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Reactivation of Aggregated Proteins by the ClpB/DnaK Bi-chaperone System

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Abstract

Protein aggregation is a common problem in protein biochemistry and is linked to many cellular pathologies and human diseases. The molecular chaperone ClpB can resolubilize and reactivate aggregated proteins. This unit describes the procedure for following reactivation of an aggregated enzyme glucose-6-phosphate dehydrogenase mediated by ClpB from *Escherichia coli* in cooperation with another molecular chaperone DnaK. The procedures for purification of these chaperones are also described.

Keywords

protein misfolding; protein aggregation; molecular chaperone; ClpB; DnaK

Introduction

Protein aggregation is a non-productive irreversible association of partially folded or misfolded polypeptides (Fink, 1998; Rousseau et al., 2006). Aggregation is a common nuisance *in vitro* in many protein production and purification procedures; *in vivo* it leads to severe cellular pathologies and is a culprit of a number of human diseases (Horwich, 2002; Valastyan and Lindquist, 2014). All forms of life employ proteins known as molecular chaperones to assist in *de novo* protein folding and to control the levels of misfolding and aggregation (Bukau et al., 2006; Hartl et al., 2011). Since protein misfolding and aggregation are often induced under stress conditions, the chaperone activity is associated with all heat-shock protein families (Hsp). The major Hsps, namely Hsp60 (bacterial GroEL), Hsp70 (bacterial DnaK), and small Hsps preferentially interact with partially folded proteins, assist in their folding, and consequently inhibit their aggregation. However, the function of these chaperones appears limited to prevention of aggregation and they cannot efficiently reactivate most preformed protein aggregates.

This unit is focused on the activity assay of ClpB, which cooperates with DnaK, its co-chaperone DnaJ, and the nucleotide-exchange factor GrpE in mediating the reactivation of

aggregated proteins (Goloubinoff et al., 1999; Motohashi et al., 1999; Zolkiewski, 1999). The energy input for protein disaggregation is obtained from the hydrolysis of ATP, which is catalyzed by ClpB and DnaK. ClpB belongs to the Hsp100 family of molecular chaperones, which is a subgroup of the AAA+ superfamily of ATPases associated with different activities (Neuwald et al., 1999). The Hsp100 chaperones are essential for stress survival of microorganisms and plants, but they do not have apparent orthologs in metazoan proteomes (Zolkiewski et al., 2012). Thus, it appears that a robust disaggregase activity of Hsp100 might be essential in those organisms that may encounter severe environmental changes during their lifetime, which cannot be avoided or neutralized by an adaptive behavior or migration. In all cases described so far, the Hsp100-mediated protein disaggregation is assisted by the co-chaperones from the Hsp70 family. The mechanism of Hsp100-mediated protein disaggregation has been extensively studied and has been described elsewhere (Desantis and Shorter, 2012; Doyle and Wickner, 2009; Saibil, 2013; Zolkiewski et al., 2012).

In this unit, we provide the description of methods for studying the reactivation of aggregated proteins in the presence of ClpB and the DnaK system from *Escherichia coli* (Basic Protocol 1). The Hsp100/Hsp70 bi-chaperone systems have been also studied in other organisms, notably yeast *Saccharomyces cerevisiae* (Bosl et al., 2006; Glover and Lindquist, 1998). The protocols described here for the *E. coli* chaperones can be readily applied to other Hsp100/70 systems with minor modifications and one important caveat: optimal reactivation of aggregated proteins requires the Hsp100 and Hsp70 partners from the same species (Miot et al., 2011). The apparent species-specificity of the bi-chaperone system is due to a direct interaction between Hsp100 and Hsp70 at the substrate-binding stage of the aggregate reactivation reaction (Rosenzweig et al., 2013). Support Protocols 1, 2 and 3 describe purification of ClpB, DnaK and GrpE, respectively, from *E. coli*.

Basic Protocol 1

Reactivation of aggregated glucose-6-phosphate dehydrogenase (G6PDH) in the presence of ClpB and DnaK/DnaJ/GrpE

This protocol outlines the procedure for detecting the aggregate-reactivation activity of ClpB in cooperation with the DnaK chaperone system. We use bacterial G6PDH as an example of a protein that becomes aggregation-prone upon chemically induced unfolding followed by a refolding attempt. This protocol is based on previously published work (Barnett et al., 2005; Diamant et al., 2000).

In the first step, the aggregates of G6PDH are produced. The G6PDH enzymatic activity is lost during aggregation. In the second step, the recovery of G6PDH activity is observed in the presence of ClpB with DnaK/DnaJ/GrpE in the ATP-containing buffer. In the following procedures, protein concentration is given in monomer units.

Materials—

Purified ClpB (see Support Protocol 1)

Purified DnaK (see Support Protocol 2; can be purchased from Enzo Life Sciences)

Purified DnaJ (see (Zylicz et al., 1989; Zylicz et al., 1985); can be purchased from Enzo Life Sciences)

Purified GrpE (see Support Protocol 3; can be purchased from Enzo Life Sciences)

Lyophilized Glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (Sigma-Aldrich).

ATP (Sigma), stock 0.2 M solution neutralized to pH 7, stored frozen

Buffer components (see below)

Equipment—

Thermostat or water bath

Microcentrifuge tubes

Ice bucket

Table-top centrifuge

Spectrophotometer (UV/Vis)

Preparation of aggregated G6PDH—

1. Mix 5 μl of 600 μM G6PDH in 50 mM Tris/HCl pH 7.5 with 5 μl of denaturation buffer. Incubate at 47 °C for 5 min. G6PDH becomes fully unfolded during this step.
2. Add 90 μl of refolding buffer, mix vigorously, and continue incubation at 47°C. As the urea concentration drops 10-fold, the conditions become favorable for refolding of G6PDH. However, a spontaneous refolding of G6PDH at 47°C is not efficient and it aggregates instead.
3. Arrest the aggregation of G6PDH by cooling the sample on ice for 2 min. Different aggregate sizes can be produced with different incubation time in the refolding buffer at 47°C (Barnett et al., 2005). For example, 15-min incubation produces large aggregates of G6PDH (M_w>1 MDa). The aggregate sample should be kept on ice, as the protein aggregation will continue, albeit at a slower rate. The sample should be mixed with a vortex shaker before further use. The final concentration of G6PDH is 30 μM.

Reactivation of aggregated G6PDH—

4. Dilute an aliquot from the sample of aggregated G6PDH 8-fold into the reactivation buffer. Incubate at 30 °C for up to 120 min. Aliquots can be withdrawn during the incubation to determine the progress of the aggregate reactivation. Aggregated G6PDH diluted into the reactivation buffer without the chaperones or without ATP can be used as a negative control.
5. Collect an aliquot from the reactivation reaction and dilute 1,000-fold into the G6PDH assay buffer. Incubate at 30 °C for 10 min. Balance the

spectrophotometer using the G6PDH assay buffer as the blank. Determine the absorption at 340 nm, which measures the amount of NADPH produced during oxidation of glucose-6-phospate catalyzed by G6PDH.

