[34] Purification and Properties of Hsp104 from Yeast

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Introduction

In this chapter we describe protocols for the purification of wild-type heat shock protein 104 (Hsp104) from *Saccharomyces cerevisiae* and a rapid procedure for the purification of a modified version of Hsp104, carrying an amino-terminal histidine extension, from *Escherichia coli.* We also describe systems for the expression of toxic HSP100 proteins and mutant Hsp104 proteins. In addition, methods for determining the ATPase activity of the purified protein and its oligomerization properties are provided. Requests for reagents employed may be made through the Web site http://http.bsd.uchicago.edu/~hsplab/index.html.

Properties of Hsp104

Biological Properties

Hsp104 is a heat-shock protein that promotes survival under extreme stresses such as heat and high concentrations of ethanol.[1,2] It appears to enhance survival by promoting the solubilization and reactivation of protein aggregates *in vivo.*[3] Hsp104 also functions in the maintenance and curing of a prion-like, protein conformation-based phenotype in yeast, referred to as [*PSI*$^+$],[4] in which the translation termination factor Sup35 is sequestered into aggregates causing ribosomes to read through stop codons.[5–8]

Biochemical Properties

Hsp104 has a calculated relative molecular weight of 102,000 and an estimated *pI* of 5.14. On the basis of the similarities between these predicted

[1] Y. Sanchez and S. L. Lindquist, *Science* **248,** 1112 (1990).
[2] Y. Sanchez, J. Taulien, K. A. Borkovich, and S. Lindquist, *EMBO J.* **11,** 2357 (1992).
[3] D. A. Parsell, A. S. Kowal, M. A. Singer, and S. Lindquist, *Nature (London)* **372,** 475 (1994).
[4] Y. O. Chernoff, S. L. Lindquist, B.-i. Ono, S. G. Inge-Vechtomov, and S. W. Liebman, *Science* **268,** 880 (1995).
[5] B. Cox, *Curr. Biol.* **4,** 744 (1994).
[6] M. F. Tuite and I. Stansfield, *Nature (London)* **372,** 614 (1994).
[7] S. V. Paushkin, V. V. Kushnirov, V. N. Smirnov, and M. D. Ter-Avanesyan, *EMBO J.* **15,** 3127 (1996).
[8] M. M. Patino, J.-J. Liu, J. R. Glover, and S. Lindquist, *Science* **273,** 622 (1996).

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values and the observed migration of Hsp104 on two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE), we assume that Hsp104 does not undergo extensive posttranslational modifications. Hsp104 has no tryptophan residues, which makes tracking the protein during chromatographic procedures difficult. Hsp104 contains two nucleotide-binding domains, which demonstrate specificity for adenine nucleotides.[9,10] Interestingly, despite its two ATP-binding domains, Hsp104 does not bind ATP–agarose (either with a short or long arm spacer; Sigma, St. Louis, MO).[11] At low protein concentrations Hsp104 oligomerizes in the presence of ATP. These oligomers are most likely hexamers, on the basis of sizing chromatography, glutaraldehyde cross-linking, and scanning transmission electron microscopy (STEM).[9] Hsp104 is an ATPase with a K_m of ~5 m*M* and a V_{max} of ~2 nmol min^{-1} μg^{-1}.[10] ATPase activity is stimulated by certain proteins and peptides.[12]

Purification of Hsp104 from Yeast

Plasmid and Strain Construction

To increase yield in *S. cerevisiae*, *HSP104* can be expressed from high-copy vectors with strong promoters (Table I). Most commonly we employ a vector in which wild-type *HSP104* coding sequences are regulated by the highly inducible glucocorticoid response elements of the p2UG vector.[13] With this construct (p2UG104),[9] Hsp104 is induced by the addition of 10 μM deoxycorticosterone to yeast cells, which also carry a plasmid encoding the mammalian glucocorticoid receptor (pG-N795).[14] To reduce degradation problems during purification, the protein is expressed in strain BJ5457 (A741; Table II), carrying a deletion of the pep4 and prb1 protease genes.[15] A variant carrying an *HSP104* deletion is employed to avoid contamination of wild-type Hsp104 when purifying mutant variants of Hsp104 or related HSP100 proteins from other organisms (A798, Table II).

Constructs for the expression of Hsp104 from the *GAL1-10* promoter are also available (104b-U; Table I). The growth of large-scale cultures in raffinose and galactose is costly, but a method to circumvent this can be

[9] D. A. Parsell, A. S. Kowal, and S. Lindquist, *J. Biol. Chem.* **269,** 4480 (1994).
[10] E. C. Schirmer, C. Queitsch, A. S. Kowal, D. A. Parsell, and S. Lindquist, submitted (1998).
[11] D. A. Parsell and S. Lindquist, unpublished observations (1990).
[12] E. C. Schirmer and S. Lindquist, unpublished observations (1995).
[13] D. Picard, M. Schena, and K. R. Yamamoto, *Gene* **86,** 257 (1990).
[14] M. Schena, D. Picard and K. R. Yamamoto, *Methods Enzymol.* **194,** 389 (1991).
[15] E. W. Jones, *Methods Enzymol.* **194,** 428 (1991).

