein's amino acid sequence contains all the information required for the correct folding of the protein.

More recently, a class of abundant and ubiquitous proteins that apparently assist and accelerate protein folding in vivo and in vitro have been discovered. These 'molecular chaperones' [2] interact with a protein to ensure that it is correctly folded and protect it from off-pathway folding events that could lead to undesirable aggregation (protein aggregation is readily observed after heating). Although molecular chaperones differ quite extensively in their sequences, structures, and mechanisms, they share common properties, mainly in that they assist in protein folding and can prevent protein aggregation. Many molecular chaperones are induced to protect cellular components against a variety of stress conditions, including heat shock (thus many chaperones are heat-shock proteins), and their expression increases dramatically after thermal stress [3–6]. In the eukaryotic cell, molecular chaperones are numerous and diverse, and they are involved in many processes and regulatory mechanisms. For example, they are involved in (i) the stabilization of 'functional protein conformers', (ii) protein conformational maturation, (iii) protein unfolding and disassembly, (iv) quality control of protein folding, and (v) the transport of propeptides destined for another cellular compartments [6–10].

1.2. Chaperonins

Molecular chaperones have been classified in many different ways. One classification is based on the sequence similarity and structure of their assembly. Chaperones with a molecular mass of approximately $60 \cdot 10^3$ rel. mol. mass form a distinct class termed ''chaperonins'' [11]. These proteins are assembled in large, multimeric, double-ring structures containing two large, solvent-filled cavities and are often referred to as 'double doughnuts'. The double-ring structure is required to provide the 'infinite dilution environment – an Anfinsen cage' [4] for protein folding as it is thought that the protein folds inside the chaperonin cavity. ATP, magnesium, and potassium ions are other cofactors usually required for chaperonin-assisted protein folding. Chaperonins have been found and isolated from organisms in all three kingdoms: prokarya, archaea, and eukarya [12]. Table 1 shows a list of those discussed in this review.

In general, chaperonins can be subdivided into two main classes. Type I chaperonins represent the bacterial, mitochondrial, and plastid forms and include GroEL from *E. coli*. These chaperonins function in conjunction with another, smaller (although also multimeric) partner, the cochaperonin, or ''release factor'' (GroES in *E. coli*). Fig. 1 shows the three-dimensional (3D) crystal structure of the best-studied chaperonin to date, GroEL from *E. coli*, and its complex with the cochaperonin, GroES. Type II chaperonins are divided into two subclasses. Type IIa chaperonins represent forms present in the archaea. Type IIb chaperonins, the TCP-1/TRiC/CCT class, are cytosolic eukaryotic chaperonins.

Although similar in size and domain organization, the two main chaperonin classes have distinct differences. The Type I chaperonin assembly is made up of 14 identical subunits arranged in two rings of seven. The majority of Type IIa chaperonins are

Table 1
Properties of chaperonins discussed in this review. Column 1 indicated the number of subunits described for each chaperonin. Column 2 shows the symmetry of the chaperonin e.g. 7/2 indicates two seven subunit rings constitute the assembly, 7/1 indicates a single seven-member ring etc. Molecular weight estimation in Column 3 is approximate and is based on an average subunit size of 60 kDa. Column 4 shows p*I* that was established experimentally

Source	Number of subunits	Symmetry	Size (kDa)	p*I*
Type I				
Bacterial (mesophilic)				
Escherichia coli	1	7/2	840	4.7
Endosymbiont	1	7/2	840	4.61
Streptomyces albus	1 (2 genes)	7/2	840	4.6
Mycobacterium leprae	1 (2 genes)	7/2	840	4.72
Mycobacterium tuberculosis	1 (2 genes)	7/2	840	4.67
Rhodobacter sphaeroides	1	7/2	840	
Bacillus subtilis	1		840	
Bacterial (thermophilic)				
Thermus thermophilus	1	7/2 and 7/1	840/420	5.1
Thermoanaerobacter brockii	1	7/1	420	
Mitochondrial				
Pumpkin cotyledon	1	7/2	840	
Mammalian	1	7/1	420	5.47
Plastid				
Brassica napus (α and β)	2	7/2	840	
Narcissus pseudonarcissus	2	7/2	840	
Type IIa				
Sulfolobus shibatae	2	9/2	1080	5.3
Thermoplasma acidophilum	2	8/2	960	
Pyrodictum occultum	2	8/2	960	
Thermococcus littoralis	2	8/2	960	4.8
Pyrococcus furiosus	2	8/2	960	4.8
Methanococcus jannashii	1	–	960	
Methanopyrus kandleri	1	8/2	960	
Type IIb				
Rabbit reticulocyte lysate	8	8/2	960	
Bovine testes	8	8/2	960	
Murine	8	8/2	960	
Yeast	8	8/2	960	

made up of two similar but different subunits, termed α and β, and have either an eight-fold or nine-fold symmetry. Recently, however, two archaeal methanogens, *Methanopyrus kandleri* [13] and *Methanococcus jannashii* [14], have been found to apparently contain a single subunit. Eukaryotic Type IIb chaperonins have a much more complex structure. They are made up of eight different but sequence-related subunits, and each of the two rings in the double doughnut assembly contains a set of all eight subunits [15].

Because chaperonins can protect enzymes from denaturation and aggregation, by catalyzing productive folding and/or preventing irreversible unfolding and aggregation, interest in these proteins has significantly increased. Purified chaperonins could be used directly as enzyme stabilizers or, if co-expressed with the enzyme of interest in a mesophilic host, could direct the correct folding and assembly of the recombinant enzyme [16]. However, because the primary function of chaperonins is to interact with proteins and peptides, the purification of chaperonins

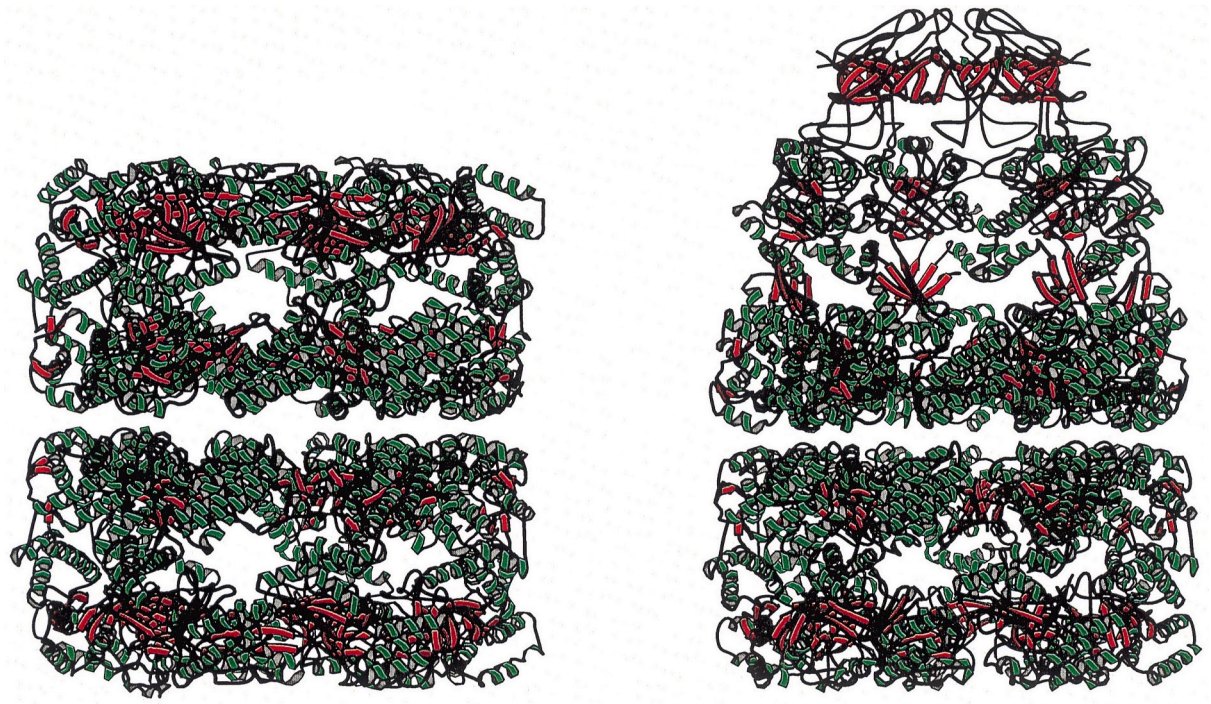


Fig. 1. The crystal structures of the best characterized chaperonin to date, GroEL from *E. coli* (left) and its complex with GroES co-chaperonin (right). The figure was produced with the program BOBSCRIPT [80] by Dr. Martin Walsh.

can be challenging. This article is concerned exclusively with the isolation and purification of chaperonins.

1.3. Properties common to chaperonins and features exploited during purification

Despite many differences, the majority of chaperonins possess several common features that can be exploited during purification. First, they are rather acidic, with a p*I* that is generally below pH 6. Second, they form very large assemblies, having a molecular weight from 800 to $1100 \cdot 10^3$ rel. mol. mass. Third, they bind and hydrolyze ATP. Fourth, they show a high affinity for unfolded polypeptides and hydrophobic compounds which enables effective purification protocols to be developed and used when chaperonins are being purified directly from a complex protein mixture. Chaperonins are also quite abundant (1–2% of total cell protein). In addition, a heat shock given to cells during the final hours of growth can significantly increase the level of chaperonin expression, as can the addition of a low concentration of ethanol/methanol to the media. Milligrams of the chaperonin can be obtained from a single preparation in a relatively short time.

As interest in these molecules has grown, many chaperonins from different organisms have been sequenced, cloned, and overexpressed in *E. coli*. This article describes chaperonin purification methods that use both the wild type organism and recombinant strains as starting material. The diversity of these chaperonins is illustrated in Table 1.