Support Protocol 1

Purification of *E. coli* ClpB

The transcript of wt *E. coli* ClpB contains an internal translation-initiation site and is expressed *in vivo* as two polypeptides: a full-length 95-kDa ClpB95 and a truncated 80-kDa ClpB80, which does not include the N-terminal domain (Squires et al., 1991; Woo et al., 1992). The biological significance of production of both ClpB isoforms has been described elsewhere (Nagy et al., 2010). The following purification procedure is valid for either wt ClpB (a mixture of ClpB95 and ClpB80) or the separated isoforms, which can be produced from the mutated expression vectors (Barnett et al., 2000). It should be noted that the capability of ClpB80 to reactivate large protein aggregates is lower than that of ClpB95 (Barnett et al., 2005).

Materials—

Expression plasmid for ClpB with IPTG-dependent promoter (as described in (Barnett et al., 2000))

Competent *E. coli* cells BL21(DE3) (available from Life Technologies and other vendors)

LB broth culture media

LB-agar plates

Ampicillin (or another antibiotic, depending on the expression plasmid)

Isopropyl β-D-1-thiogalactopyranoside (IPTG)

Glycerol

Polyethyleneimine

Q Sepharose Fast Flow (GE Healthcare Life Sciences)

Ammonium sulfate

Superdex 200 prep grade (GE Healthcare Life Sciences)

SDS sample buffer

SDS-PAGE running buffer

SDS-PAGE molecular weight standards

Coomassie blue gel staining solution

Gel destaining solution

Buffer components (see below)

Dialysis tubing

Concentrator (optional)

Equipment—

Autoclave

Shaker-incubator

SDS-PAGE equipment

Low-pressure chromatography system with a gradient maker

Centrifuges

Transformation of E. coli—

1. Transform *E. coli* BL21(DE3) with the expression plasmid using the protocol supplied with the competent cells. The protocol involves heat-shock of the bacterial sample at 42°C after adding plasmid DNA. Use LB-agar plates with an appropriate antibiotic selection. For the pET-20b plasmid described in (Barnett et al., 2000), use 0.1 mg/ml ampicillin.

Colony selection and testing of protein expression—

2. Pick up to 10 single *E. coli* colonies after transformation and grow overnight in 1 ml LB with 0.1 mg/ml ampicillin at 37 °C with shaking 225 rpm. Dilute each culture 20-fold into two tubes with LB + ampicillin and incubate for 1 hr at 37 °C with shaking. Induce protein expression by adding 0.4 mM IPTG to one of the tubes for each colony culture. The other tube will be a negative control for the expression induction. Continue incubation for 2 hr. Collect aliquots from each culture, mix with SDS-sample buffer, and boil for 5 min. Run SDS-PAGE, stain the gel with Coomassie blue dye and destain. Observe the presence of a protein band close to 100 kDa molecular weight standard. The band corresponds to ClpB95 and should be stronger in the induced samples, as compared to the non-induced controls. Select bacterial strains producing high amounts of ClpB95. Produce glycerol stocks by mixing the selected cultures 1:1 with 80% sterile glycerol. Store the bacterial stocks at −80°C.

Protein expression—

3. Inoculate 50 ml LB + ampicillin with bacterial from a frozen stock. Grow the culture overnight at 37°C with shaking 225 rpm. On the following morning, dilute the culture into 1 liter LB + ampicillin (preheated to 37°C). Incubate the culture for 1 hr at 37°C with shaking 225 rpm. Collect an aliquot before induction as the control sample for SDS-PAGE analysis. Induce protein expression with 0.4 mM IPTG. Grow the culture for 2 hr. Collect and aliquot for SDS-PAGE analysis.

Cell collection and lysis—The following steps should be performed in the cold-room at 4 °C or on ice.

4. Collect the bacterial cells by centrifugation at 7,000g for 20 min at 4°C using a pre-chilled rotor. Discard the supernatant. Weigh the centrifuge bottles to estimate the mass of the collected bacteria. Bacterial pellets may be frozen at −20°C if needed. Using a glass spatula suspend the pellets in Buffer A (10 ml of buffer per gram of bacterial cells). The suspension will be quite viscous and cloudy. Disrupt cells by sonication. Sonicate using 15-sec pulses with 15-sec intervals to avoid heating the sample. The sample should gradually loose its viscosity. Centrifuge the sample at 20,000g for 45 min. Save the supernatant.

Nucleic acid precipitation—

5. Slowly add polyethyleneimine to the final concentration of 0.04% with gentle stirring using a glass spatula. The solution will turn cloudy. Centrifuge the sample at 20,000g for 60 min. Save the supernatant. Collect an aliquot for SDS-PAGE analysis.

Ion-exchange chromatography—

6. Measure the volume of the solution from the previous step and add solid KCl to 0.1 M. Equilibrate a column packed with Q Sepharose anion-exchange resin in Buffer B. Load the sample onto the column and wash with Buffer B. Elute the proteins with a salt gradient 0.1–0.4 M KCl. Collect elution fractions and analyze them with SDS-PAGE. Pool the fractions containing the highest amount of ClpB95.

Ammonium sulfate precipitation—

7. Measure the volume of the sample. Add solid ammonium sulfate to 60% saturation at 0°C while stirring slowly (APPENDIX 3F). The solution should turn cloudy. Centrifuge the sample at 14,000g for 20 min. Discard the supernatant. Dissolve the pellet in 2 ml of Buffer B.

Gel-filtration chromatography—

8. Equilibrate a column packed with Superdex 200 in Buffer B. Load the sample onto the column. Depending on the column volume, the sample may have to be split into portions. Do not load more than 5% of the column volume for a single run. Elute the proteins with Buffer B and collect fractions. Analyze the fractions with SDS-PAGE. Pool the fractions containing ClpB of the highest purity.

Final protein concentration, dialysis, and storage—

9. Precipitate the protein with ammonium sulfate (see above) or use a concentrator to bring the sample volume to ~2 ml. Dialyze extensively in Buffer C with gentle stirring. Save the final dialysate buffer as a spectrophotometric reference. Centrifuge the dialyzed sample at 14,000g for 20 min to remove any precipitated protein. Determine the protein concentration by measuring absorption at 280 nm. The absorption coefficient of ClpB is 0.38 cm^2/mg. Split the sample into 0.1–0.2 ml aliquots and store at −20°C. Avoid storage at −80°C. The sample

with 10% glycerol will not be deep-frozen at −20°C, which preserves the activity of ClpB. ClpB can be stored at −20°C for several years without a significant loss of activity.