TABLE I
PLASMIDS FOR EXPRESSION OF Hsp104 IN YEAST

Accession number	Plasmid	Promoter	Selection	Copy number	Induction by:	Product	Refs.
9029	p2UG	*GRE-CYC1*	URA3, Amp^R	High (2 μm)	GR/DOC[a]	—	[b]
5316	p2UG104	*GRE-CYC1*	URA3, Amp^R	High (2 μm)	GR/DOC[a]	Hsp104	[c]
5228	pG-N795	*GPD*	TRP1, Amp^R	High (2 μm)	Constitutive	GR	[d]
5800	104b-U	*GAL1–10*	URA3, Amp^R	Low (CEN6, ARS4)	Galactose	Hsp104	[e,f]
5306	pYS104	*HSE*	URA3, Amp^R	Low (CEN6, ARS4)	Heat	Hsp104	[g]
5632	PLH102	*GPD*	URA3, Amp^R	High (2 μm)	Constitutive	Hsp104	[h]

[a] GR/DOC: Glucocorticoid receptor (GR), which is constitutively expressed in cells, is activated by addition of deoxycorticosterone (DOC).

[b] D. Picard, M. Schena, and K. R. Yamamoto, *Gene* **86,** 257 (1990).

[c] D. A. Parsell, A. S. Kowal, and S. Lindquist, *J. Biol. Chem.* **269,** 4480 (1994).

[d] M. Schena, D. Picard, and K. R. Yamamoto, *Methods Enzymol.* **194,** 389 (1991).

[e] E. C. Schirmer, S. Lindquist, and E. Vierling, *Plant Cell* **6,** 1899 (1994).

[f] E. C. Schirmer, C. Queitsch, A. S. Kowal, and S. Lindquist, submitted (1997).

[g] Y. Sanchez and S. L. Lindquist, *Science* **248,** 1112 (1990).

[h] L. Henninger and S. Lindquist, unpublished observations (1993).

found in Joshua-Tor *et al.*[16] Hsp104 can also be induced to high levels in wild-type cells with a heat shock of 37–39° for 90 min. (A plasmid containing Hsp104 behind its natural heat shock promoter is pYS104).[1] Hsp104 can be expressed constitutively at a high level from the GPD promoter (PHL102; Table I),[17] but this protein seems to have a lower specific activity in thermotolerance than Hsp104 induced by heat stress.[18] The reason for this is unclear; however, pending its resolution we recommend the GRE expression system. It yields the highest expression (with the exception of the GPD expression system): 5- to 10-fold greater than that observed in heat-treated cells.[9]

Solutions and Equipment

Deoxycorticosterone (Sigma), 10 m*M* (1000×) in ethanol
Yeast–peptone–dextrose (YPD, 10×), per liter: 100 of yeast extract, 200 g of Bacto-peptone, 200 g of dextrose, 0.4 g of adenine sulfate; components become soluble during autoclaving
KCl stock (2.5 *M*)
Buffer A (10×): 0.5 *M* Tris (pH 7.7), 20 m*M* ethylenediaminetetraacetic acid (EDTA), 100 m*M* $MgCl_2$, 50% (v/v) glycerol. Add 1.4 m*M*

[16] L. Joshua-Tor, H. E. Xu, S. A. Johnston, and D. C. Rees, *Science* **269,** 945 (1995).
[17] L. Henninger and S. Lindquist, unpublished observations (1993).
[18] S. Lindquist and G. Kim, *Proc. Natl. Acad. Sci. U.S.A.* **93,** 5301 (1996).

TABLE II
STRAINS FOR PURIFICATION OF Hsp104 FROM YEAST

Accession number	Strain	Genotype	Refs.
A741	BJ5457	*α, ura3-52, trp1, lys2-801, leu2Δ1, his3Δ200, pep4::HIS3, prb1Δ1.6R, can1, GAL*	[a]
A798	BJ5457HSP104::LEU2	*α, ura3-52, trp1, lys2-801, leu2Δ1, his3Δ200, pep4::HIS3, prb1Δ1.6R, can1, GAL, hsp104::LEU2*	[b]
A750	BJ5457/p2UG104, pG-N795	*α, ura3-52, trp1, lys2-801, leu2Δ1, his3Δ200, pep4::HIS3, prb1Δ1.6R, can1, GAL*, carrying plasmids p2UG104 and pG-N795	[b]

[a] E. W. Jones, *Methods Enzymol.* **194,** 428 (1991).
[b] D. A. Parsell, A. S. Kowal, and S. Lindquist, *J. Biol. Chem.* **269,** 4480 (1994).