2. Type I chaperonins: bacterial and organelle

2.1. Purification of chaperonins from mesophilic bacteria

The best-characterized chaperonin to date, GroEL from *E. coli*, is a Type I chaperonin. The *groE* locus was first described by Georgopoulos et al. [17], who reported that mutations in the *groE* operon interfered

with the assembly of the heads of bacteriophage lambda and T4, although the reason for this was unclear. Subsequent studies on the assembly of an important chloroplast enzyme, ribulose bisphosphate carboxylase (RUBISCO), revealed that an accessory protein was required for its correct assembly [18]. It was not until 1988 [11] that the homology between these two proteins was discovered. This observation was the impetus for the tremendous amount of work on chaperonins that has been done over the past decade. To simplify purification, numerous laboratories have cloned and overexpressed the GroEL in *E. coli*. Several purification schemes have been developed for the GroEL chaperonin [19–23]. We found that the following method [23,24] works very well for purifying wild type *E. coli*, particularly if a mild heat shock (42°C for 1 h) is given before harvesting the cells. We routinely use the *E. coli* B K12 strain. Cells are grown in 2×TY medium to a density of OD_{600nm} 1.5–2.0 and harvested by centrifugation at 6000 rpm for 8 min. Typical cell yield is more than 3 g of cells per liter of culture. The cell paste is resuspended in an extraction buffer: 50 m*M* of Tris/HCl buffer at pH 7.5, containing 1 m*M* of EDTA and 1 m*M* of DTT (Buffer A). After sonication on ice, cell debris is removed by centrifugation (12 000 rpm for 30 min). This clarified cell extract is then applied to an anion-exchange column (FastQ Sepharose, 200 cm^3; Pharmacia) equilibrated with buffer A, and proteins are eluted with a linear gradient of 0–1 *M* NaCl (1 l) at 6 ml/min. GroEL elutes between 200 and 250 m*M* of NaCl. Fractions containing the chaperonin are identified by using both nondenaturing PAGE (4–15% gel) and SDS–PAGE (12.5% gel) and are concentrated by using a pressurized stirred cell that uses a $100 \cdot 10^3$ rel. mol. mass cut-off membrane (Amicon). In our laboratory, we routinely concentrate 500 ml to approximately 10–15 ml by using this method. It often results in chaperonin concentrations as high as 100–200 mg/ml and exploits the chaperonin's high solubility at neutral pH. The ability to concentrate the chaperonin to such concentrations increases resolution and recovery during the second chromatographic step, gel permeation chromatography. We use a 150 cm×2.5 cm Sephacryl 300 (Pharmacia) column. We have found that the inclusion of methanol 20% (v/v) in the elution buffer (Buffer A+250 m*M* of NaCl) increases the purity of the chaperonin being prepared during this separation. Exactly why this occurs is not clear; however, it seems that presence of methanol triggers the release of GroEL-bound proteins and peptides. At this point, the GroEL is at least 90% pure. After concentration and subsequent dilution of the GroEL-containing fractions, a final HR 16/10 MonoQ column (Pharmacia) is run by using the same buffer system with a linear gradient of 200–350 m*M* of NaCl. GroEL elutes between 225 and 260 m*M* of NaCl. Fractions containing the chaperonin can be pooled and concentrated to 200 mg/ml or higher by using centrifugal concentrators (Filtron or Amicon). The highest GroEL concentration on record in our laboratory is 285 mg/ml. The determination of chaperonin concentration, purity, and storage conditions is discussed later in this article. This method works well for purifying GroEL from overproducing strains and from wild type cells, and it is easily carried out in 2 or 3 days. Fig. 2 compares a two-dimensional (2D) gel of GroEL purified from wild type *E. coli* by means of the above method with the crude extract.

Commonly, purification methods for recombinant GroEL exploit the large size of chaperonins. Early methods often employed sucrose/glycerol gradients [11,25], which are cumbersome and difficult to scale up. They have been superseded by gel permeation chromatography, particularly since new, higher-capacity, high-resolution resin with greater mechanical strength and faster flow-rates has become available.

A major problem encountered during the purification of all types of chaperonins is the copurification of bound protein substrates that are unfolded during the physical stress of the purification procedure. The inclusion of 20% methanol in chromatography buffers ensures sufficient purity for the chaperonins we routinely purify. However, several other strategies have been employed to circumvent the copurification problem. One method is described by Schmidt et al. [26] and Fisher [27]. First the resin was treated with 6 *M* of guanidinium/HCl and equilibrated with 20 m*M* of Tris/HCl at pH 7.5, containing 5 m*M* of $MgCl_2$. Then GroEL was batch-treated with Affigel-blue under conditions that do not allow the chaperonin to bind to the resin. After incubating for 15–30 min at room temperature, GroEL was eluted

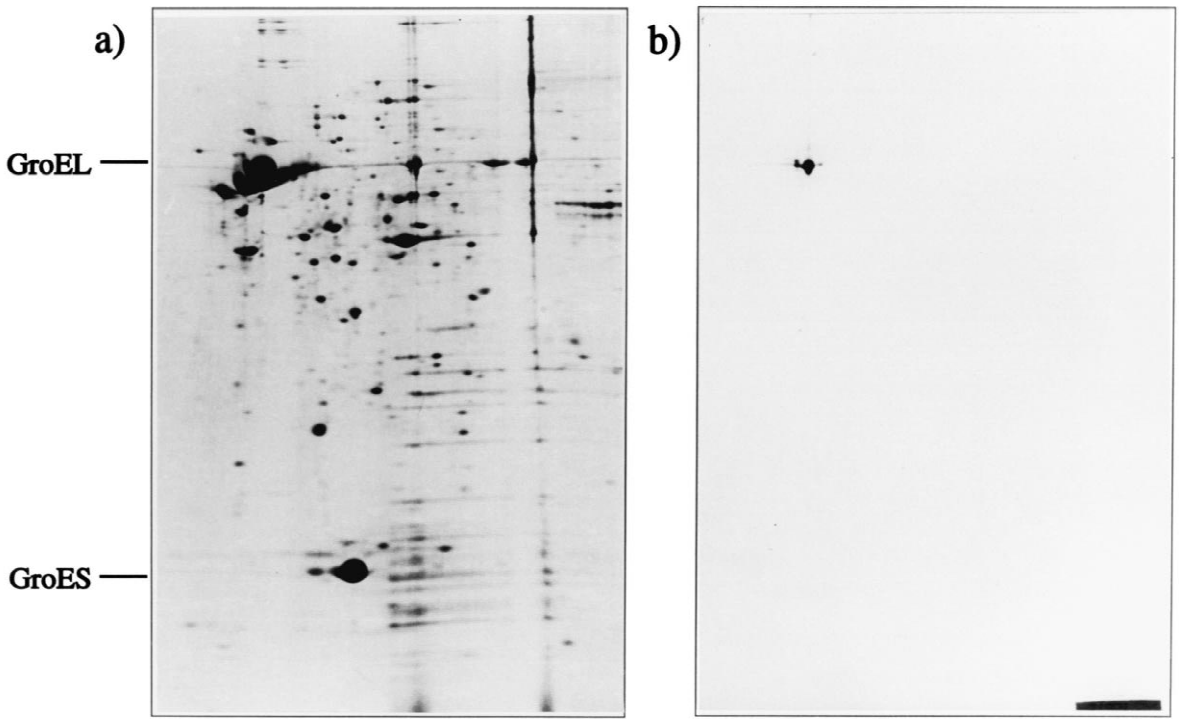


Fig. 2. 2D gel electrophoresis of: (a) cell extract from *E. coli* showing the level of expression of GroEL upon heat induction; (b) pure GroEL sample separated under identical conditions. In both cases charge separation is from left to right and molecular weight separation is from the top to the bottom.

from the resin and found to be a single band on denaturing polyacrylamide gels. According to Fisher [27], this method removed all traces of the enzyme glutamine synthase from the GroEL preparation, as judged by the absence of tryptophan fluorescence.

Chaperonins show weak ATPase activity in vitro, and the release of bound protein substrate depends on nucleotide hydrolysis. For that reason, an ATP-agarose column has been used as the final purification step. Zahn et al. [28] described the evaluation of four ATP columns that differed in the method of attachment of the ATP and in the spacer length between ATP and the agarose. They found that GroEL binds to a column only when ATP is attached through the adenine base with the C_8 spacer. The fact that GroEL binds to ATP-agarose is surprising since the ATP binding sites are inside the chaperonin cavity and not likely to be accessible to ATP crosslinked to the column. Further experiments showed that GroEL binds to the column only in the presence of Mg^{2+} ions and only when both the ribose-triphosphate group and amino group of the purine ring are accessible. Zahn et al. [28] reported that without the ATP-agarose step, 2D crystals of GroEL could not be formed.

Investigation of Type I chaperonins has not been exclusive to *E. coli* GroEL. Several other purification procedures were developed for chaperonins from several sources. A few of the more unusual methods are described below.

The only protein produced by an intracellular bacterial symbiont of the pea aphid is a 14mer of a $59 \cdot 10^3$ rel. mol. mass polypeptide. Named symbionin, it was found to be homologous to GroEL chaperonin [29,30]. The researchers started from a complex material and achieved a very high yield by using the following method. Frozen pea aphid tissue was homogenized in 150 m*M* of Tris/HCl at pH 6.4 containing 300 m*M* of KCl, 1 m*M* of EDTA, and 500 m*M* of PMSF. After clarification by filtration and centrifugation, ammonium sulfate was added. The proteins that were fractionated between 40% and

50% saturation were recovered as a crude symbionin fraction. After dialysis, an aliquot was taken and layered over a linear density gradient of 10–30% sucrose and centrifuged for 20 h in a Beckman SW-28 rotor at 25 000 rpm. Proteins in the gradient fractions were subjected to SDS–PAGE. Symbionin was identified by means of immunoblotting that used antiserum raised against the protein. The final symbionin yield was 2.9 mg per 10 g of frozen aphid. The symbionin was stable for more than a year when stored at −20°C in the presence of 50% glycerol.

Another interesting divergence in the Type I chaperonins is the existence of two GroEL genes in the organism. One is in the same operon with the smaller cochaperonin GroES. The other is a separate, homologous, but nonadjacent GroEL-like gene. This observation has been reported for *Streptomyces albus* [31], *Mycobacterium leprae* [32], and *Mycobacterium tuberculosis* [33]. Separating these two homologous chaperonins from the same organism proved to be challenging. Nevertheless, Kong et al. [33] derived an efficient protocol that successfully exploits the differences in amino acids between the two chaperonin homologues. One chaperonin has a histidine-rich C-terminal tail (DHDHHHGHAH) instead of the MGGM motif commonly found in Type I chaperonins. The chaperonin with the MGGM motif does not bind to the Ni^{2+} column, whereas the other chaperonin containing the histidine-reach sequence does. Kong et al. [33] used immobilized metal ion affinity chromatography (IMAC) as an effective purification step. Briefly, crude bacterial lysates were loaded onto a nitrilotriacetic acid resin column in a guanidine/HCl/urea/phosphate buffer system, as described by the manufacturer (Qiagen). After the column was washed with a buffer containing no guanidine/HCl at pH 8.0 and then incubated in the same buffer at a lower pH (7.0) overnight, the proteins were eluted in the same buffer at pH 5.9. Fractions containing the $60 \cdot 10^3$ rel. mol. mass protein were pooled, dialyzed against PBS, and lyophilized.

In some cases, the ability of a chaperonin to bind to a cochaperonin in the presence of ATP has been used to enhance the chaperonin's purification. For example, Terlesky and Tabita [34] used sucrose density gradients in the presence of 5 m*M* of ATP to successfully isolate the chaperonin/cochaperonin complex from *Rhodobacter sphaeroides*, a purple, nonsulfur, photosynthetic bacterium. This is well illustrated in the SDS–PAGE gels shown in Fig. 3 where the effect of ATP on the purification is shown. Comparison of panel A (no ATP) with panel B (ATP) shows that in the presence of ATP the cochaperonin migrates with the chaperonin and remains at the bottom of the gradient. They then used green

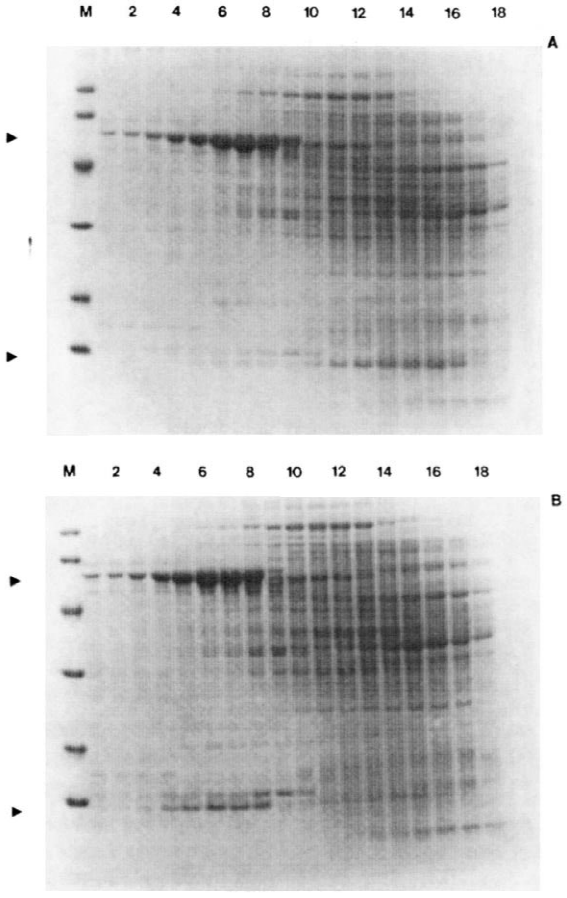


Fig. 3. Sucrose density gradient centrifugation of cell extracts from heat-shocked (40°C) photolithoautotrophically grown *R. sphaeroides* HR in the presence and absence of 5 m*M* ATP. (A) SDS–PAGE, using 11.5% acrylamide gels of samples taken from fractions after sucrose density gradient centrifugation of cell extract (12.3 mg of protein) in the absence of ATP. (B) SDS–PAGE of samples of fractions isolated following sucrose density gradient centrifugation of cell extracts in the presence of 5 m*M* ATP. Fraction 1 is from the bottom of the gradient. Arrows indicate the location of cpn60 and cpn10. *M* denotes the molecular weight standards. This figure was reproduced with kind permission from Ref. [34].