Support Protocol 2

Purification of *E. coli* DnaK

We followed the published method (Cegielska and Georgopoulos, 1989) with some changes. All steps (except MONO Q) are carried out between 0–4 °C.

Materials—

Expression vector

E. coli competent cells

LB-agar (see recipe below)

ATP

ammonium sulfate ($AmSO_4$)

antibiotics

Coomassie Brilliant Blue G

dimethylsulfoxide (DMSO)

ethylenediaminetetraacetic acid (EDTA)

EDTA-free protease inhibitor tablet (Roche, cat. no. 11 836 170001)

Glycerol

HEPES buffer

Isopropyl β-D-1-thiogalactopyranoside (IPTG)

magnesium chloride ($MgCl_2$)

2-mercaptoethanol (2-ME)

potassium chloride (KCl)

protease inhibitor PMSF

sodium dodecylsulphate (SDS)

sucrose

TRIS base

TRIS HCl

ATP-agarose (Sigma, cat. no. A2767)

Dialysis tubing, 10 kDa cut-off

Falcon tubes

Petri dishes

French Press (AMINCO, Silver Spring, Maryland)

MONO Q GL column (GE Healthcare Life Sciences, cat. no. 17-5166-01)

Gel electrophoresis apparatus

Bovine gamma globulin as a standard

Pre-cast 10% polyacrylamide gels

High speed, refrigerated centrifuge

UV-visible spectrometer

Protein assay kit (Bio-Rad)

Transform E. coli—

1. Transform pMSK (Amp^r) plasmid bearing *dnaK* gene under an IPTG promoter into *E.coli* BB1553 competent cells (Δ*dnaK52*::Cm^r derivative of MC4100 with *sidB1* secondary mutation) (Dean and James, 1991; Montgomery et al., 1999). Use a basic protocol for bacterial transformation and heat shock for 90 sec at 42°C. Spread bacteria on LB agar plate supplemented with ampicillin and chloramphenicol to 100 μg/ml and 50 μg/ml, respectively, for overnight growth.

Select colonies and test protein expression—

2. On the next day, pick up several single colonies and make 5-ml cultures of individual colonies in LB media supplemented with ampicillin and chloramphenicol at final concentration 100 μg/ml and 50 μg/ml, respectively.
3. Grow bacteria at 30°C for a few hours, until optical density (OD) at λ=600 nm is at least 0.6.
4. Take two 1-ml aliquots from each sample and store the rest at 4°C. Return 1-ml cultures to 30°C.
5. Add IPTG to final concentration 0.4 mM to one of two 1-ml aliquots and grow cells for another 4 hr.
6. Centrifuge cells, remove supernatant and dissolve pellet in 50 μl of SDS polyacrylamide gel electrophoresis (PAGE) loading buffer (in reducing conditions).
7. Run PAGE (induced samples vs. un-induced) to make sure that production of DnaK has been induced. Use 10% or 12% gel. Stain gel with 0.01% Coomassie blue.

   DnaK runs at ~70 kDa. Consider the possibility that synthesis of other chaperones maybe induced, as well.

8. Choose the best colony to prepare a glycerol stock: mix 500 µl of un-induced culture with 500 µl of sterile 50% glycerol, mix well and store at −80°C. (Fig. 28.10.1A).

Starter culture and glycerol stock—

9. Use the rest of 5 ml culture, or inoculate frozen cells to make a starter culture.

Culture for protein purification—

10. Use 3 ml culture to inoculate 1 liter culture, and grow until OD_{600} equals 0.6. Add IPTG to final concentration 0.4 mM and incubate another 4–6 hr. Avoid raising the temperature above 30°C.

Harvest bacteria—

11. Harvest cells by centrifuging 20 min at 6,000 rpm (rotor JA 14 for Beckman centrifuge JA-21). Wash the pellet with Buffer S. The pellet can be stored at −20°C for a few weeks.

Lyse cells with a French Press—

12. Resuspend the cell pellet in the Buffer S (3 ml/g cells). Add two volumes of French Press Lysis Buffer. Add (every 30 min) freshly prepared PMSF to final concentration 100 µM.

13. Use 1,200 psi of pressure. Collect lysate in an ice-cold tube.

    A French Press will disrupt bacterial cells without damaging DnaK.

14. Transfer to Sorvall tubes, and centrifuge in a Sorvall SS-34 rotor at 17,000 rpm for 40 min. Save supernatant, discard pellet, measure volume of the supernatant.

Ammonium sulfate ($AmSO_4$) precipitation—

15. Determine the volume (ml) of ice cold saturated $AmSO_4$ solution to add for 65% saturation using the following equation: Vo x 1.86, where Vo is the volume of supernatant.

16. Slowly add the $AmSO_4$ while stirring the solution (0°C). Incubate for 15 min more with stirring.

17. Transfer to Sorvall tubes, balance tubes, and spin (Sorvall SS-34 rotor; 17,000 rpm) for 90 min. Resuspend pellets with 5 ml Buffer A/pellet.

18. Combine solubilized pellets in one tube; keep on ice.

Dialysis—

19. Use 10 kDa cutoff tubing. Wet tubing with distilled water for 10 min prior to dialysis. Dialyze against Buffer A (three changes of 4 liters each).

ATP-Agarose Affinity chromatography—

20. Place 5 ml of ATP-agarose slurry into a 50-ml Falcon tube. Add 20 ml buffer A to the Falcon tube, then place the tube on ice.

21. Pour the suspension into a 1 × 7 cm column with the stopcock open until the agarose settles. Leave 1–2 ml of liquid over the top of the resin.

22. Add 5 ml of protein supernatant to the top of the ATP column, let flow through at 0.5 ml/min. (Collect flow through.)

23. Wash column with 10 column volumes of low-salt Buffer A.

24. Elute with 6 mM ATP-$MgCl_2$. Collect 10 1-ml fractions. Measure OD (λ=295 nm) of each fraction.

    Several of the fractions will have high OD, whereas the others will have low OD.

25. Repeat steps 22–24 until all of the solution is passed over the column.

26. Pool the fractions with high OD. Keep on ice.

Precipitate DnaK using ammonium sulfate—

27. Determine the volume (ml) of ice-cold saturated ammonium sulfate ($AmSO_4$) to add for 70% saturation using this equation: Vo x 2.33, where Vo is the volume of supernatant. Slowly add the $AmSO_4$ while stirring the solution of protein. Incubate for 15 min more with stirring.