2-mercaptoethanol and 1 m*M* 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF; protease inhibitor) fresh before use. *Note:* Owing to the high cost of AEBSF, it is replaced with 1 m*M* PMSF (phenylmethylsulfonyl fluoride) for dialysis.

Phosphate (pH 6.8) stock, 0.5 *M*: Roughly 49% (w/v) Na_2HPO_4 plus 51% (w/v) NaH_2PO_4

Buffer B: 50 m*M* potassium phosphate (pH 6.8), 5% (v/v) glycerol, 1.4 m*M* 2-mercaptoethanol, 1 m*M* AEBSF

Additional protease inhibitors: Added from concentrated stock solutions to the following final concentrations: 12 μg/ml (peptstatin A, in ethanol); 7 μg/ml (leupeptin, in H_2O); 2 μg/ml (aprotinin in 10 m*M* HEPES); 1 m*M* benzamidine (in H_2O); 1 μ*M* sodium metabisulfate (in H_2O)

Bead-Beater (BioSpec Products, Bartlesville, OK): Available with 350-ml cup or 15-ml cup. This apparatus is in essence a blender with a 4-mm-thick Teflon blade and an outer shell surrounding the lysis chamber to accommodate an ice–water bath. For much smaller preparations a Mini-Beadbeater-8 cell disrupter (BioSpec Products) accommodates 2-ml microcentrifuge tubes.

Growth of Cells for Protein Purification

A high cell density and a high ratio of glass beads to cells in the lysis step greatly enhance the efficiency of breakage. The procedure detailed below is for 50 g (wet weight) of packed cells, but it can readily be scaled for smaller or larger preparations.

Strain A750 is grown at 25° in minimal medium containing dextrose (2%, w/v), ammonium sulfate (0.5%, w/v), yeast nitrogen base without amino acids (0.17%, w/v), adenine (10 mg/liter), arginine (50 mg/liter), lysine (50 mg/liter), methionine (20 mg/liter), phenylalanine (50 mg/liter), threonine (100 mg/liter), tyrosine (50 mg/liter), aspartic acid (70 mg/liter), leucine (100 mg/liter), and histidine (20 mg/liter). The absence of tryptophan and uracil forces maintenance of the GRE-HSP104 and GR (glucocorticoid receptor) expression plasmids. Reversion of the *trp* allele in this strain is sometimes problematic; strains should be monitored for Hsp104 expression competence before preparing large-scale cultures.

An initial overnight culture of cells in midlog phase (~ 2–5×10^6 ml^{-1}) is used to inoculate four 6-liter flasks, each containing 1.5 liters of medium. After dilution, the growth rate slows to a doubling time of ~3 hr; however, as cells again reach midlog phase, the doubling time reaches ~2.3 hr. When cultures reach midlog phase, Hsp104 is induced with deoxycorticosterone at a final concentration of 10 μM. After 8 additional hours of incubation, the medium is supplemented with 10× YPD and cells are incubated an additional 10–15 hr. The rich medium allows cells to reach a higher stationary-phase density, and plasmid loss in the absence of selection is not high during this short period. Cells (final density, ~ 1–3×10^8 cells/ml) are collected by centrifugation (4500 rpm for 20 min at 4°). Cell pellets are resuspended in ice-cold water, combined, and subjected to an additional round of centrifugation. The gram weight of the cell pellet is measured, and cells are resuspended by adding <50% gram weight (i.e., for 160 g of cells add 80 ml) of buffer A. Protein can be purified with equal success from cells lysed immediately or cells frozen with liquid nitrogen. To freeze cells, the slurry (in buffer A) is dripped into a bath of liquid nitrogen. Frozen droplets are removed to a storage container (keep the lid loose initially to prevent explosions from the vaporizing nitrogen) and stored at −80° until use.

Lysis of Cells

Cells resuspended in buffer A are supplemented with additional protease inhibitors (see Solutions and Equipment). Densely resuspended cells (165 ml) are mixed with 150 ml of glass beads (425–600 μm in diameter; acid washed) and placed in a 350-ml Bead-Beater cup with an ice–water bath contained in the outer shell. Cells are blended 12 times for 30 sec each, with a 90-sec recovery period between pulses to recool the inner chamber. If frozen cells are used, they should be thawed by constant stirring of the frozen droplets in a warm water bath, making certain all cells are kept cold. Cells may be lysed when frozen clumps of cells are less than 0.5

cm in diameter (in this case the initial blending pulse can be extended to 2 min without significant heating of the lysate). Lysates are removed from the beads and the beads are washed with ice-cold H_2O (one-third the lysate volume). Lysate and wash are accrued and subjected to centrifugation (18,000 rpm for 20 min at 4°). Supernatants are diluted 1 : 1 with buffer A before being applied to columns.