A-agarose chromatography to remove the contaminating RUBISCO enzyme, which binds to this column under the conditions used. A final HR 10/10 MonoQ column yielded pure chaperonin and cochaperonin.

The purification of the chaperonin/cochaperonin complex as the initial purification stage has also been described for the chaperonins from *Bacillus subtilis* [35]. The researchers cloned the chaperonin and cochaperonin from *B. subtilis* and overexpressed it in *B. subtilis*. The *B. subtilis* groE operon, under the control of the *sacB* promoter, was induced by adding 2% sucrose, and the cells were incubated for 2 h. Cells were recovered by centrifugation, and the clarified cell extract was applied to a 5–20% linear sucrose density gradient. The gradient fractions containing the GroEL/GroES complexes were identified by SDS–PAGE, and the complexes were precipitated with 60% (w/v) ammonium sulfate for 1 h at 4°C. Only *B. subtilis* chaperonin/cochaperonin complexes were isolated by this method. The complexes were then further separated by native agarose gel electrophoresis in TBE buffer (89 m*M* of Tris/HCl at pH 8.3 containing 89 m*M* of boric acid and 2.5 m*M* of EDTA). Two distinct bands were observed. Each was eluted by diffusion into buffer. The eluted proteins were precipitated with 50% ammonium sulfate (w/v) and collected by centrifugation. The pellets were dissolved in an appropriate buffer and found to be about 90% pure.

2.2. Purification of chaperonins from thermophilic bacteria

Chaperonins are expressed at high levels in thermophilic organisms, including thermophilic bacteria. To date, the best studied thermophilic Type I chaperonin is that from the thermophilic eubacterium *Thermus thermophilus* [22,24,36]. This organism grows optimally at 70–75°C. When given a heat shock (80°C for 1 h), chaperonin production is increased quite substantially. Because this chaperonin is a thermophilic organism, and therefore its enzymes (including proteases) are inactive at room temperature, all further steps in the purification procedure can be carried out at room temperature. A method similar to the one that was described for GroEL can be used to purify this chaperonin (ref. [24] and p. 7 this review). Fig. 4 shows the chromatographs from a typical purification run. Pure chaperonin fractions were concentrated by using centrifugal concentrators (Filtron or Amicon). Table 2 gives a summary of purification steps.

Another purification method for the chaperonin from *T. thermophilus* was described by Taguchi et al. [36]. In this case, the clarified cell extract was fractionated first with ammonium sulfate (30–60% saturation). The precipitate was dissolved and applied to an anion-exchange column. The use of a weak anion-exchange resin, DE52 cellulose, allowed the chaperonin/cochaperonin complex (called ''holochaperonin'') to be purified directly from the bacterium. This initial chromatographic step was followed by gel filtration on a Sepharose CL6B column, where the holochaperonin eluted at void volume. The fractions containing the holochaperonin were stored as a 60% ammonium sulfate suspension at 4°C. The yield was good; about 200 mg of holochaperonin was obtained from 100 g of wet cells. Because the GroEL/GroES complex is often of interest with respect to the functional aspects of the chaperonin folding cycle, this method may be particularly useful.

The chaperonin from *T. thermophilus* has been cloned and overexpressed in *E. coli* [37,38]. The purification procedure for recombinant *T. thermophilus* HB8 chaperonin cloned into *E. coli* was developed in our laboratory [24]. Two strains were created: one in which just the chaperonin (cpn60) gene was expressed, and a second in which the chaperonin (cpn60) and cochaperonin (cpn10) from *T. thermophilus* were coexpressed on a single plasmid [38]. Interestingly, purification of the recombinant *T. thermophilus* chaperonin from these two strains resulted in some significant differences in the protein's behavior. The thermophilic chaperonins appeared to be highly expressed in *E. coli* and were folded and assembled at 37°C.

Typically, one of the most powerful steps in the purification of a thermophilic protein overexpressed in a mesophilic host like *E. coli* is heat denaturation at 60–70°C. For example, after 5 min at 62°C, 85% of *E. coli* proteins precipitate [39]. Initially, we used the strain harboring only the chaperonin cpn60, heated the extract, and, after centrifugation, applied the extract to a Fast Q column. The procedure was

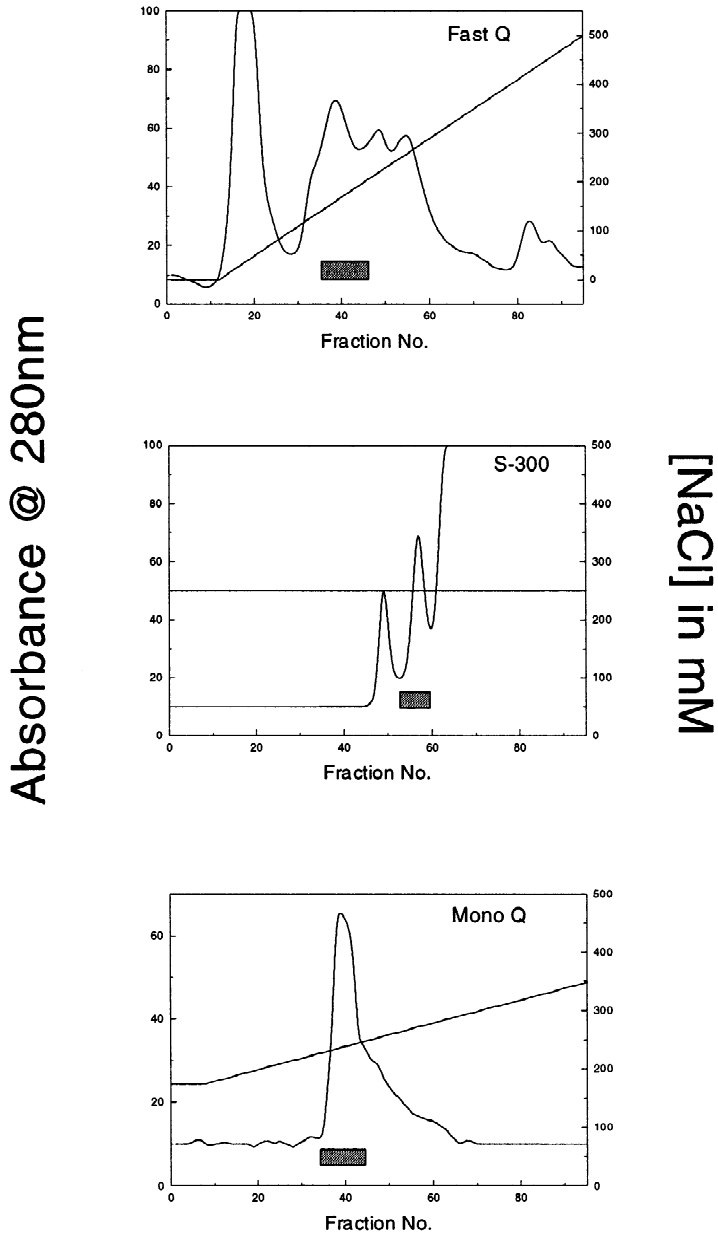


Fig. 4. Summary of the chromatographic steps in purification of Type I chaperonin from *T. thermophilus* on FastQ, Sephacryl 300, and MonoQ 16/10 columns. Absorbance at 280 nm and concentration of NaCl is plotted for each chromatographic separation. Bar indicates fractions in each step that contained chaperonin and were pooled [24].

essentially the same as that described above for the *E. coli* GroEL chaperonin (Ref. [24] and p. 7 this review). However, we tested the purity of the chaperonin samples by using 2D gels and silver staining and found numerous contaminants. Further gel permeation chromatography on a Superdex 200 column (Pharmacia) showed that the small-molecular-weight contaminants coeluted with a high-molecular-weight complex. The contaminating proteins were likely to be tightly bound, unfolded *E. coli*

Table 2
Summary of total yield of chaperonin from *T. thermophilus* (42.5 g of cells)

	Volume (ml)	A_{280}	Chaperonin (mg)
Crude extract	210	672	~92.0[a]
Fast Q	130	114	81.5
S-300	35	13.6	74.2
Mono Q	44	8.1	62.3

[a] Estimated from gel electrophoresis.

proteins that had been captured by the thermophilic chaperonin during the heating step. In the absence of the thermophilic co-chaperonin (cpn10) and ATP, denatured proteins remained irreversibly bound to the recombinant thermophilic chaperonin during purification. We resolved this problem by omitting the heating step and adding 20% methanol to the buffers. We also noted that the yield of purified recombinant tetradecameric chaperonin was low and that there was always a high percentage of monomers and single rings in the preparations. This result agrees with that found by other researchers, who reported an equilibrium between monomers, heptamers, and tetradecamers [40]. We found that heptameric single rings, when incubated with ATP and/or cpn10, dimerised to form a double-ring chaperonin. This occurrence was also reported for another thermophile, *Thermoanaerobacter brockii* [41]. When we purified the cpn60 from the strain containing both cpn60 and cpn10, we found that this equilibrium shifted toward the tetradecamer.

Another potential problem is contamination of the thermophilic chaperonin (or indeed any chaperonin overexpressed in *E. coli*) with endogenous GroEL. This is of particular concern since the expression of heterologous proteins could have been stressful to the cell, initiating a stress response and inducing GroEL production. However, we found that the level of GroEL was not elevated in the cells and also that the small difference in the p*I* and amino acid composition of the two chaperonins was sufficient to separate them chromatographically in an anion-exchange column and on an analytical scale on native polyacrylamide gels.