28. Transfer to Sorvall tubes, balance tubes, and centrifuge for 90 min at 17,000 rpms (Sorvall SS-34 rotor). Resuspend pellets with 5 ml MONO Q (low salt) buffer/pellet. Combine solubilized pellets in one tube; keep on ice. Dialyze the resuspended pellets overnight against 3 liters of low-salt MONO Q buffer.

MONO-Q (anion exchange chromatography)—

29. Inject 5 ml of DnaK solution over the MONO Q column that is connected to a high performance liquid chromatography system or a fast protein liquid chromatography system. Wash the column with 20 ml of low-salt MONO Q buffer.

30. Elute with a 50–500 mM KCl gradient at room temperature. Measure OD at λ=254 nm.

31. Assay fractions by SDS-PAGE to determine which fractions contain DnaK. Pool fractions containing DnaK and dialyze for a total of 48–72 hr against four changes of 4 liters Buffer B.

    DnaK, purified by this method, appears as a single band on a Coomassie Blue-stained SDS PA gel (Fig.1B).

32. Determine protein concentration using a Bio-Rad protein assay kit, with bovine gamma globulin as a standard.

Final dialysis, OD_{280}/OD_{260} ratio, storage—

34. Dialyze protein into Buffer B until OD_{280}/OD_{260} ratio ≥1.6.

35. For kinetic experiments, keep purified DnaK on ice. Add fresh ice daily, and use within a week or two. Otherwise, add glycerol to 10% (v/v), freeze, and store up to 6 months −80°C.

Support Protocol 3

Purification of *E. coli* GrpE

The following procedure is based on the published method (Zylicz et al., 1987) with some changes. All steps are carried out between 0°C and 4°C.

Materials—

Expression vector

E. coli competent cells

LB-agar (see recipe)

ATP

ammonium sulfate ($AmSO_4$)

antibiotics

Coomassie Brilliant Blue G

dimethylsulfoxide (DMSO)

ethylenediaminetetraacetic acid (EDTA)

EDTA-free protease inhibitor tablet

Glycerol

HEPES buffer

Bovine gamma globulin as a standard

L-arabinose

magnesium chloride ($MgCl_2$)

2-mercaptoethanol (2-ME)

potassium chloride (KCl)

phenylmethysulfonyl fluoride (PMSF)

sodium dodecylsulphate (SDS)

sucrose

TRIS base

TRIS-HCl

Affi-Gel 10 resin (Bio-Rad, cat. no. 153-6099)

Dialysis tubing, 10 kDa cut-off

Falcon tubes

Petri dishes

French Press (AMINCO, Silver Spring, Maryland)

MONO Q GL column (GE Healthcare Life Sciences, cat. no. 17-5166-01)

Gel electrophoresis apparatus

Pre-cast 10% or 12% polyacrylamide gels

High speed, refrigerated centrifuge

UV-visible spectrometer

Protein assay kit (Bio-Rad)

Transform E. coli—

1. Transform pWKG20 (Amp^r) plasmid bearing *grpE* gene under an arabinose P_{BAD} promoter (a generous gift from Dr. William L. Kelley (University of Geneva, Division of Infectious Diseases, Switzerland), into *E.coli* DA259 competent cells (C600 ΔgrpE::ΩCm^R thr::Tn10) (constructed by Georgopoulos and described in (Sugimoto et al., 2008)). Use a basic protocol for bacterial transformation and heat shock for 60 seconds at 42°C. Spread bacteria over LB agar plate supplemented with ampicillin and chloramphenicol to final concentrations 100 μg/ml and 50 μg/ml, respectively, for overnight growth.

Select colonies and test for protein expression—

2. On the next day, pick up several single colonies and make 5-ml cultures in LB media supplemented with ampicillin and chloramphenicol at final concentrations of 100 μg/ml and 50 μg/ml, respectively.
3. Grow bacteria at 37 °C for a few hours, until optical density (OD) at λ=600nm is at least 0.6.
4. Take two 1 ml aliquots from each sample and store the rest at 4 °C. Return 1ml cultures to 30 °C.
5. Add L-arabinose to final concentration 0.2% to one of two 1-ml aliquots and grow cells for another four hours.
6. Centrifuge cells, remove the supernatant and dissolve the pellet in 50 μl sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis (PAGE) loading buffer (in reducing conditions).
7. Run SDS-PAGE (induced samples vs. un-induced) to make sure DnaK was expressed. Use 10% or 12% gel. Stain gel with 0.01% Coomassie Brilliant Blue.

GrpE migrates at ~24 kDa. DnaK may also be induced (Fig. 2, panel A).

8. Choose the best colony to make your glycerol stock: mix 500 µl of un-induced culture with 500 µl of sterile 50% glycerol, mix, and store at −80 °C.

Prepare starter culture and glycerol stock—

9. Use the rest of the 5 ml culture, or inoculate frozen cells to make a starter culture.

Culture for protein purification—

10. Use 3 ml culture to inoculate 1 liter culture, and grow until OD_{600} equals 0.6. Add L-arabinose to final concentration 0.2% and incubate for another 4–6 hr. Growing bacteria at 30 °C increases the yield of protein.

Harvest bacteria—

11. Harvest cells by centrifuging at 6,000 rpm for 20 min (rotor JA 14 for Beckman centrifuge JA-21). Wash the pellet with Buffer S.

    The pellet can be stored at −20°C for a few weeks.

Lyse cells using a French Press—

12. Resuspend the cell pellet in the Buffer S (3 ml/g cells). Add two volumes of French Press Lysis Buffer. Add (every 30 min) freshly prepared PMSF to final concentration 100 µM.

13. Use 1,200 psi of pressure. Collect lysate in an ice-cold tube.

    A French Press provides quick and efficient disruption of the bacterial cells without damaging GrpE.

14. 14. Transfer to Sorvall tubes, and centrifuge in a Sorvall SS-34 rotor at 17,000 rpm for 40 min. Save supernatant, discard pellet, measure the volume of the supernatant.

Ammonium sulfate precipitation—

15. Slowly add solid ammonium sulfate to final concentration of 0.4 g/ml while stirring the solution on ice. Incubate for 15 min with stirring.

16. Transfer to Sorvall tubes, balance tubes, and spin (Sorvall SS-34 rotor; 17,000 rpms) for 90 min at 4°C.

17. Remove supernatant and resuspend pellet in a minimal volume of Buffer B (no KCl), discard supernatant.

Dialysis—

18. Use 10-kDa cutoff tubing. Wet tubing with distilled water for 10 min prior to dialysis. Dialyze against Buffer B with 50 mM KCl (three changes of 2 liters each, for at least 12 hr).