Chromatography

All columns should be maintained at 4° through the procedure and all buffers, equipment, and other reagents cooled before use. As Hsp104 has no tryptophan and produces a weak signal at 280 nm, all fractions should be kept until the quality of peak fractions has been confirmed by analysis by 10% (w/v) SDS–PAGE. The range indicated for elution of peak fractions covers the wider range observed from multiple preparations. In individual purifications, Hsp104 has sometimes eluted closer to one or the other end of this range. Hsp104 accumulates to 5–10% of the total cellular protein, and the first two columns each yield about 10-fold purification; so Hsp104 eluted from the first DEAE column is 90–95% pure. The last two columns increase this to >98%.

1. *Affi-Gel Blue:* Lysate from 50 g of cells is applied onto a 30-ml Affi-Gel Blue (Bio-Rad, Hercules, CA) column preequilibrated in buffer A with a flow rate of 70 ml/hr. At a similar flow rate, the column is washed with five column volumes of buffer A (150 ml) followed by buffer A containing 100 m*M* KCl (150 ml). Protein is eluted with 100 ml of buffer A containing 1 *M* KCl. The eluant is dialyzed for 4 hr to overnight against buffer A containing 1 m*M* PMSF and cleared by centrifugation.

2. *DEAE I:* The dialyzed eluant from the Affi-Gel Blue column is applied onto a 15-ml DEAE column (Pharmacia, Piscataway, NJ) preequilibrated in buffer A at a flow rate of 50 ml/hr. The column is washed with 75 ml of buffer A and protein is eluted with a 200-ml linear gradient of 0–500 m*M* KCl in buffer A. Fractions eluting between 70 and 140 m*M* KCl are enriched in Hsp104 and these are accrued and dialyzed against buffer B.

3. *Hydroxyapatite:* Accrued fractions from the DEAE column are applied onto a 15-ml hydroxyapatite column preequilibrated in buffer B at a flow rate of 20 ml/hr. After washing with five column volumes of buffer B, proteins are eluted with a linear gradient of buffer B from 50–400 m*M* potassium phosphate, pH 6.8. Fractions eluting between 135 and 180 m*M* phosphate are pooled and precipitated with addition of solid ammonium sulfate (crushed to powder with a mortar and pestle) to 70% of saturation.

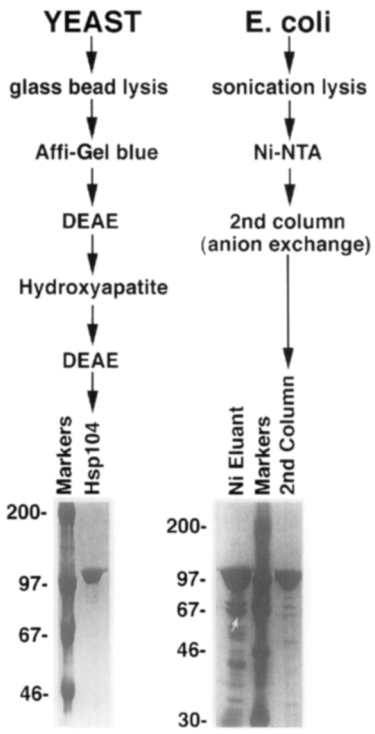

FIG. 1. Flow chart of Hsp104 purification procedures from yeast and *E. coli.* The purity of the final products is shown at the bottom. The arrow in the Ni eluant lane indicates common degradation products of Hsp104, which react with antibodies against Hsp104.

The precipitate is collected by centrifugation (15,000 rpm for 20 min at 4°), resuspended in buffer A, and dialyzed to completion against buffer A.

4. *DEAE II:* Insoluble material is removed by centrifugation and proteins are applied onto a second 15-ml DEAE column at a flow rate of 50 ml/hr. The column is washed with 200 ml of buffer A, followed by elution with a linear gradient of 50 to 300 m*M* KCl in buffer A. Hsp104 elutes from the column at a salt concentration of 105–150 m*M*. The purity of the preparation is shown in Fig. 1.

Yields and Calculating Protein Concentration

Every gram of packed cells yields roughly 0.5 mg of purified Hsp104 protein, as assessed by amino acid analysis and using a calculated extinction coefficient $\varepsilon = 31{,}900\ M^{-1}$ at 276 nm[19] (there is no difference between

[19] H. Edelhoch, *Biochemistry* **6,** 1948 (1967).

native and denatured Hsp104 at this wavelength). The Bradford assay gives values roughly twice this when using BSA (bovine serum albumin) as a standard.