The purification of chaperonin 60 and chaperonin 10 from the anaerobic thermophile *T. brockii* is based on a different principle, mainly the affinity of chaperonins for hydrophobic surfaces [42]. The organism grows to high cell densities at 68–70°C. About 10 g of wet cells can be obtained per liter of culture [43]. In contrast to other Type I chaperonins, *T. brockii* is purified as a heptamer (i.e., a single ring). The researchers developed a purification protocol that employs hydrophobic and anion-exchange chromatography. They screened several hydrophobic adsorbents for binding the chaperonin. The best matrix was found to be thioethers linked with C_4 and C_6 spacers. These materials were made by coupling butane and hexane thiols to epichlorhydrin-activated Sepharose CL-4B (Pharmacia), and mercaptobenzinidazole (Aldrich) linked to divinylsulphone-activated Sepharose. The chaperonin was also found to bind to both the C_4 and C_6 in the presence of Na_2SO_4; however, the C_6 adsorbent had a higher capacity. In the absence of Na_2SO_4, the chaperonin remained bound to the C_6 column but was eluted from the C_4 column. Use of the C_6 column in the absence of Na_2SO_4, followed by elution with 2-propanol, resulted in excellent purification. However, the capacity of the column was limited to 20 mg of extract protein applied per cm^3 of C_6 resin. The chaperonin could be eluted from the C_6 column by using hydrophobic weakening agents. The researchers found that 20% 2-propanol was the best eluant [43]. For larger-scale purifications, C_6 adsorption could be carried out batchwise, followed by transferring the resin with bound chaperonin to a column for elution. The 2-propanol-eluted fraction was typically about 70% pure, as estimated by SDS–PAGE. This fraction was purified further by anion-exchange chromatography that used Q Sepharose (Pharmacia) After fractions containing the chaperonin were concentrated and dialyzed, the preparation was estimated to be 95–98% pure. To remove the remaining contaminants, high-performance anion-exchange chromatography that used the Bio-Pro Q column was carried out. Some hydrophobic interaction with the matrix was observed,

which caused multiple artifactual peaks and poor recovery. To resolve this problem, chromatography was performed in a buffer containing 20% 2-propanol. After the sample was applied (up to 20 mg of sample per a 5 cm^3 matrix), a gradient of NaCl was applied, with the main chaperonin component eluting at approximately 300 m*M* of NaCl. In addition, chaperonin heterogeneity, varying from one preparation to another, was apparent because of the charge variants of the chaperonin; no traces of contaminating proteins were present in the main peaks as judged by SDS–PAGE and silver staining. When this method was used, the total yield of pure chaperonin from *T. brockii* was typically 40 mg from 20 g cells. Table 3 summarizes the purification of this chaperonin.

The purification of the heptameric chaperonin from *T. brockii* is more complex than other methods described. One reason is that the size of the chaperonin cannot be exploited as an efficient purification step. The authors used the same method to successfully purify chaperonins from related anaerobic extreme thermophiles; the yields and purity of the final preparations were similar to those achieved previously [42]. However, this method was found to be unsuitable for extracts from bacteria with functional tetradecamer chaperonins (*E. coli* and *Zymomonas mobilis*). Perhaps the method could also be applied to purify heptameric chaperonins from mitochondria (see below).

2.3. Purification of chaperonins from mitochondria

Less information is available on the purification of mitochondrial Type I chaperonins than for other types of chaperonins. In some cases, they are purified from whole cells [44]. However, it is more efficient to use a mitochondrial fraction as the starting material. In general, methods used to isolate the Type I chaperonin from mitochondria are similar to the methods described for bacterial chaperonins. For example, chaperonin was purified from the mitochondrial fraction of a pumpkin cotyledon extract by using ammonium sulfate precipitation followed by glycerol gradient sedimentation [45]. The authors used an antiserum raised against GroEL to identify fractions containing the chaperonin, and they identified it as a tetradecamer by using the molecular weight standard in the glycerol sedimentation. It is not clear how pure the protein was after this procedure.

The mammalian mitochondrial chaperonin has been cloned and overexpressed in *E. coli* [46]. The purification protocol is briefly described below. The clarified cell extract was applied to an HR MonoQ column (Pharmacia). A linear gradient of 0–1.0 *M* of NaCl was run, and fractions containing the chaperonin (as demonstrated by SDS–PAGE) were pooled, adjusted to 500 m*M* of NaCl, and applied to a 1×3-cm column of ATP-agarose (Sigma ATP attached through C-8 linker). Purified mitochondrial chaperonin was eluted with 3 m*M* of ATP. The authors found two well-resolved peaks of chaperonin on the FastQ column, one eluting at 90–140 m*M*, and the second eluting at 270–350 m*M*. Only the chaperonin that eluted in the second peak bound to the ATP-agarose column, so it was used for further purification. Further investigation of this fraction showed that the recombinant mammalian chaperonin was a heptamer (a single ring) that was functional in vitro. The fractions that eluted from the Fast Q at the lower salt concentration did not bind to the ATP-agarose column, and their properties were not explored further. However, the two peaks of chaperonin observed on the ion-exchange column could represent monomeric and heptameric states. We have noted a similar pattern in some chaperonins that we purify on a regular basis: the monomer and

Table 3
Summary of the purification procedure for the chaperonin from *T. brockii by* Truscott et al. [42]. Protein concentration was measured by the dye binding method except for fraction 4 which was measured by absorbance at 205 nm. Starting material was 20 g of cell paste

Fraction	Volume (ml)	Protein (mg)
Crude Extract	100	1200
Non-adsorbed portion from C_6 column	200	900
Protein eluted from C_6 column with 20% *p*-propanol	35	90
Chaperonin from Q-Sepharose column	15	40

heptamer elute at lower salt concentrations than does the tetradecamer.

2.4. Purification of chaperonins from plastids

In support of the endosymbiont theory of organelle evolution, plastids of plant cells contain a chaperonin with a sequence that is very homologous to that of the bacterial Type I chaperonins. Indeed, the discovery of this chaperonin – the RUBISCO binding protein – in plant chloroplasts by Barraclough and Ellis [18] played a historically important role in defining the function of chaperonins. Plastid chaperonins can be considered the most complex of the Type I chaperonins. They contain two subunits, α and β, similar to Type IIa chaperonins, but their sequence similarity classifies them as Type I chaperonins.

As it was for mitochondria, the initial purification step is subcellular fractionation. The objective is to isolate the organelle of interest. This step is usually achieved by means of a Percoll gradient for chloroplasts [47] or a sucrose step gradient, as described, for chromoplasts [48]. After this initial step, plastid chaperonins can be purified by methods similar to those used for bacterial and mitochondrial chaperonins. For example, Viitanen et al. [47] used an HR MonoQ column followed by ATP-agarose chromatography. As a final step, they used a TSK 3000 gel filtration column to obtain pure chaperonin, which was stable when stored at 12 mg/ml in 50 m*M* of Tris/HCl containing 300 m*M* of NaCl and 5% glycerol at −80°C. They found that the pea chaperonin contained roughly equal amounts of the α and β subunits. The α and β subunits of *Brassica napus* were cloned and expressed individually in *E. coli* [49]; however, their purification has not been described.

Bonk et al. purified the plastid chaperonin from chromoplasts of *Narcissus pseudonarcissus* [48] obtained from the stroma fraction by means of sucrose gradient sedimentation. After precipitation in ammonium sulfate (30–70%), the procedure consisted of two chromatographic steps: gel filtration and anion exchange. The researchers identified the chaperonin by immunoblotting it with antisera produced against chromoplast chaperonin. They have also purified mitochondrial plant chaperonin by using the same method on the mitochondrial fraction obtained during the subcellular fractionation step.

3. Type IIa chaperonins: archaeal

3.1. Purification of chaperonin from archaea

The first member of the Type IIa chaperonins to be characterized in depth [50,51] was a heat shock protein, TF55, from the thermophilic archaebacterium *Sulfolobus shibatae.* Electron microscopy revealed that this chaperonin has the characteristic double doughnut of the GroEL Type I chaperonin. However, there are nine subunits in each ring. Furthermore, the sequence of this chaperonin is not very homologous to that of the Type I chaperonins but is very homologous to that of TCP-1, a protein essential in yeast. The extra four subunits per chaperonin double-ring structure increase its size to $1020 \cdot 10^3$ rel. mol. mass and allows chromatographic methods based on size (such as gel permeation chromatography and sucrose/glycerol gradient centrifugation) to be exploited. To date, no cochaperonin or release factor has been documented in these organisms. It appears that these chaperonins function quite differently to Type I chaperonins.

Purification of TF55 was described by Trent et al. [51] and comprises two major steps. Cells of *S. shibatae* were grown at 75°C as described in [52], then harvested and resuspended in a buffer containing 50 m*M* of HEPES at pH 7.5 and 5 m*M* of $MgCl_2$. The cells were then lysed by adding Triton X-100 until a final concentration of 0.1% (v/v) was reached. The clarified cell extract was applied to a Q-Sepharose fast-flow (Pharmacia) FPLC column. Fractions containing TF55 that were eluted between 150 and 200 m*M* of NaCl were concentrated by ultrafiltration (Centricon 30; Amicon) and further fractionated by sedimentation in a 10–30% (v/v) glycerol gradient centrifuged at 25 000 rpm for 20 h at 4°C in a SW27 rotor (Beckman). This procedure yielded pure chaperonin, as demonstrated by electrophoresis. (Total protein yield was not discussed, however.) This chaperonin could also be purified by a protocol similar to that described earlier for GroEL and *T. thermophilus*. Fig. 5 shows the elution profiles

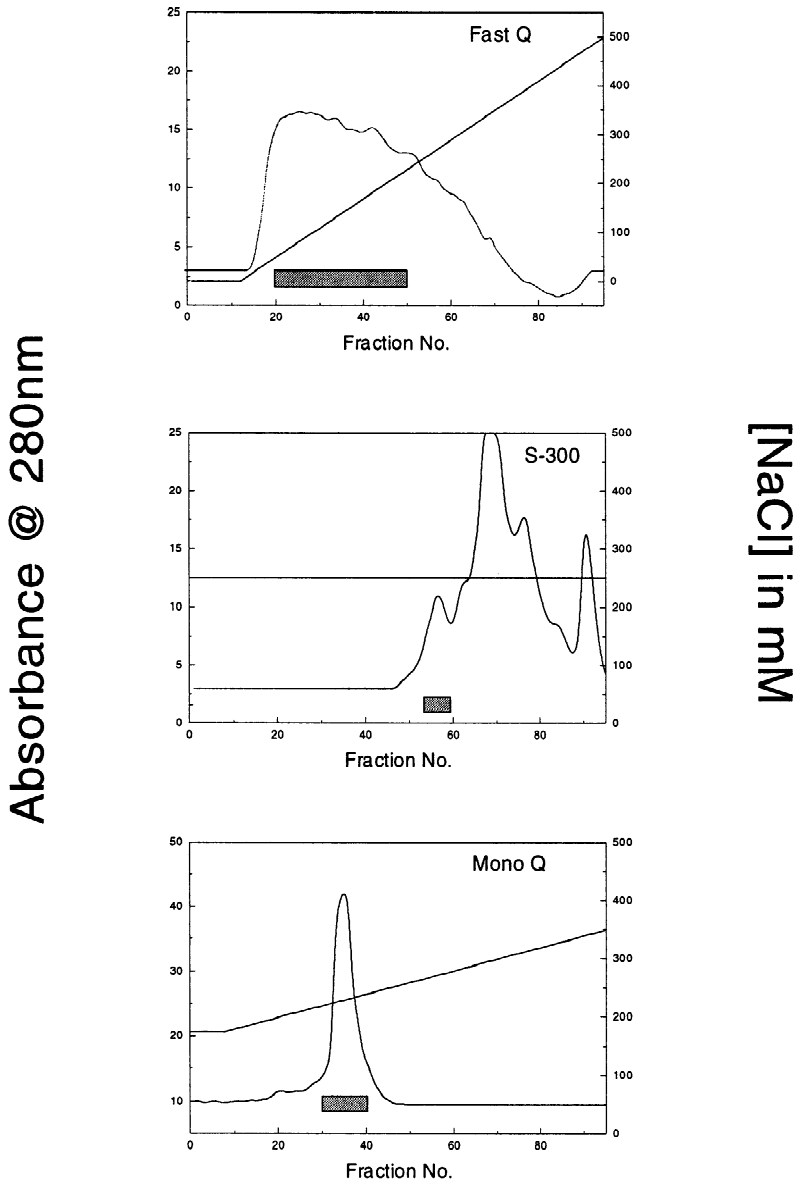


Fig. 5. Summary of the chromatographic steps in purification of Type IIa chaperonin from *S. shibatae* on FastQ, Sephacryl 300, and MonoQ 16/10 columns. Absorbance at 280 nm and concentration of NaCl is plotted for each chromatographic separation. Bar indicates fractions in each step that contained chaperonin and were pooled [24].

from the chromatographic steps using this method [24].