DnaK-Agarose Affinity chromatography

19. Conjugate purified DnaK to Affi-Gel 10 resin (Bio-Rad) according to the manufacturer's protocol.

20. Spin the dialyzed solution (from step 18) to remove precipitates. (Sorvall SS-34 rotor; 17,000 rpm for 10 min.)

21. Equilibrate a 20-ml DnaK affinity column with Buffer B. Load the clarified solution of GrpE to the column at a flow rate of 10 ml/hr. Wash the column with 5 column volumes (CV) of 50 mM KCl in Buffer B, two CVs of 0.5 M KCl in Buffer B, and two CVs of 2 M KCl in Buffer B.

22. Re-equilibrate the column with three CV of 50 mM KCl in Buffer B.

23. Elute GrpE with 10 mM ATP 50 mM KCl, 20 mM $MgCl_2$ in Buffer B (pH adjusted to 7.6). Continue eluting until $OD_{280} < 0.05$.

24. Pool the high OD fractions. Keep on ice.

MONO-Q (anion exchange chromatography)—

25. Dialyze the pooled fractions of GrpE overnight against two changes of 4 liters of low salt MONO Q buffer.

26. Inject 5 ml of GrpE solution over the MONO Q column (Pharmacia, HR 5/5) that is connected to an HPLC or FPLC system. Wash the column with 20 ml of low salt MONO Q buffer.

27. Elute with a 50–500 mM KCl gradient at room temperature. Measure OD at λ=254 nm.

28. Assay fractions by SDS-PA gel electrophoresis to determine which fractions contain GrpE. Pool fractions containing GrpE and dialyze against two changes of 4 L Buffer C.

29. GrpE, purified by this method, appears as a single band on a Coomassie Blue stained SDS PA gel (Fig. 28.10.2, panel B). Determine protein concentration using a Bio-Rad protein assay kit (Bio-Rad, catalog number 500-0006), with Bovine gamma globulin as a standard (Standard I, catalog number 500-0005).

Aliquot and store—

30. Add glycerol to final concentration 10% before freezing, make 1 ml aliquots and store up to 6 months at −80°C.

REAGENTS AND SOLUTIONS

Unless noted otherwise, the following buffers can be stored for 6 months at 4°C

Basic Protocol 1

Denaturation buffer—

10 M urea, 40 mM dithiothreitol (DTT), 15% glycerol.

Urea should be prepared fresh and filtered through a deionizing resin (for example, Bio-Rad AG 501-X8) to remove isocyanate.

Note: another common protein denaturant, guanidinium chloride inhibits the ATPase of *E. coli* ClpB (Zolkiewski, 1999) and other Hsp100 chaperones (Grimminger et al., 2004) and cannot replace urea in this procedure.

G6PDH assay buffer—

50 mM Tris/HCl pH 7.8, 5 mM $MgCl_2$, 2 mM glucose-6-phosphate (Sigma), 1 mM $NADP^+$ (Sigma).

Reactivation buffer—

Refolding buffer containing 5 mM ATP, 2 μM ClpB, 1 μM DnaK, 0.5 μM DnaJ, and 0.5 μM GrpE.

Refolding buffer—

50 mM triethanolamine/Cl pH 7.5

20 mM $Mg(OAc)_2$

30 mM KCl

1 mM β-mercaptoethanol

1 mM EDTA

Support Protocol 1

ClpB Buffer A—

50 mM Tris-HCl, pH 7.5

10% glycerol (v/v)

1 mM EDTA

1 mM DTT

ClpB Buffer B—

Buffer A with 0.1 M KCl

ClpB Buffer C—

50 mM Tris-HCl, pH 7.5

10% glycerol (v/v)

20 mM $MgCl_2$

0.2 M KCl

1 mM EDTA

1 mM DTT

Support Protocols 2 and 3

Buffer S—

50 mM Tris-HCl, pH= 8.0

10% sucrose (w/v)

DnaK Buffer A—

25 mM HEPES, pH = 8.0

10 mM KCl

1 mM EDTA

5 mM 2-mercaptoethanol (2-ME)

DnaK Buffer B—

25 mM HEPES, pH 7.0

50 mM KCl

5 mM $MgCl_2$

5 mM 2-ME

French Press Lysis Buffer—

0.3 M KCl

10 mM 2-mercaptoethanol (2-ME)

10 mM EDTA

1% streptomycin sulfate (to precipitate DNA and decrease viscosity)

1 tablet of EDTA-free protease inhibitor cocktail per 30 ml of the buffer

GrpE Buffer B—

25 mM TRIS-HCl, pH 7.6

1 mM EDTA

5 mM 2-ME

10% glycerol (v/v)

GrpE Buffer C—

25 mM HEPES, pH 7.5

50 mM KCl

1mM EDTA

5 mM 2-M

LB broth (Sambrook J, 1989)—

Recipe for 1 liter. Dissolve the following compounds in 1 liter of distilled or deionized water.

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g

For LB-agar, add 15 g of agarose before autoclaving.

Sterilize in an autoclave (20 min, 15 psi liquid cycle) For LB-agar, gently swirl the solution to distribute the agar; add antibiotics at 50°C. Pour into petri dishes.

MONO Q (low salt) buffer—

25 mM TRIS, pH = 7.8

50 mM KCl

1 mM EDTA

5 mM 2-ME

MONO Q (high salt) buffer—

25 mM TRIS, pH = 7.8

500 mM KCl

1 mM EDTA

5 mM 2-ME

PMSF stock solution—Dissolve 3–4 mg of PMSF in 1 ml DMSO. Prepare fresh solution each time.

COMMENTARY

Background Information

The protocol described in this unit has been used to detect the disaggregase activity of ClpB and to test the activity of a number of ClpB variants (Barnett et al., 2005; Nagy et al., 2010; Nagy et al., 2009; Zhang et al., 2012). The method is similar to those used by other authors (Diamant et al., 2000). Analogous protocols have been described for different aggregated protein substrates: firefly luciferase (Zolkiewski, 1999) and malate dehydrogenase (Nagy et

al., 2010). The protocol can be applied in biochemical tests of the Hsp100 and Hsp70 activities *in vitro*.

Although the ClpB-mediated and DnaK-assisted reversal of protein aggregation is robust and reproducible, the reaction has not been widely used to disaggregate proteins expressed in bacteria as inclusion bodies. It should be stressed that an efficient control of protein aggregation *in vivo* requires a balance between the amounts and activities of different chaperones. Indeed, overexpression of ClpB in *E. coli* increases the extent of aggregation of heterologous proteins (Kedzierska and Matuszewska, 2001), most likely due to a production of increased amounts of unfolded polypeptides, which overload the remaining chaperones. It has been shown that solubility of recombinant proteins can be enhanced to some extent by a coordinated overproduction in *E. coli* of four chaperone systems: ClpB, GroEL/GroES, DnaK/DnaJ/GrpE, and the small Hsps IbpA/IbpB (de Marco et al., 2007). The *in vitro* procedure described in this unit can be used to recover soluble protein from insoluble aggregates for the purpose of detecting enzymatic activity of the protein of interest. It should be stressed, however, that the procedure has not been scaled-up to produce large amounts of soluble proteins of interest.