Scaling for Larger or Smaller Preparations

For large-scale cultures, a 20-liter polypropylene carboy is used to grow cultures. Sterile silastic tubing is connected through an adaptor in the lid. One end is attached to filtered house air, and the other end extends to the bottom of the carboy. Sampling of the culture density is effected through a second access point in the lid of the carboy, which also allows the exchange of CO_2. A 200-ml overnight culture is used to inoculate a 20-liter culture. The culture is grown in a 20-liter polypropylene carboy at room temperature; effort should be made to maintain the room at approximately 25° as the doubling time of the cells is much longer at lower temperatures. The rate of growth can be increased with a space heater next to the carboy. Aeration should be maintained at a level that prevents cells from settling, but does not cool them (house air is typically lower than room temperature).

For small-scale cultures a 15-ml Bead-Beater chamber is available to maintain high cell density in minimal volumes; other apparatuses exist for use with microcentrifuge tubes.

Purification of HSP100 Proteins from Other Species in Yeast

Yeast Expression Systems for Toxic Proteins

Expression of some HSP100 proteins (e.g., Hsp101 from *Arabidopsis thaliana*,[20] Hsp101 from *Glycine max*,[21] and Hsp100 from *Leishmania major*[22]) is apparently toxic to *E. coli*. To facilitate cloning in *E. coli*, a modified *HSP100* gene is employed to reduce basal expression. In this system, the sequence around the initiating AUG is changed to a context unfavorable for expression in *E. coli*, but still capable of strong induction in yeast. Specifically, the *HSP100* gene is modified to contain a polylinker site (*Bam*HI) followed by three guanine nucleotides directly in front of the initiating AUG, and this is inserted into a pRS313-based vector[23] carrying *URA3* as the selectable marker and the *GAL1-10* promotor in the polylinker (104b-U; Table I).[20] This plasmid also has reduced basal expression from the *GAL* promoter during growth in raffinose, allowing yeast cells carrying

[20] E. C. Shirmer, S. Lindquist, and E. Vierling, *Plant Cell* **6,** 1899 (1994).
[21] Y.-R. J. Lee, R. T. Nagao, and J. L. Key, *Plant Cell* **6,** 1889 (1994).
[22] A. Hubel, S. Brandau, A. Dresel, and J. Clos, *Mol. Biochem. Parasitol.* **70,** 107 (1995).
[23] R. S. Sikorski and P. Hieter, *Genetics* **122,** 19 (1989).

toxic HSP100 varaints to be grown in raffinose before galactose induction. For protein purification, a protease-disrupted strain carrying an *hsp104* deletion should be used (BJ5457HSP104::LEU2; Table II).

When expressing HSP100 proteins from other organisms using this system, attention should be paid to issues of codon usage and translation termination sequences. Because procedures for the purification of Hsp104 depend on anion-exchange chromatography and the middle region of HSP100 proteins is both highly charged and variable in size between subtypes, it is likely that the methods presented here will be applicable only for members of the same subtype. A method for purification of ClpA (an HSP100 protein that lacks the charged middle region) has been published by Maurizi *et al.*[24]

Vector to Facilitate Production of Mutant hsp104 Proteins

A modified *HSP104* gene, $HSP104_R$, facilitates the cloning and analysis of Hsp104 mutants. This vector contains unique restriction sites approximately every 500 bp throughout the coding sequence of Hsp104 that do not change the encoded amino acids or significantly alter codon usage. For mutagenesis studies, for example, the segment of interest containing the mutation is sequenced, excised, and inserted into an unmutagenized version of $HSP104_R$ (104b-U; Table I) to ensure that no unintended mutations are present.

Protein Purification from *Escherichia coli*

Plasmid and Strain Constructions

Adding a short stretch of histidine residues to the end of a protein allows rapid purification in one step because histidine residues have a high affinity for nickel resins. Hsp104 protein carrying a six residue (6*x*) histidine extension at its amino terminus is as stable and functions in thermotolerance as well as wild-type Hsp104 in yeast (Hsp104 with a carboxy-terminal 6*x*-histidine expression functions in thermotolerance, but is less stable).[25] *In vitro,* Hsp104 protein with a 6*x*-histidine extension purified from *E. coli* exhibits ATP hydrolysis similar to that of wild-type Hsp104 purified from yeast, whether the histidine extension is cleaved from the protein or not.[12] The modified protein also assembles into oligomers indistinguishably from the wild-type yeast protein.[26]

[24] M. R. Maurizi, M. W. Thompson, S. K. Singh, and S.-H. Kim, *Methods Enzymol.* **244,** 314 (1994).

[25] D. A. Parsell and S. Lindquist, unpublished observations (1993).

[26] A. S. Kowal and S. Lindquist, unpublished observations (1996).