Purification of the chaperonin from another *Sulfolobus* strain that also shows the nine-fold symmetry, *Sulfolobus solfataricus* [53], uses a TMAE-650-(S) tentacle anion-exchange column. The authors used a linear sucrose density gradient to separate the chaperonin on the basis of its size. The final step was a Superose 6 gel filtration column. Purity was monitored by SDS–PAGE on 10% gels by using the Tris/Tricine buffer method described by Schaegger and von Jagow [54].

Originally, it was reported that the chaperonin from *S. shibatae* [50] and *S. solfataricus* [53] was a homooligomeric assembly, similar to its prokaryotic counterparts. However, as interest in Type IIa chaperonins grew, it became clear that the two *Sulfolobus* strains – like *Pyrodictum occultum* [55], *Thermoplasma acidophilum*[56], *Pyrococcus furiosis* [57], and *Thermococcus littoralis* [24,58] – were expressing chaperonin that was composed of two similar but distinct subunits, termed α and β.

Some Type IIa chaperonins from archaea have eight-fold symmetry. They are often referred to as thermosomes. The best-studied of these is the chaperonin from *Thermoplasma acidophilum* [56]. Purification is straightforward; a Fractogel EMD TMAE-600S column is used for the first step, followed by a glycerol gradient. The final step is a Superose 6 gel permeation column. All purification steps were monitored by electron microscopy as well as SDS–PAGE and native gel electrophoresis. Fig. 6 shows an SDS gel of the thermosome complex at different stages in the purification protocol. The final yield of pure chaperonin was 1–2 mg from 2.5-g cells. Native gel electrophoresis of this chaperonin by means of a Tris/glycine buffer system showed the presence of two different bands. These were excised from the gel and separately analyzed by SDS–PAGE. In both cases, two thermosome subunits were detected. The authors also found that the SDS–PAGE Laemmli buffer system cannot resolve the thermosome subunits [56]. In contrast, the SDS–PAGE Tris/Tricine buffer system [54] provides better resolution. Note that when Type IIa and Type IIb chaperonins are compared, the reverse is true; for Type IIb chaperonins, the SDS–PAGE Laemmli buffer system provides better resolution for different subunits.

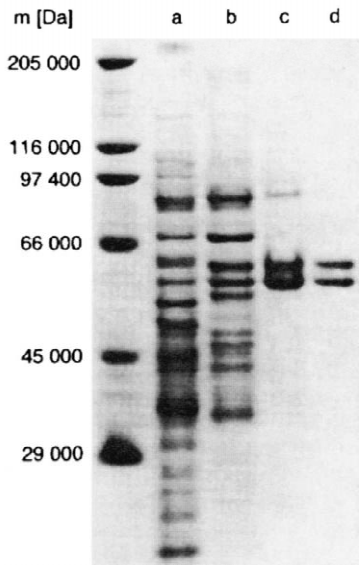


Fig. 6. Thermosome complex at different stages of purification. Aliquots containing approximately 20 μg protein were loaded on each lane of a 10% SDS/polyacrylamide gel run in Tris/Tricine buffer according to Schägger and v. Jagow [36]. Lane a, membrane-free cell lysate; lane b, pooled thermosome-containing fractions from the TMAE-600S column; lane c, pooled fractions from the glycerol gradient; lane d, fraction of the Superose 6 column. Molecular mass standards are shown on the left side of the gel. This figure was reproduced with kind permission from Ref. [56].

Another thermophilic archaea, *Pyrodicticum occultum,* also has eight-fold symmetry. Its purification is described by Phipps et al. [55]. Again, ion-exchange is the first step in purification. However, the paper by Phipps et al. [55] describes the use of a linear density gradient consisting of 10–30% (w/v) sucrose and 5–10% (w/v) glycerol as the second stage in the procedure. After this procedure fractions containing chaperonin were pooled and resolved on a linear gradient of 18–37% (w/v) glycerol in TN buffer containing 50 m*M* of Tris/HCl at pH 7.0 and 50 m*M* of NaCl. The chromatographs obtained from these three steps are shown in Fig. 7. This figure also shows the correlation of the chaperonin's activity, in this case ATPase activity, with chromatographic protein profiles. In the final stage, a pool of the desired fractions was chromatographed on an HR MonoS cation-exchange FPLC column (Pharmacia). The chaperonin passed through the column without binding and was collected in the flow-through. Fractions were analyzed at each stage by electron microscopy and SDS–PAGE. Purified chaperonin was stable for several months when stored in TN buffer and 30% glycerol at 0°C. Although this method provided pure chaperonin, the duplication of the gradient sedimentation steps seems superfluous.

A large-scale method for purification of 700 mg of chaperonin from *Thermococcus littoralis* is described by Joachimiak et al. [24]. DEAE Sepharose fast flow was used at a 9 l gradient from 0 to 500 m*M* of NaCl. Fractions (100 ml) containing the chaperonin

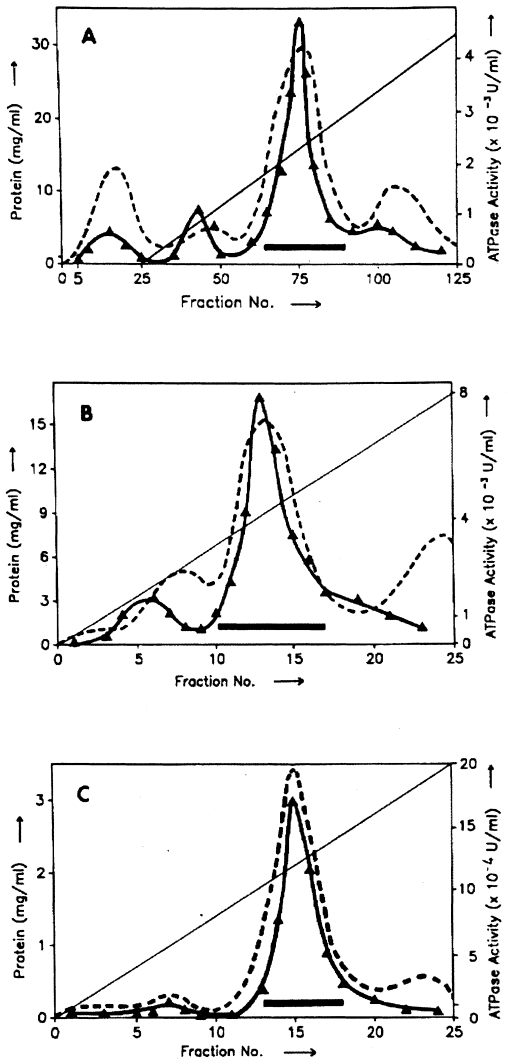


Fig. 7. Purification of the ATPase complex from *P. occultum*. (A) DEAE–Sephacel chromatography of membrane-free cell lysate. Bed volume=100 ml. Elution was achieved with a gradient of 50–500 m*M* KCl, represented by the thin line. The complex eluted between 200 and 300 m*M* KCl. Volume/fraction=8 ml. (B) Sucrose-glycerol density gradient centrifugation of pooled fractions from the DEAE column. The linear gradient of 10–30% sucrose and 5–10% glycerol is indicated by the thin line. The complex eluted in the 18–21T sucrose/7–8% glycerol range. Volume/fraction=0.5 ml. (C) Glycerol density gradient centrifugation of pooled fractions from the sucrose-glycerol gradient. The linear gradient of 18–37% glycerol is represented by the thin line. The complex eluted in the 28–32% glycerol range. Volume/fraction=0.5 ml. Fractions were assayed for ATPase activity at 100°C (▲ – ▲) and their protein content estimated by absorbance at 280 nm (– – –). A solid bar indicates fractions pooled at each stage. This figure was reproduced with kind permission from Ref. [55].

as determined by SDS–PAGE were combined and concentrated to approximately 30 ml by ultrafiltration. This concentrated sample of chaperonin was then applied to a column of Superdex 300 (Pharmacia). A yield of 1 mg of chaperonin per gram of wet cells was routinely achieved. This method also works well for the purification of *Pyrococcus furiosus* [59].

3.2. Conformational differences of the Type IIa chaperonins

A number of reports provide evidence that the Type IIa chaperonins exist in two slightly different conformations, often referred to as open and closed. As described above for *Thermoplasma acidophilum*, these have different mobilities on nondenaturing polyacrylamide gels [61,62,64]. We originally thought that the two conformations represented the empty chaperonin assembly and a chaperonin-substrate complex. However, excision of the bands and subsequent 2D electrophoresis indicated that no additional proteins were present in the slower-migrating band. To purify open and closed forms, we used preparative nondenaturing PAGE. The preparative cell model 491 from BioRad was used in a Tris/phosphate buffer at pH 7.5 according to the manufacturer's instructions. The method resulted in a very high resolution, and we were able to obtain the pure open and closed forms on the milligram scale, suitable for CD and EM studies (Fig. 8). Highly overloaded 2D gels, coupled with silver staining, did not reveal the presence of other contaminating polypeptides. The only difference in the preparations was in their structures, and the difference was disclosed by both CD and EM. The slower-moving band on nondenaturing PAGE is in the open conformation, but its polypeptide composition is identical, as revealed by 2D gel electrophoresis.