Critical Parameters

Basic Protocol—The rate of protein aggregation strongly depends on multiple parameters including protein concentration, temperature, and the ionic strength of solution. Obviously, an aggregation-prone protein is not in a state of equilibrium and its biochemical properties depend on time. Thus, in order to obtain reproducible results in the chaperone-mediated aggregate reactivation assay, it is essential to control the experimental conditions during production of aggregates, including the time of protein refolding. Care should be taken to vortex the aggregated sample periodically to prevent precipitation and sedimentation of aggregated G6PDH to the bottom of the test tube, as this would make the activity determination inaccurate.

Support Protocol 2—DnaK is a weak ATPase. The ATPase activity of DnaK requires potassium (KCl). KCl must be in the buffer otherwise DnaK is inactive; therefore, do not substitute NaCl for KCl.

Troubleshooting

In case of low aggregate reactivation rates and/or yields, the ATPase activity of ClpB should be tested. The ClpB ATPase is a prerequisite for its chaperone activity (Barnett et al., 2000). In our studies, we used a colorimetric assay for determination of inorganic phosphate production from ATP (Zolkiewski, 1999). The assay is based on formation of phosphomolybdate complex and its reaction with malachite green dye, which produces a color change and an absorption increase at ~640 nm (Hess and Derr, 1975; Lanzetta et al., 1979). The ATPase activity of purified ClpB at 37°C should be around 0.5 nmol Pi produced by pmole of ClpB per hour.

Anticipated Results

The rate and yield of aggregate reactivation depends on the properties of the aggregates, primarily their size. For large aggregates of G6PDH prepared as described in this protocol, the enzymatic activity is undetectable in the aggregated sample. In the presence of ClpB and the DnaK system, the G6PDH activity recovers up to ~10% of the native control within 60 min of the reaction (Barnett et al., 2005; Diamant et al., 2000).

Time Considerations

Protein expression and purification are the most time-consuming procedures. It takes several days to purify each of the chaperones from *E. coli* frozen stock. Once the chaperones are produced, the aggregate reactivation procedure can be completed within a few hours.

Acknowledgments

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Literature Cited

Barnett ME, Nagy M, Kedzierska S, Zolkiewski M. The amino-terminal domain of ClpB supports binding to strongly aggregated proteins. J. Biol. Chem. 2005; 280:34940–34945. [PubMed: 16076845]

Barnett ME, Zolkiewska A, Zolkiewski M. Structure and activity of ClpB from Escherichia coli. Role of the amino-and -carboxyl-terminal domains. J. Biol. Chem. 2000; 275:37565–37571. [PubMed: 10982797]

Bosl B, Grimminger V, Walter S. The molecular chaperone Hsp104--a molecular machine for protein disaggregation. J. Struct. Biol. 2006; 156:139–148. [PubMed: 16563798]

Bukau B, Weissman J, Horwich A. Molecular chaperones and protein quality control. Cell. 2006; 125:443–451. [PubMed: 16678092]

Cegielska A, Georgopoulos C. Functional domains of the Escherichia coli dnaK heat shock protein as revealed by mutational analysis. J. Biol. Chem. 1989; 264:21122–21130. [PubMed: 2531744]

de Marco A, Deuerling E, Mogk A, Tomoyasu T, Bukau B. Chaperone-based procedure to increase yields of soluble recombinant proteins produced in E. coli. BMC Biotechnol. 2007; 7:32. [PubMed: 17565681]

Dean DO, James R. Identification of a gene, closely linked to dnaK, which is required for high-temperature growth of Escherichia coli. J. Gen. Microbiol. 1991; 137:1271–1277. [PubMed: 1655950]

Desantis ME, Shorter J. The elusive middle domain of Hsp104 and ClpB: location and function. Biochim. Biophys. Acta. 2012; 1823:29–39. [PubMed: 21843558]

Diamant S, Ben-Zvi AP, Bukau B, Goloubinoff P. Size-dependent disaggregation of stable protein aggregates by the DnaK chaperone machinery. J. Biol. Chem. 2000; 275:21107–21113. [PubMed: 10801805]

Doyle SM, Wickner S. Hsp104 and ClpB: protein disaggregating machines. Trends Biochem. Sci. 2009; 34:40–48. [PubMed: 19008106]

Fink AL. Protein aggregation: folding aggregates, inclusion bodies and amyloid. Fold. Des. 1998; 3:R9–R23. [PubMed: 9502314]

Glover JR, Lindquist S. Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins. Cell. 1998; 94:73–82. [PubMed: 9674429] The first in vitro reconstitution of the protein disaggregating system of yeast chaperones.

Goloubinoff P, Mogk A, Zvi AP, Tomoyasu T, Bukau B. Sequential mechanism of solubilization and refolding of stable protein aggregates by a bichaperone network. Proc. Natl. Acad. Sci. U. S. A.

1999; 96:13732–13737. [PubMed: 10570141] The first description of the bacterial protein disaggregating system of chaperones.

Grimminger V, Richter K, Imhof A, Buchner J, Walter S. The prion curing agent guanidinium chloride specifically inhibits ATP hydrolysis by Hsp104. J. Biol. Chem. 2004; 279:7378–7383. [PubMed: 14668331]

Hartl FU, Bracher A, Hayer-Hartl M. Molecular chaperones in protein folding and proteostasis. Nature. 2011; 475:324–332. [PubMed: 21776078]

Hess HH, Derr JE. Assay of inorganic and organic phosphorus in the 0.1–5 nanomole range. Anal. Biochem. 1975; 63:607–613. [PubMed: 1122033]

Horwich A. Protein aggregation in disease: a role for folding intermediates forming specific multimeric interactions. J. Clin. Invest. 2002; 110:1221–1232. [PubMed: 12417558]

Kedzierska S, Matuszewska E. The effect of co-overproduction of DnaK/DnaJ/GrpE and ClpB proteins on the removal of heat-aggregated proteins from Escherichia coli DeltaclpB mutant cells--new insight into the role of Hsp70 in a functional cooperation with Hsp100. FEMS Microbiol. Lett. 2001; 204:355–360. [PubMed: 11731148]

Lanzetta PA, Alvarez LJ, Reinach PS, Candia OA. An improved assay for nanomole amounts of inorganic phosphate. Anal. Biochem. 1979; 100:95–97. [PubMed: 161695]

Miot M, Reidy M, Doyle SM, Hoskins JR, Johnston DM, Genest O, Vitery MC, Masison DC, Wickner S. Species-specific collaboration of heat shock proteins (Hsp) 70 and 100 in thermotolerance and protein disaggregation. Proc. Natl. Acad. Sci. U. S. A. 2011; 108:6915–6920. [PubMed: 21474779]

Montgomery DL, Morimoto RI, Gierasch LM. Mutations in the substrate binding domain of the Escherichia coli 70 kDa molecular chaperone, DnaK, which alter substrate affinity or interdomain coupling. J. Mol. Biol. 1999; 286:915–932. [PubMed: 10024459]

Motohashi K, Watanabe Y, Yohda M, Yoshida M. Heat-inactivated proteins are rescued by the DnaK.J-GrpE set and ClpB chaperones. Proc. Natl. Acad. Sci. U. S. A. 1999; 96:7184–7189. [PubMed: 10377389] The first description of the bacterial protein disaggregating system of chaperones.