The plasmid employed for purification in *E. coli,* pETH6104b, is a modified pET28a expression vector (Novagen, Madison, WI), with the T7 epitope tag removed. pETH6104b contains $HSP104_R$ coding sequences under inducible control of the T7 promoter for expression using the system developed by Studier *et al.*[27] This plasmid produces an Hsp104 protein carrying a 6*x*-histidine extension at the amino terminus that can be cleaved from the protein using thrombin.[28] The selectable marker for the plasmid is kanamycin. The plasmid is transformed into pLysS cells, which contain a plasmid encoding T7 lysozyme that is selected for with chloramphenicol.[29] We have also had success purifying Hsp104 using the pJC45 vector system (see Clos and Brandau[30]).

Purification of Hsp104 from *E. coli* using pETH6104b is simple and fast. A disadvantage is that a higher level of degradation of Hsp104 occurs in *E. coli* than in yeast. The process of reducing these degradation products substantially reduces yields. Degradation products are minimized by inducing Hsp104 when cells are at a low density, for a short period (proteases are induced in late-log phase).

Buffers and Solutions

LB medium (per liter): 10 g of Bacto-tryptone, 5 g of NaCl, 5 g of yeast extract
Kanamycin, 50 mg/ml (1000×, in H_2O)
Chloramphenicol, 34 mg/ml (1000×, in ethanol)
Isopropyl-β-D-thiogalactopyranoside (IPTG, 1 *M*; Sigma) (1000× in H_2O)
Imidazole (pH 8.0) stock: 1 *M*
Nickel buffer (5×): 100 m*M* Tris (pH 8.0), 2 *M* NaCl. Imidazole and AEBSF are added before use
Nickel binding buffer: 20 m*M* Tris (pH 8.0), 400 m*M* NaCl, 0.01% (v/v) Triton X-100, 10 m*M* imidazole, 1 m*M* AEBSF
Buffer Q (10×): 200 m*M* Tris (pH 8.0), 5 m*M* EDTA, 50 m*M* $MgCl_2$
NaCl stock: 5 *M*

Growth of Bacterial Cells Containing Hsp104 Proteins

A 4-ml overnight culture of *E. coli* cells is used to inoculate 1 liter of LB medium containing kanamycin (50 μg/ml) and chloramphenicol (34 μg/

[27] F. W. Studier, A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorf, *Methods Enzymol.* **185,** 60 (1990).
[28] E. C. Schirmer, J. R. Glover, and S. Lindquist, unpublished observations (1995).
[29] F. W. Studier, *J. Mol. Biol.* **219,** 37 (1991).
[30] J. Clos and S. Brandau, *Protein Expression Purif.* **5,** 133 (1994).

ml). Cells are grown to an A_{595} of <0.4 (1-cm light path). Typically, this requires ~3 hr. Expression of Hsp104 protein is then induced by the addition of IPTG to a final concentration of 1 m*M*. Incubation is continued for 1 hr and cells are collected by centrifugation (in a cooled rotor as described above for yeast). Cells must be processed immediately because Hsp104 activity is lost when *E. coli* cells are frozen.

Cell Lysis

Cells are resuspended as a thick slurry in a minimal volume of cold nickel binding buffer and transferred to siliconized microcentrifuge tubes. (Hsp104 binds to the walls of polypropylene tubes.)[25] Cells are sonicated in a Branson sonicator (Branson Ultrasonic Corp., Danbury, CT) with a microtip adaptor using 3 cycles of 40 pulses each at a 90% duty cycle with cooling for several minutes on ice between cycles. Before applying to the nickel matrix, lysates are cleared of cellular debris by centrifugation (14,000 rpm for 20 min at 4°).

Column Chromatography

1. *Nickel–NTA:* Lysates containing 6*x*-histidine tagged proteins are applied onto a Ni^{2+}–NTA–agarose (nickel–nitrilotriacetic acid–agarose; Qiagen, Chatsworth, CA) column preequilibrated in nickel binding buffer. For every liter of cells ~0.75 ml of packed matrix is used. After washing with 20 column volumes of nickel binding buffer, proteins are eluted with the same buffer containing 220 m*M* imidazole. For every liter of cells, roughly 10 mg of Hsp104 is obtained from the nickel matrix purification step.

2. *Anion-exchange medium:* Several different anion-exchange media can be used for the second step in purification: POROS HQ columns can be resolved using FPLC, HPLC, or perfusion chromatography systems; Resource-Q columns can be resolved using a peristaltic pump or FPLC; and DEAE columns can be resolved with gravity flow or a peristaltic pump.