3.3. Purification of α and β subunits of archaeal chaperonins

The second subunit in the chaperonin was initially detected by one-dimensional SDS–PAGE by using the Tris/Tricine buffer system [60,61] on 12% gels in the PHAST system (Pharmacia). The resolution was much higher when larger 10–15% gradient gels

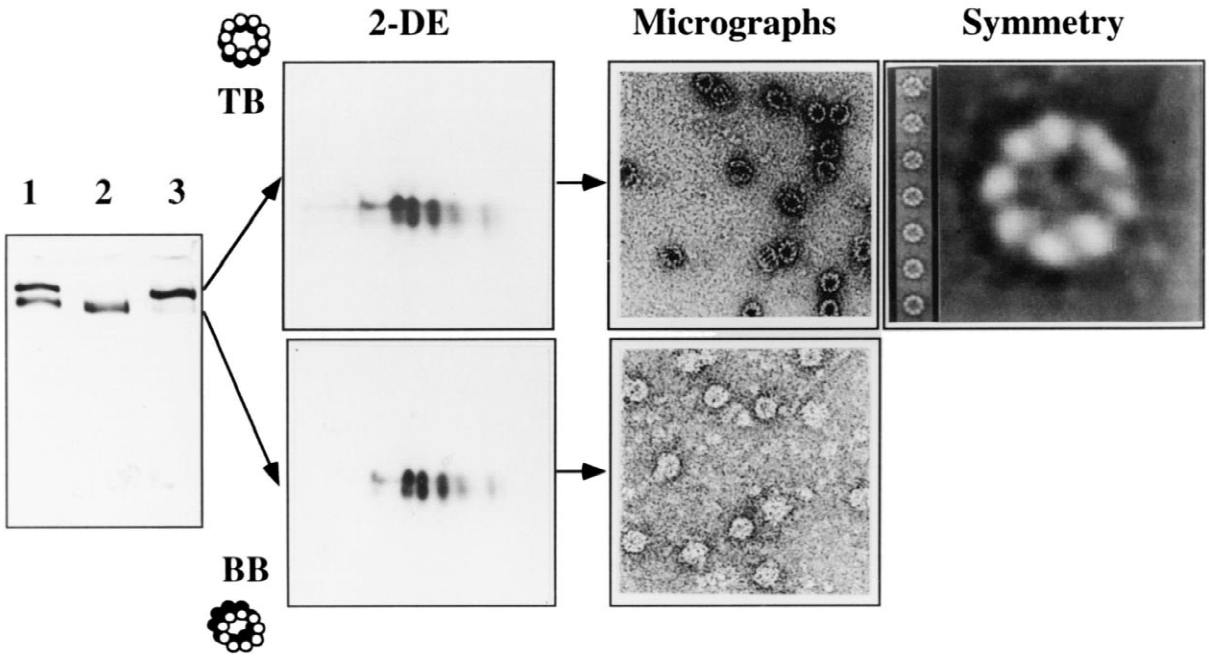
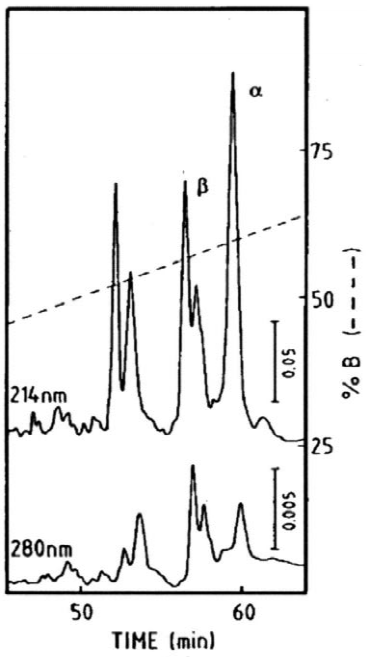


Fig. 8. Separation of the chaperonin complexes from *S. shibatae* on polyacrylamide gel electrophoresis under native conditions. Lane 1: pure chaperonin obtained from freshly grown culture of *S. shibatae* separated on 6% native gels; lane 2: top band (TB, open complex); lane 3: bottom band (BB, closed complex). Protein bands were stained with silver. 2-DE panel: chaperonin bands were excised, electroeluted and run on 2-DE gel electrophoresis under denaturing conditions, as indicated by arrows; both complexes show identical subunit composition. Micrographs panel: electron micrographs of open (TB) and closed (BB) complexes. Symmetry panel: rotationally averaged electron micrograph of seven open complexes (from an electron micrograph similar to that shown in the top micrograph panel); shows chaperonin complexes with a well defined central cavity and 9-fold symmetry [61].

(BioRad) were used in the Tris/Tricine buffer system. Further, 2D electrophoresis of the purified chaperonin from *S. shibatae*, in which isoelectrofocusing (IEF) was used in the first dimension and a 10–20% gradient with the Laemmli gel system was used in the second dimension, clearly showed the heterogeneity in both the charge and subunit size of the purified Type IIa chaperonin. This mass and charge heterogenity can be seen in Fig. 8, panel 2 quite clearly. Further genetic studies of *S. shibatae* allowed the second gene to be identified and subsequently cloned and overexpressed in *E. coli* [60]. This interesting example shows the power of different buffer systems and the gel porosity of SDS–PAGE in the resolution of very similar polypeptide chains. It is always useful to test several conditions to find the most optimal procedure for the protein of interest.

After the discovery of two very similar chaperonin subunits in *S. shibatae*, we attempted to purify each subunit. SDS–PAGE showed that, after the initial FastQ column, both chaperonin subunits were eluted with 75–150 m*M* of NaCl. To separate the α and β subunits, the small difference in p*I* was exploited, and the salt gradient was optimized on HR 10/10 MonoQ or Resource Q columns. Using this approach made it possible to obtain pure α and β subunits in sufficient quantity for biochemical characterizations and of sufficient purity for crystallization experiments [60,61].

The heterooligomeric nature of the chaperonin from *S. solfataricus* was confirmed by Knapp et al. [62]. These authors detected the second subunit by reversed-phase HPLC and the traces, together with SDS–PAGE gels are shown in Fig. 9. From the chromatograph it is clear that two species (α and β) are present, however they are almost indistinguishable on the gel. Their chaperonin purification method



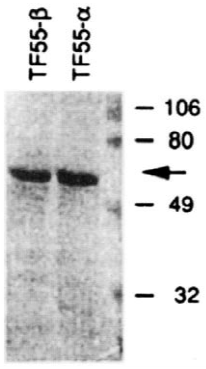


Fig. 9. Separation of the α and β subunits on a C_4 reversed-phase HPLC column. (top) Chromatogram showing the α subunit clearly separated from the β subunit. The other peaks correspond to peptides or non-protein substances not visible in the SDS–PAGE (not shown). (bottom) SDS–11%PAGE of the subunits purified by HPLC (shown by an arrow). Molecular weight markers (rel. mol. mass) are indicated. This figure was reproduced with kind permission from Ref. [62].

was somewhat different than those described above. The cells were grown at 75°C; however, a heat shock (90 min at 88°C) was given before harvesting to maximize the amount of chaperonin transcribed. Proteins in the clarified cell extract were precipitated with ammonium sulfate and separated by centrifugation. The chaperonin was found in the pellet resulting from the addition of 0.35 g of $(NH_4)_2SO_4$ per milliliter of lysate. This pellet was dissolved in a neutral buffer and dialyzed overnight. The dialysate was applied to a HR MonoQ 10/10 column (Pharmacia), and the chaperonin was eluted with a linear gradient of NaCl. Pooled fractions were concentrated in a stirred cell (Amicon) and subsequently applied to a linear 10–30% (v/v) glycerol gradient. Additional purification and separation of the α and β subunits was achieved by reversed-phase (C_4)-HPLC. Chaperonin (200 μl; 100 μg) purified through the glycerol gradient step was dialyzed against 50 m*M* of $(NH_4)HCO_3$ at pH 7. After the addition of 40 μl of formic acid, the dialyzate was applied to a C_4 column (Vydac 214TP54, 5 μm, 4.6 mm×250 mm), equilibrated with 0.1% trifluoro acetic acid (TFA) (0 to 70% over 70 min at 1 ml/min), and monitored at 214 and 280 nm.

3.4. Purification of cloned Type IIa chaperonins

There are few reports of the direct purification of subunits of Type IIa chaperonins from the wild type organism (as described above); however, the cloning and overexpression of Type IIa chaperonin subunits in *E.coli* has produced much purer subunits. Several laboratories have cloned the α and β genes from a number of archaeal species and expressed them separately and together in *E coli*. It was shown that homooligomeric assemblies of α or β subunits have the same symmetry as the heterooligomeric α/β assembly [61]. The expression and purification of chaperonin subunits pose interesting questions. For example, will the Type IIa chaperonins, expressed together in the same cell, assemble into their native multimeric structure? Will the multimeric assembly form when just α or β is overexpressed individually? These are important questions from a purification point of view, since the large size of the chaperonins is a major characteristic exploited in their purification. Furthermore, if these chaperonin assemblies are formed in the *E. coli* cell, will they have a collection of indigenous *E. coli* proteins bound irreversibly to them? An additional problem is the separation of the native *E coli* GroEL from the Type IIa chaperonin.

To circumvent these problems, several different

cloning strategies have been used. In cloning the α and β subunits of *T. acidophilum* [56] and *S. shibatae* [60], a histdine$_6$ tag was used to enable purification. This strategy has the benefit of allowing the subunit to be purified as a $60 \cdot 10^3$ rel. mol. mass monomer if assembly in *E. coli* does not occur. It also has the added advantage of separating the Type IIa chaperonin monomers from *E. coli* GroEL, which does not bind to the Ni^{2+} column. Waldmann et al. [63] used a standard procedure for Ni^{2+} chelate affinity chromatography to purify the His$_6$-tagged α and β proteins from *T. acidophilum*. Briefly, a cell free extract was diluted twofold with the column-equilibration buffer (20 m*M* of sodium phosphate, 500 m*M* of NaCl, 1 m*M* of imidizole at pH 7.0). The solution was then applied to a Fractogel EMD chelate column (8 cm×1.5 cm, Merck). Proteins were eluted with a 30-ml gradient of 1–500 m*M* imidizole in the equilibration buffer. Fractions containing the chaperonin were pooled, concentrated by ultrafiltration in Centriprep 10 and Centricon 30, and loaded on a Superose 6 gel filtration column running at room temperature in Tris/HCl at pH 7.5 containing 150 m*M* of NaCl, 1 m*M* of EDTA, and 5% glycerol at a flow-rate of 0.5 ml per min. Purification was monitored by Tris/Tricine SDS–PAGE and nondenaturing PAGE. However, Waldmann et al. [63] reported that the binding efficiency of the His$_6$-tagged chaperonin to the affinity column was rather low, even when a large column was used. Purification of the *S. shibatae* α subunit also proved challenging. As in the GroEL chaperonin assembly, the C-termini of both subunits are believed to project toward the inner cavity of the toroid, resulting in poor accessibility for the column-bound Ni^{2+} ion. However, sufficient quantities of protein were obtained to biochemically characterize the molecule. In both cases, the His$_6$ tag did not prevent assembly, and homooligomeric assemblies composed of α and β subunits could be detected in the cytoplasm of *E. coli*. This finding allowed a second size-exclusion step to be used in the purification procedure. Waldmann et al. [63] reported that the chaperonin from *T. acidophilum* purified by metal chelate chromatography was contaminated with small amounts of *E. coli* proteins, as seen by SDS–PAGE. One was identified as *E. coli* GroEL by immunostaining; presumably, it was a result of copurification, but it is not clear why, since GroEL does not bind to the metal column itself. Other minor bands were found to be degradation products of the Type IIa chaperonin itself. However, the preparations were at least 96% pure. Both the α and β homooligomeric chaperonin complexes of *T. acidophilum* were stable for a few days at 4°C. However, longer storage increased the amount of degradation, particularly of the β subunit. The lower stability of the β subunit was also observed during nondenaturing PAGE at a pH of 8.8. Interestingly, we have observed that the α/β subunits of the chaperonin from *S. shibatae* also show degradation upon storage.

The α and β Type IIa chaperonins from the hyperthermophilic archaea *Thermococcus* strain KS-1 [64] and *Thermococcus litoralis* [58] have been cloned and overexpressed in *E. coli*. No His$_6$ tag was implemented in this cloning strategy. In the case of the *Thermococcus* KS-1 strain, the clarified cell extract was first heated to 70°C for 30 min to precipitate the majority of *E. coli* proteins. After the precipitated proteins were removed by centrifugation, ammonium sulfate was added to the supernatant until it was 30% saturated. Hydrophobic interaction chromatography (Ether-Toyopearl) was used as a first chromatographic step. The chaperonins were eluted with a linear gradient of ammonium sulfate from 30–0% saturation. Dialyzed fractions containing the chaperonin were applied to a ResourceQ column (Pharmacia) equilibrated with buffer A and eluted with a linear gradient of 0–500 m*M* of NaCl in the same buffer. Fractions containing the chaperonin subunits were pooled, concentrated by ultracentrifugation, and then applied to a gel filtration column (Shodex PROTEIN KW803). The recombinant chaperonin, either all α or all β, eluted in the void volume. Yoshida et al. [64] found that both the recombinant all-α chaperonin and the all-β chaperonin precipitated during the concentration step. To dissociate the aggregated homooligomeric assemblies, various detergents and organic solvents were added. If it was added during gel filtration, methanol (15%, v/v) was found to dissociate the aggregated α subunit assembly. Unfortunately, the β subunit assembly remained aggregated under all conditions tested.