Nagy M, Guenther I, Akoyev V, Barnett ME, Zavodszky MI, Kedzierska-Mieszkowska S, Zolkiewski M. Synergistic cooperation between two ClpB isoforms in aggregate reactivation. J. Mol. Biol. 2010; 396:697–707. [PubMed: 19961856]

Nagy M, Wu HC, Liu Z, Kedzierska-Mieszkowska S, Zolkiewski M. Walker-A threonine couples nucleotide occupancy with the chaperone activity of the AAA+ ATPase ClpB. Protein Sci. 2009; 18:287–293. [PubMed: 19177562]

Neuwald AF, Aravind L, Spouge JL, Koonin EV. AAA+: A class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. Genome Res. 1999; 9:27–43. [PubMed: 9927482]

Rosenzweig R, Moradi S, Zarrine-Afsar A, Glover JR, Kay LE. Unraveling the mechanism of protein disaggregation through a ClpB-DnaK interaction. Science. 2013; 339:1080–1083. [PubMed: 23393091] Demonstration of a direct interaction between ClpB and DnaK.

Rousseau F, Schymkowitz J, Serrano L. Protein aggregation and amyloidosis: confusion of the kinds? Curr. Opin. Struct. Biol. 2006; 16:118–126. [PubMed: 16434184]

Saibil H. Chaperone machines for protein folding, unfolding and disaggregation. Nat. Rev. Mol. Cell Biol. 2013; 14:630–642. [PubMed: 24026055]

Sambrook J, FE.; Maniatis, T. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 1989.

Squires CL, Pedersen S, Ross BM, Squires C. ClpB is the Escherichia coli heat shock protein F84.1. J. Bacteriol. 1991; 173:4254–4262. [PubMed: 2066329]

Sugimoto S, Saruwatari K, Higashi C, Sonomoto K. The proper ratio of GrpE to DnaK is important for protein quality control by the DnaK-DnaJ-GrpE chaperone system and for cell division. Microbiology. 2008; 154:1876–1885. [PubMed: 18599817]

Valastyan JS, Lindquist S. Mechanisms of protein-folding diseases at a glance. Dis. Model. Mech. 2014; 7:9–14. [PubMed: 24396149]

Woo KM, Kim KI, Goldberg AL, Ha DB, Chung CH. The heat-shock protein ClpB in Escherichia coli is a protein-activated ATPase. J. Biol. Chem. 1992; 267:20429–20434. [PubMed: 1400361]

Zhang T, Ploetz EA, Nagy M, Doyle SM, Wickner S, Smith PE, Zolkiewski M. Flexible connection of the N-terminal domain in ClpB modulates substrate binding and the aggregate reactivation efficiency. Proteins. 2012; 80:2758–2768. [PubMed: 22890624]

Zolkiewski M. ClpB cooperates with DnaK, DnaJ, and GrpE in suppressing protein aggregation. A novel multi-chaperone system from Escherichia coli. J. Biol. Chem. 1999; 274:28083–28086. [PubMed: 10497158] The first description of the bacterial protein disaggregating system of chaperones.

Zolkiewski M, Zhang T, Nagy M. Aggregate reactivation mediated by the Hsp100 chaperones. Arch. Biochem. Biophys. 2012; 520:1–6. [PubMed: 22306514]

Zylicz M, Ang D, Georgopoulos C. The grpE protein of Escherichia coli. Purification and properties. J. Biol. Chem. 1987; 262:17437–17442. [PubMed: 2826421]

Zylicz M, Ang D, Liberek K, Georgopoulos C. Initiation of lambda DNA replication with purified host- and bacteriophage-encoded proteins: the role of the dnaK, dnaJ and grpE heat shock proteins. EMBO J. 1989; 8:1601–1608. [PubMed: 2527744]

Zylicz M, Yamamoto T, McKittrick N, Sell S, Georgopoulos C. Purification and properties of the dnaJ replication protein of Escherichia coli. J. Biol. Chem. 1985; 260:7591–7598. [PubMed: 3889001]