POROS HQ: Protein eluted from the nickel column is dialyzed against buffer Q. Before injection, protein is filtered through a low protein binding Millex-GV 0.22-μm pore size filter unit (Millipore, Bedford, MA). The POROS 20 HQ column (PerSeptive Biosystems, Framingham, MA) is first equilibrated with 20 column volumes of buffer *Q*. The sample is applied and the column is washed with 20-column volume of 60 m*M* NaCl in buffer Q. Hsp104 is eluted from the column using a 20-column volume linear gradient from 60 to 660 m*M* NaCl in buffer Q. Hsp104 elutes from the matrix at ~300 m*M* NaCl.[31]

[31] M. Ramakrishnan, D. Hattendorf, J. Glover, E. C. Schirmer, and S. Lindquist, unpublished observations (1997).

Resource Q: A Resource-Q (Pharmacia) FPLC column functions similarly to the POROS column, but using a gradient from 50 to 900 m*M*.

DEAE: Protein eluted from the nickel column is dialyzed against buffer A and applied onto a 5-ml DEAE column (Pharmacia) preequilibrated with buffer A. Hsp104 is eluted with a 50–300 m*M* KCl gradient in buffer A (as for the fourth column in the yeast purification protocol).

If the eluant from the nickel column is less clean than that shown in Figure 1, then a third column step may be necessary. Note: Some lower molecular weight contaminants may not be observed by SDS–PAGE unless acrylamide concentrations ≥12% are used. To remove these contaminants, the eluant from the anion exchange column is diluted in buffer Q and applied to a POROS-Heparin column. The column is washed with 10-column volumes of buffer Q and Hsp104 is eluted with a 0–450 m*M* NaCl gradient in buffer Q (Hsp104 elutes at ~300 m*M* salt).[31]

DEAE requires less specialized equipment, but Resource-Q and POROS yield similar results and provide better purification and recoveries than DEAE. Owing to the loss of material in separating contaminants and Hsp104 degradation products, the final yields of purified full-length Hsp104 from 1 liter of starting culture are typically only 1–4 mg.

The removal of the 6*x*-histidine extension using thrombin cleavage requires low temperatures and high salt concentrations to limit the activity of the enzyme because, under optimal conditions, it also cleaves Hsp104.

Storage of Purified Hsp104 Protein

Concentration of Hsp104 Protein

Hsp104 binds to many types of concentrating media such as Amicon Centricon and Centriprep concentrators, but Ultrafree-15 centrifugal filter devices with a molecular weight cutoff of 30,000 (Millipore) exhibit low binding and Hsp104 can be concentrated to >20 mg/ml in these devices without precipitating from solution. Hsp104 can also be concentrated by precipitation with ammonium sulfate without loss of activity as described earlier for the yeast protein purification following the hydroxyapatite column step.

Short-Term Storage of Purified Hsp104

Hsp104 protein purified from yeast retains full ATPase activity when stored on ice in buffer A with 10% (v/v) glycerol for 1 month and loses only 50% activity after 9 months. Little if any degradation is observed over this time. However, if stored in ATPase assay buffer (see below), a

physiological buffer lacking glycerol, wild-type Hsp104 loses ~50% of its ATPase activity within several weeks and mutant variants of Hsp104 typically lose an even greater percentage of activity.[12]

Long-Term Storage of Purified Hsp104

Hsp104 purified from yeast and flash-frozen in liquid nitrogen can be stored indefinitely at −80° in buffer A containing 10% (v/v) glycerol with little or no loss of activity. Hsp104 retains ATPase activity when concentrated by lyophilization.[25] If shipped on dry ice, tubes should be sealed with Parafilm to prevent the introduction of CO_2, which precipitates Hsp104. Repeated freeze–thaw cycles reduce the activity of Hsp104 as previously noted for the related HSP100 protein, ClpA.[24]

Assays to Test Activity of Purified Hsp104

ATPase Assays

Hsp104 hydrolyzes ATP under a variety of buffer and pH conditions. The ATPase activity reaches a maximum at pH 6.5, drops to half this level at pH 7.5, and increases again to an intermediate level at pH 9.[10] Activity is generally tested in 20 m*M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES, pH 7.5), 140 m*M* KCl, 15 m*M* NaCl, 10 m*M* $MgCl_2$, and ATP, pH 7.5. The assay should be performed in siliconized Eppendorf tubes, or in the presence of 0.02% (v/v) Triton X-100 to prevent Hsp104 binding to the walls of the tubes.[25] A reaction mix is made from 10× stocks of the individual buffer components and Hsp104. This is aliquoted to tubes on ice and peptides and other varied components are added. A typical assay volume is 25 μl and contains 1 μg of Hsp104. The tubes are preincubated at 37° for 1 min and ATP is added to start the reaction. The reaction is incubated at 37° for 7 min and then terminated by addition of 800 μl of Malachite Green reagent [0.034% (w/v) Malachite Green (Sigma), 1.05% (w/v) ammonium molybdate, 1 *M* HCl, filtered to remove insoluble material].[32] After 1 min at room temperature, color development is stopped by addition of 100 μl of 34% (w/v) citric acid. Two hundred microliters of the sample is removed to 96-well assay plates (Costar, Cambridge, MA) and the A_{650} determined with a Molecular Devices (Palo Alto, CA) V_{max} kinetic microplate reader with SoftMax software. Values are calibrated against KH_2PO_4 standards and corrected for phosphate released in the absence of Hsp104. Hsp104 cleaves ATP at a rate of ~2 nmol min^{-1} μg^{-1}.[10]