Purification of the α and β subunits from *T. litoralis* was carried out by a similar heat denaturation step, followed by anion-exchange, FastQ Sepharose (Pharmacia), gel filtration, and high-resolution anion-exchange (HR MonoQ, Pharmacia), as described by Joachimiak et al. [24]. Both the α and β subunits formed assemblies in *E. coli,* and a sufficient amount and quality were obtained for crystals. Fig. 10 shows a comparison of some Type I and Type IIa chaperonins.

4. Type IIb chaperonins: from cytosol of eukarya

This article has a separate section on the eukaryotic cytosolic chaperonin, since it is more complex than Type I and Type IIa chaperonins. This chaperonin is composed of eight sequence-related subunits, and it plays a specialized function in the cell. In the true spirit of chaperone nomenclature, it has many aliases. It was first described by Willison

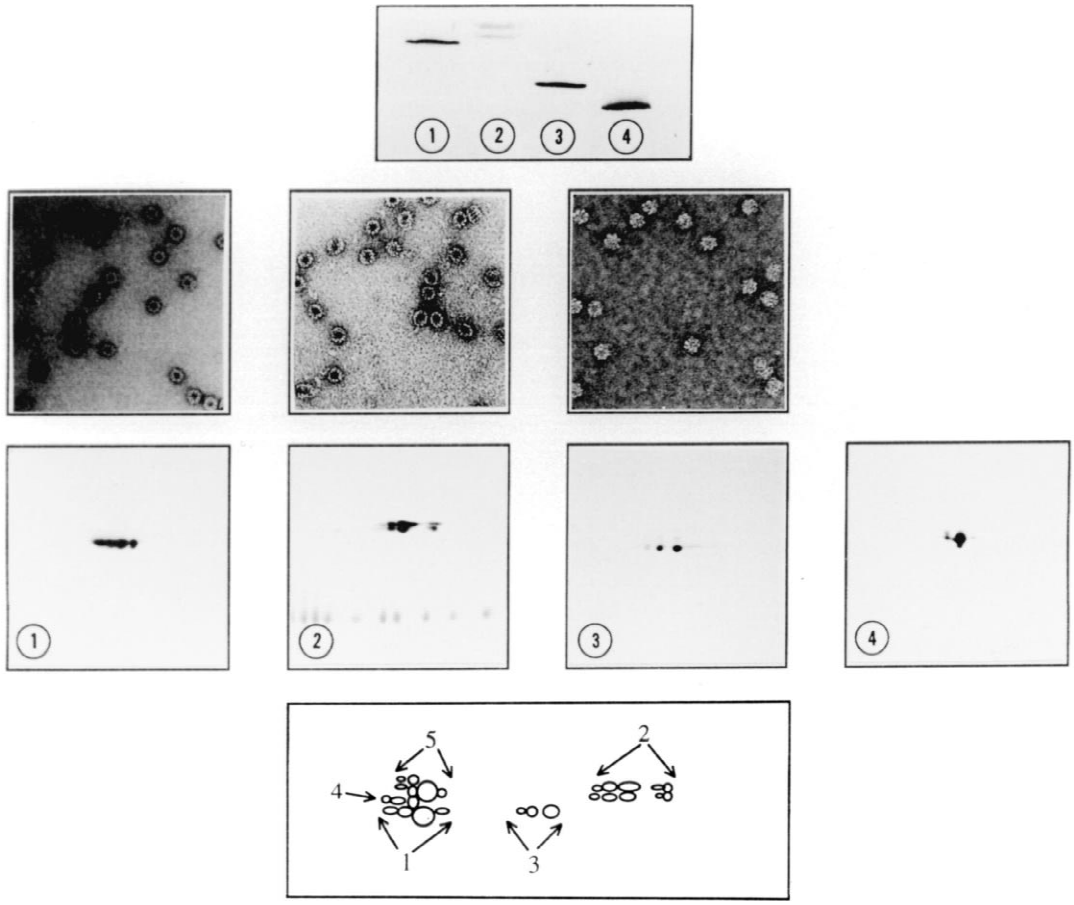


Fig. 10. Electron microscopic images and polyacrylamide electrophoresis of purified chaperonins from bacterial and archaeal sources. Top panel shows separation of chaperonins on 4–15% polyacrylamide gel under native conditions. The middle panel shows 2D gel electrophoresis and corresponding electron microscopic images of four chaperonins from bacterial and archaeal sources. The chaperonin complex from *S. shibatae* sample was run on a 2D electrophoresis gel, with charge standards shown as a line of protein spots below the chaperonin sample. The bottom panel shows the relative position of five different chaperonins on 2D gel electrophoresis. The observed charge and mass polymorphism of thermophilic archaeal chaperonins is contrasted with more homogenous preparations of bacterial chaperonins derived from mesophilic and thermophilic sources. The numbering scheme for chaperonins is as follows: (1) *T. litoralis,* (2) *S. shibatae,* (3) *T. thermophilus,* (4) *E. coli,* and (5) *P. furiosus* [24]

et al. [65] as the protein TCP-1 (for tail-less complex polypeptide), although, at that time, it was a protein of unknown function. Then it was discovered to be a chaperonin [66,67] and was subsequently found to contain several different subunits [51,68] named TRiC (TCP-1 Ring Complex). At least eight different subunits were found in this chaperonin, and a new name, CCT (chaperonin containing TCP-1), was derived [69].

4.1. Purification of recombinant Type IIb chaperonins

Numerous purification methods have been developed for this interesting protein. However, unlike the other chaperonins, even though this protein is quite abundant in the cell, the final yield of purified chaperonin is rather small. Isolation of the Type IIb chaperonin from rabbit reticulocyte lysate was first described by Gao et al. [66]. The starting material was clarified reticulocyte lysate, which was applied to HR MonoQ (Pharmacia) ion-exchange chromatography. The column was developed with a 65 ml buffer of 10 m*M* of Tris/HCl at pH=7.2 containing 10 m*M* of KCl, 2 m*M* of EGTA, and 1 m*M* of DTT, and the protein was eluted with a linear gradient of 20–500 m*M* of $MgCl_2$. An activity assay, which measured the folding of ^{32}P-labelled denatured β-actin, was used to detect the chaperonin. The fraction containing the chaperonin was then subjected to ATP-agarose chromatography (ATP coupled through C_8, Sigma). The β-actin folding assay was again used to detect chaperonin activity. In the final step, gel filtration on a Superose 6 column (Pharmacia) was used. The purified assembly was composed of at least five different polypeptides. The authors did not give information on the final yield.

The Type IIb chaperonin was also purified directly from bovine testes by Frydman et al. [68]. Approximately 40 g of material was homogenized, clarified by centrifugation, and applied to an HR MonoQ 16/10 column (Pharmacia). Bound proteins were eluted by using 350 ml linear gradient of 100–400 m*M* of NaCl. The presence of a TCP-1 chaperonin was determined by western blotting that used the monoclonal rat anti-mouse TCP-1 antibody 91A [65]. The pooled fractions of the major TCP-1 peak (150 mg of protein) eluting at 285 m*M* of NaCl were concentrated by ultrafiltration on a $100 \cdot 10^3$ rel. mol. mass filter (Millipore) and were separated on a 10–40% sucrose gradient. Fractions containing the chaperonin were pooled (18 mg of protein), and sucrose was removed by dilution and ultracentrifugation. The $MgCl_2$ concentration was adjusted to 5 m*M* in pooled fractions containing TCP-1 and applied to a 10 ml ATP-Agarose column (C_8, Sigma). After the column was washed with buffer, bound protein was eluted with a gradient of 0–5 m*M* of ATP in the same buffer. ATP was removed by a desalting step. Aliquots were stored at −80°C. The final yield of purified TRiC was 8–12 mg per 40 g of fresh tissue.

Type IIb chaperonin from both reticulocyte lysate and bovine testes was purified by using the above method. Rommelaere et al. [15] found that the chaperonin assembly contained eight distinct subunits, one of which was TCP-1. These authors also described the purification of the individual subunits by using reversed-phase HPLC (Waters) on a C_4 column (Vydac, The Separations Group). A gradient of 1% acetonitrile per min. in 0.1% trifluoro acetic acid was developed, and eluted proteins were detected at 214 nm. Fig. 11 shows the peak assignment from their HPLC profile and the corresponding sample run on a SDS–PAGE gel. In order to establish possible sequence relationships isolated polypeptides were oxidized with performic acid, dried, and resolubilized in 4 *M* of urea per 100 m*M* of Tris at pH 8.0. Before treatment with proteases, they were diluted to a 1:4 ratio in 100 m*M* of Tris at pH 8.0. All subunits were digested with trypsin except the two polypeptides present in peak 6, which were treated with endoproteinase Lys-C. All proteolytic digests were performed according to manufacturer's recommendations. Peptide mixtures were purified by C_{18} (Vydac) reversed-phase HPLC as above, and selected peptides were sequenced using automated Edman degradation on an Applied Biosystems sequencer. It was found that the subunits were sequence-related, implicating the existence of a multi-gene family.

Murine Type IIb chaperonin has also been characterized [70]. The researchers used Hep-2 cells or testis germ cells as a starting material for chaperonin purification. All steps were carried out at 4°C. After extraction, a cocktail of protease inhibitors was added to the cell-free extract before being layered