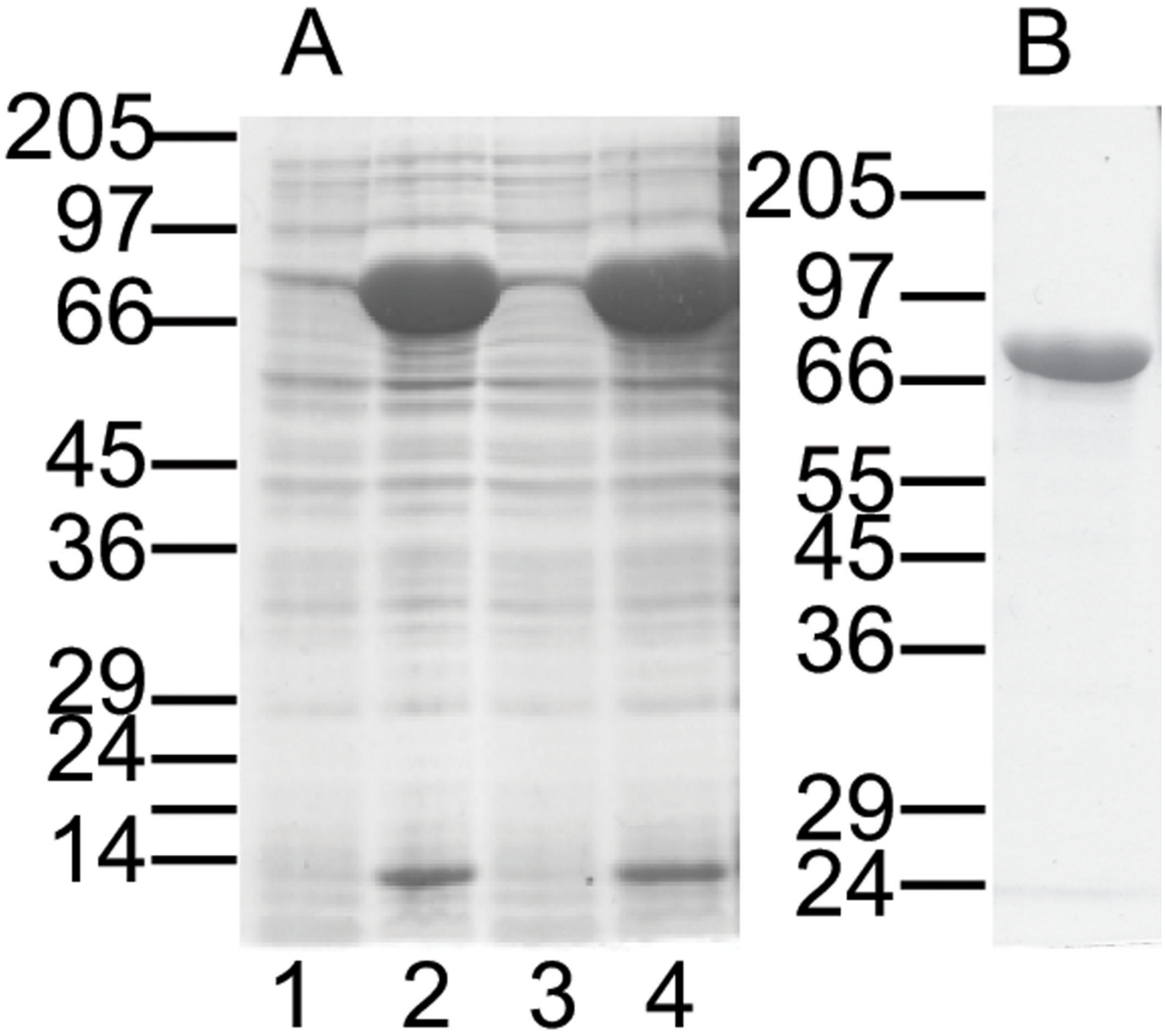

Figure 28.10.1. DnaK purification

SDS-PAGE analysis of DnaK expression and purification. 10% SDS polyacrylamide gel with reducing conditions. Panel A shows the protein profile of two colonies, without induction with IPTG (lanes 1 and 3) or induced with 0.4mM IPTG (lanes 2 and 4). Panel B shows DnaK purified according to the protocol described here.

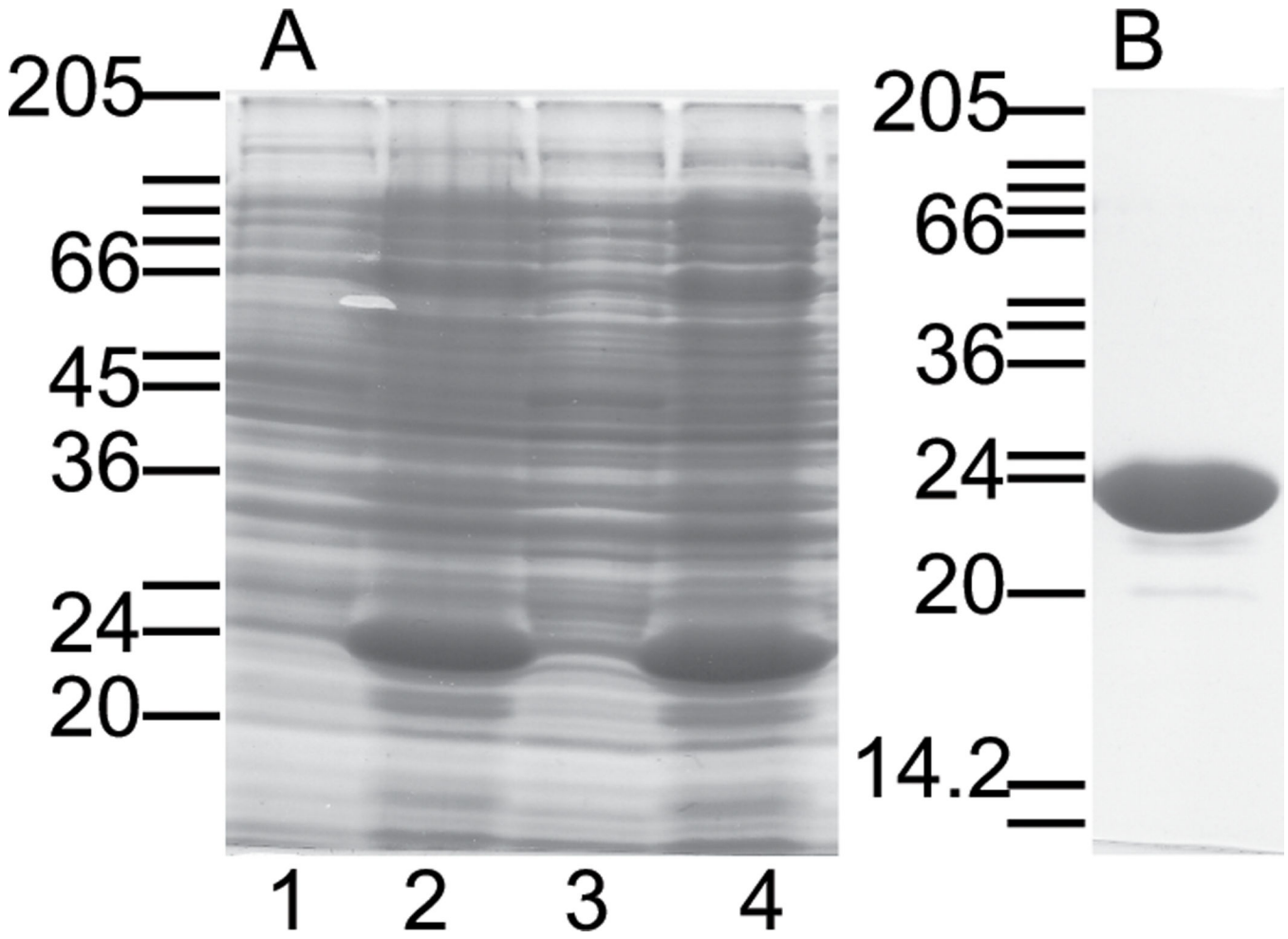

Figure 28.10.2. GrpE purification

SDS-PAGE analysis of GrpE expression and purification. 10% (A) or 12% (B) SDS polyacrylamide gel with reducing conditions. Panel A shows the protein profile of two colonies: no induction (lanes 1 and 3); induced with 0.2% of L-arabinose (lanes 2 and 4). Panel B shows GrpE purified according to the protocol described here.