[32] T. P. Geladopoulos, T. G. Sotiroudis, and A. E. Evangelopuolos, *Anal. Biochem.* **192,** 112 (1991).

Peptide-stimulated ATPase activity is assayed by replacing 1/10 of the reaction volume H_2O with either peptide at 2 mg/ml or a control of the buffer in which the peptide is dissolved. At a final concentration of 200 μg/ml, poly (L-lysine) (M_r 33,000 Sigma) should increase the ATPase activity of Hsp104 roughly sevenfold, and the oxidized chain B of insulin (Sigma) should increase the ATPase activity of Hsp104 roughly 50%.[12]

Oligomerization

Hsp104 is dialyzed against 20 m*M* HEPES (pH 7.5), 2 m*M* EDTA, 200 m*M* KCl, 10 m*M* $MgCl_2$, 2 m*M* dithiothreitol (DTT). Protein is diluted to a final concentration of 0.0425 mg/ml and incubated with 1–5 m*M* nucleotides or appropriate controls on ice for 10 min. Precipitated material is removed from reactions by centrifugation (14,000 rpm for 10 min at 4°) and the supernatant is aliquoted into tubes for each time point (100 μl/tube). Cross-linking is initiated by the addition of 4 μl of a 2.6% (v/v) glutaraldehyde stock (freshly prepared from an EM-grade stock 50% solution; Electron Microscopy Sciences, Ft. Washington, PA), and is allowed to proceed for 2 min to 1 hr. The reaction is quenched by the addition of 100 μl of 1 *M* glycine. Samples are then placed on ice, 75 μg of a carrier peptide is added (insulin chain B, oxidized; Sigma), and cross-linked proteins are precipitated with trichloroacetic acid (TCA; final concentration, 10%, v/v). No difference in precipitated protein is observed whether stopped reactions are incubated on ice for 1 or 12 hr. Samples are centrifuged

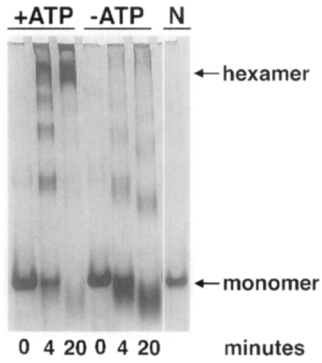

FIG. 2. Example of cross-linking gel to assess Hsp104 oligomerization. Hsp104 was mixed with nucleotide or buffer and cross-linked with glutaraldehyde as described in text. In the presence of ATP, most of the Hsp104 assembles into oligomers over time. In the absence of ATP, although some oligomers are observed, most of the Hsp104 remains monomeric. N indicates the migration of Hsp104 that was not incubated with glutaraldehyde. The expected migration Hsp104 monomers and hexamers is indicated on the basis of the migration of cross-linked phosphorylase *b* standards on the same gel.

(14,000 rpm for 30 min at 4°) and washed several times with 100% ethanol. The pellets are dried and suspended in sample buffer as described in Sigma Technical Bulletin MWS-877X (according to the method of Weber and Osborn[33]) and analyzed by 3.5% (w/v) SDS–PAGE, using Bio-Rad minigels, which require 3.5 hr to resolve samples (Fig. 2). Cross-linked phosphorylase *b* standards (Sigma) are resolved on the same gels to estimate molecular weights. Gels are stained with silver using the procedure of Morrissey.[34]

Acknowledgments

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[35] SecB: A Chaperone from *Escherichia coli*

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SecB is a molecular chaperone in *Escherichia coli* that is dedicated to the facilitation of the export of a number of proteins destined for the periplasmic space or the outer membrane. This role in export is demonstrated *in vivo* by the accumulation of pulse-labeled precursor species in a strain that is devoid of SecB[1] and *in vitro* by showing that SecB is required for translocation of precursors into inverted vesicles of cytoplasmic membrane.[2] Like all proteins classified as molecular chaperones, SecB has the ability to bind selectively and with high affinity to polypeptides that are in a nonnative state. SecB binds precursor polypeptides and maintains them in a state competent for translocation through the cytoplasmic membrane. Translocation cannot occur if the polypeptide is either folded or aggre-

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