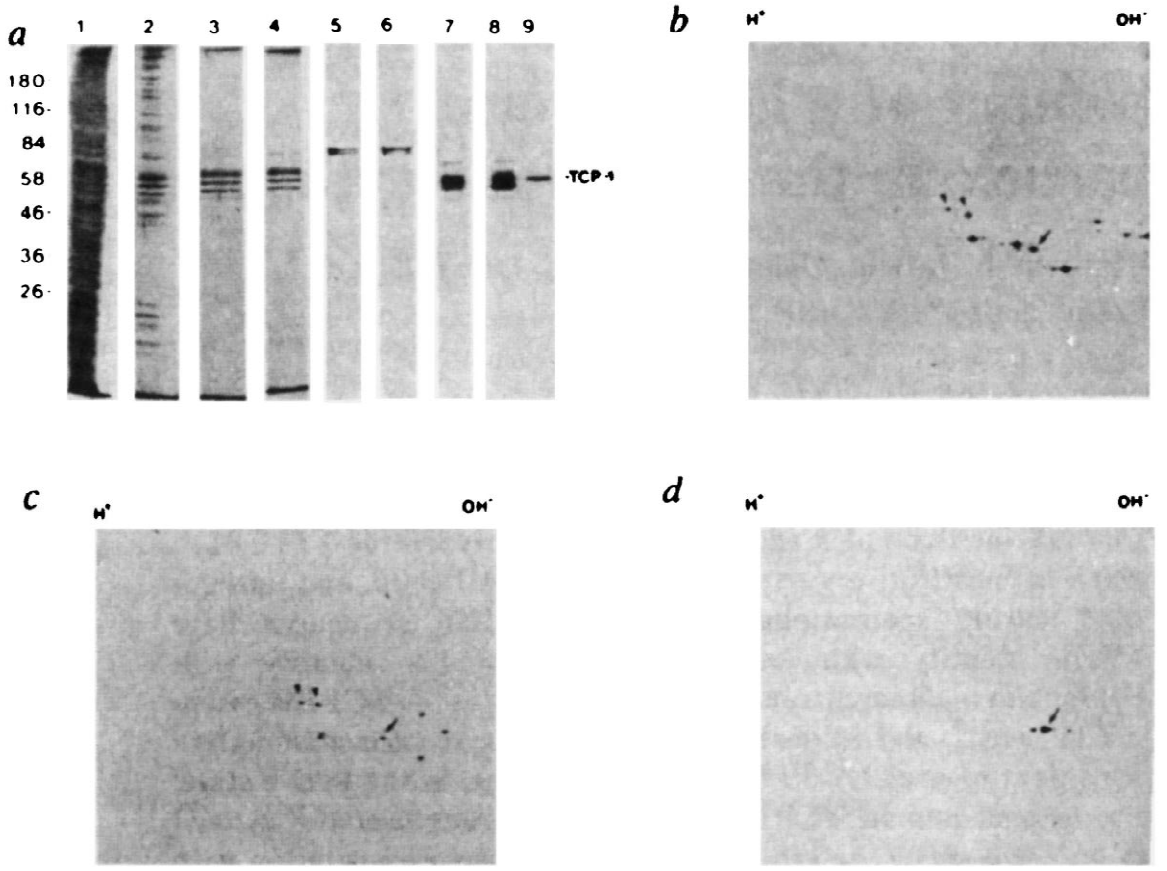


Fig. 11. Purification of TCP1 as a protein complex by a two-step method of sucrose gradient fractionation followed by either DE-52-cellulose anion-exchange or ATP affinity chromatography. A Silver staining of SDS–PAGE-separated samples from the purification of murine testis germ bell TCP1 complex; PNS (lane 1), pooled 19.5–21.5% sucrose gradient fractions (lane 2: TCP1 peak elutes after DE-6\52 and ATP-agarose chromatography (lanes 3 and 4, respectively). Samples obtained from ATP-agarose chromatography (lane 5) or immunoprecipitation with anti-TCP1 antibody 91A (lane 6) were western blotted and probed with monoclonal mouse anti-Hsp70. ^{35}S-methionine-labeled proteins with BHK or murine testis germ cells were immunoprecipitated with anti-TCP1 antibody 19A, separated by SDS–PAGE and autoradiographed as shown in lanes 7 and 8, respectively. Immunoprecipitation in mixed micelle buffer of the same germ cell preparation used in lane 8 with anti-TCP1 antibody 91A identifies TCP1 in the complex (lane 9), b, c, d, Hep.2 cell (b) or germ cell (c,d) TCP1 complex purified by ATP-agarose chromatography, separated by two-dimensional isoelectric focusing SDS–PAGE and silver stained (b, c) or transferred to nitrocellulose and probed with anti-TCP1 antibody 91A to identify TCP1. D, The Hsp70 proteins are indicated by arrow-heads and TCP1 by an arrow. This figure was reproduced with kind permission from Ref. [70].

onto a prechilled, continuous sucrose gradient (10.2–40%) (w/v) and fractionated by ultracentrifugation. Fractions containing TCP-1 (19.5–21.5% of sucrose) were pooled and dialyzed against a column equilibration buffer and loaded onto ATP-agarose (C_8, Sigma) or DE-52 cellulose (Whatman) columns (4 mm×200 mm). Columns were eluted with linear gradients of 0–15 m*M* of ATP or 0–0.4 *M* of NaCl, respectively, in five bed-volumes of equilibration buffer. The eluate was analyzed by immuno dot blots to determine the peak fractions containing TCP-1. Chaperonin eluted at 60 m*M* of NaCl (DE-52) and 3 m*M* of ATP (ATP-agarose). Fig. 12 shows the summary of purification steps by electrophoresis (a) together with the identification of TCP-1 polypeptides by immunostaining (b–c). Each purification from 1×10^9 germ cells resulted in yields of about 300 μg, and each purification from tissue culture

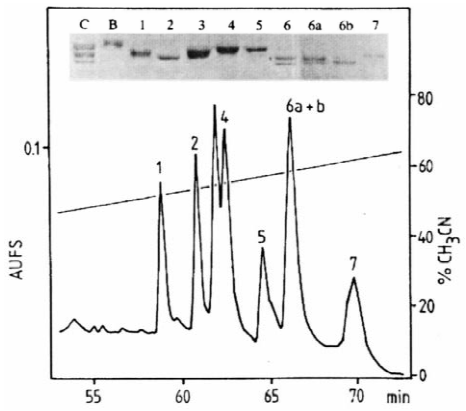


Fig. 12. C_4 reversed-phase HPLC separation profile of subunits from rabbit reticulocyte chaperonin. Only the relevant portion of the chromatogram is shown. Absorption in absorbance units full scale was monitored at 214 nm, and the $\%CH_3CN$ gradient is indicated. (Inset) Purified peptides analyzed by SDA/10% PAGE and stained with Coomassie Brilliant Blue. Lane numbers refer to the order of elution; 6a and 6b represent aliquots of preparative runs in which front and tail fractions of peak 6 were collected separately. Lane C contains the initial material loaded on the column, and lane B contains bovine serum used as a standard. This figure was reproduced with kind permission from Ref. [15].

cells resulted in 60–100 μg per 2×10^8 cells. Recovered samples were either used immediately for further analysis or snap frozen in liquid N_2 and stored at −70°C. This method was also used successfully to purify the Type IIb chaperonin from yeast [71].

The most recent purification report on a Type IIb chaperonin [72] discusses a different approach from those described above. These authors purified the chaperonin from rabbit reticulocyte lysate in three steps: ammonium sulfate precipitation (30–50% fraction), centrifugation into a sucrose cushion, and chromatography on heparin-Sepharose. This method substantially increased the final yield of chaperonin (0.1–0.25 mg per milliliter of packed cells). The method is also quite rapid when compared with the others, which may be a reason that a higher yield was obtained. The Type IIb chaperonin assembly is very unstable and will rapidly disassemble into monomers [61]. When this occurs, one of its most powerful purification properties ''its large size'' is lost. This reduces the yield of the Type IIb chaperonin. Norcum [72] showed that the method yields homogenous material with no evidence of partial proteolysis or contaminating proteins. The chaperonin was active as judged by its ATPase activity and ability to refold denatured proteins. The difficulty in purifying large amounts of Type IIb chaperonin is coupled with its relative instability when compared with Type I and IIa chaperonins. The reasons for this instability are only partly understood [61]

5. Determination of chaperonin activity, concentration, and purity

Many different assays can be used to identify chaperonins during purification. ATP hydrolysis is a simple and rapid method used to detect chaperonins [55,73,74]. ATP-dependent binding of the GroES co-chaperonin can be used to detect Type I chaperonins [75]. Protein refolding assays are a very powerful method of chaperonin identification; numerous enzyme targets have been used successfully [4,22], although assays that involve Type IIa and Type IIb chaperonins are not always straightforward [61,76]. An assay which measures protein assembly, developed by Fisher [27] is another highly specific test of chaperonin activity. Immunoassays are often used because of the variety of monoclonal and polyclonal antibodies available commercially [22,50,65,69] and ease of use. These assays, coupled with chaperonin migration on the native PAGE, are very effective in identifying chaperonins during all purification steps and allowing for sample characterization.

The concentrations of pure chaperonins can be determined by using UV spectroscopy and calculated extinction coefficients [77]. The fact that Type I chaperonins and also some subunits of Type IIa and Type IIb chaperonins have no tryptophan means that their absorbance at 280 nm is remarkably low for proteins of such a large size. This fact also biases detection during purification, when an UV detector is commonly used to monitor the protein peaks. In our experience, a small protein peak can reveal tens of milligrams of chaperonin. It is therefore advisable to run gels over the complete chromatographic run, especially if the protein appears to be 'lost'.

Determination of chaperonin purity has also proved challenging to standardize. The very nature of the molecule's function means that it promiscuously binds unfolded proteins, peptides, and, indeed, amino acids. However, we have found that high-resolution 2D electrophoresis, coupled with sensitive silver staining [78], is an excellent indicator of protein purity. We also check for the absence of nucleotides in our preparations by employing spectroscopy at 260 nm. One excellent method of chaperonin purity evaluation is described by Smith and Fisher [79]. They used second derivative spectral analysis to ensure the absence of tryptophan in a purified preparation of GroEL. Measuring tryptophan fluorescence is also a very good and highly sensitive method for detecting impurities in chaperonin samples that do not contain endogenous tryptophan [22].

6. Storage of purified proteins

When they were cited in the literature, we have described the storage conditions for the chaperonins discussed in the text. In our experience, GroEL can be stored for several months at 4°C in a Tris/HCl buffer at pH 7.5, containing 250 m*M* of NaCl, 1 m*M* of EDTA, and 5 m*M* of DTT. Both wild type and recombinant chaperonins from *T. thermophilus* can be stored in the same buffer at either room temperature or 4°C. Thermophilic Type IIa chaperonins can also be stored at room temperature; however, the subunits are less stable, and it is much better if they are stored at 4°C. We and others have also successfully stored Type I and II chaperonins in 50% glycerol at −80°C for extended periods without significant degradation or loss of activity. It seems that Type IIb chaperonins are the most unstable chaperonins; they must be frozen at −70°C in 50% glycerol as soon as possible after purification.

7. Conclusions

The protocols described were used to purify many different chaperonins to homogeneity on a milligram scale (Table 1). The best-characterized chaperonin to date is GroEL. It can be used as a reference protein in both method development and subsequent biochemical and structural analyses. In general, purification procedures were designed to exploit several properties of the chaperonins. These proteins are acidic, and they bind well to a strong ion-exchange quaternary amine-type matrix. Chaperonins form double-ring structures that are very large – from 800 to $1100 \cdot 10^3$ rel. mol. mass in molecular weight. Because chaperonins are one of the most abundant, large, soluble proteins in cells, gel permeation chromatography is extremely effective in separating them from most of the other cellular proteins. These proteins are weak ATPases and therefore bind also to ATP-agarose. Chaperonins bind hydrophobic regions of unfolded proteins, and, as a consequence, they bind to hydrophobic matrices. However, this property is also detrimental, because unfolded proteins and peptides will remain bound during purification. To remove these bound proteins and peptides, permeation chromatography can be carried out in the presence of alcohols.

The purification of functional chaperonins from bacteria, archaea, and eukarya is highly relevant to the issue of chaperonin-assisted protein folding and protein aggregation. To date, most mechanistic studies have focused on GroEL and related cofactors and examined the ability of the chaperonin to protect and refold chemically denatured protein substrates in depth. The use of other analogs of GroEL, such as those described herein, will allow the protection and refolding of a greater number of protein substrates to be examined. Although much is known about GroEL structure and function, attempts to fully characterize the eukaryotic cytosolic chaperonin (CCT) have proved difficult, presumably because of the instability of the Type IIb chaperonin. The archaeal Type IIa chaperonins show a high degree of homology to Type IIb chaperonins, but they contain only one or two related subunits. They therefore provide a simpler system by which to examine the structure and function of Type II chaperonins and their role in protein folding. There are still many unanswered questions with regard to the role and the function of Type II chaperonins. Hopefully, the availability of large quantities of highly purified archaeal chaperonins will help in the characterization of this mechanism.

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