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GroE-dependent expression and purification of pig heart mitochondrial citrate synthase in *Escherichia coli*

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Abstract

Citrate synthase (CS) is a dimeric, mitochondrial protein, composed of two identical subunits (M_r 48 969 each). The nuclear-encoded α-helical protein is imported into mitochondria post-translationally where it catalyses the first step of the citric cycle. Furthermore, the pathway of thermal unfolding as well as the folding pathway was studied extensively, making CS a well-suited substrate protein for studying chaperone function. In chaperone research the quality of the substrate proteins is essential to guaranty the reproducibility of the results. In this context, we here describe the GroE-enhanced recombinant expression and purification of CS. CS was expressed in *E. coli* by using an arabinose regulated T7 promotor. Under standard expression conditions only insoluble, inactive CS was detected. Interestingly, the expression of soluble and active CS was possible when GroEL/GroES was co-expressed. Furthermore, a shift to lower expression temperatures increased the amount of soluble, active CS. We describe for the first time, the purification of CS in soluble and active form by following a CiPP strategy (capture, intermediate purification, polishing). After the initial capturing step on DEAE-Sephacel the protein was further purified on a Q-Sepharose column. After these two steps of anion-exchange chromatography a final size-exclusion chromatography step on a Superdex 75-pg column yields CS with a purity over 99%. Using this expression and purification strategy 1 mg CS per g *E. coli* wet weight were purified.

Keywords: *Escherichia coli*; Citrate synthase; Enzymes

1. Introduction

Citrate synthase (CS) is a dimeric, mitochondrial protein, composed of two identical subunits (M_r 48 969 each). It catalyzes the first step of the citric acid cycle, the condensation of oxaloacetic acid and acetyl-CoA to citrate and coenzyme A [1]. The enzyme is a nuclear-encoded α-helical protein which is translated in the cytosol by free ribosomes and imported into the mitochondria post-translationally in an unfolded state. The three-dimensional structure was solved at 2.7 Å by X-ray crystallography [2,3], revealing a high α-helical content. Folding in vivo takes place in the mitochondrial matrix. At elevated temperatures, CS looses its activity very rapidly with a midpoint of the transition at 48 °C [4–7]. This inactivation is accompanied by structural changes in the molecule [6]. Addition of the substrates oxaloacetic acid and acetyl-CoA stabilizes the enzyme, shifting the midpoint of the thermal unfolding transition to 66.5 °C [5,6]. This stabilization is due to huge conformational changes induced by substrate binding

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[2,3,8]. During inactivation CS unfolds via a dimeric inactive and several monomeric intermediates [9–11]. The monomeric intermediates are highly prone to irreversible aggregation [9–11]

Because these folding and unfolding pathways of CS in vitro are very well analyzed, CS became one of the major substrate proteins to study chaperone functions [12,13]. One of the best characterized chaperones is GroEL/GroES from *E. coli* [14,15]. In vivo, the GroE system is essential for viability. It seems to be involved in the folding of 5–10% of the polypeptide chains to their native, three-dimensional structure [16]. Under stress conditions the GroE complex maintain viability of the cell by stabilizing unfolding proteins or by keeping unfolding intermediates in a reactivatable state and thereby preventing irreversible aggregation. GroEL is a tetradecameric molecule consisting of two heptameric rings of identical subunits stacked back to back [15].

Concerning the area of chaperone research highly purified and active CS is necessary, to ensure the reproducibility of inactivation and reactivation measurements [7]. Commercially available CS is usually purified from pig hearts. This CS normally is supplied in ammonium sulfate solution and therefore inactive state. Because of the inhibitory effect of ammonium sulfate on thermal denaturation of CS, dialysis against non-interfering buffer systems is necessary. Furthermore, the purity of the available CS from several suppliers is not sufficient for chaperone research, making a further purification necessary.

The recombinant expression and purification of CS in soluble and active form would overcome these problems. Using a temperature-dependent T7 promotor, Evans et al. [17] were able to express small amounts of active CS in *E. coli*. But, as far as known to the authors, no purification strategy to obtain recombinant expressed CS in active form has been described yet. In this study we demonstrate that the co-expression of GroE raises both the amount and activity of recombinant expressed CS. We describe a purification strategy for active and highly pure CS, suitable for chaperone research. Furthermore, the co-expression strategy allows the co-purification of GroEL and GroES. Thus, all three proteins can be purified from one expression culture.

2. Experimental

2.1. Cloning

For cloning of the CS-gene without mitochondrial leader sequence in pBAD the CS-gene was amplified by PCR, using Pwo-Polymerase Kit (Roche, Basel, Switzerland) according to manufactors instructions in a Primus 25 PCR-cycler (MWG, Ebersberg, Germany). Fifty ng of CS-cDNA template vector [16] and the primer pair TGACCCATGGCTGCTTCTTCCACGAACTTA and GATCGAATTCTTATCACTTAGAGTCCACAAGTTTTATC were used. The resulting PCR-product was purified using High Pure PCR-Product Purification Kit (Roche, Basel, Switzerland) and treated with the restriction enzymes *Nco*I and *Eco*RI for 2.5 h at 37 °C. After a further purification the fragment was ligated into a equaly restricted pBAD vector using T4-Ligase (Promega, Madison, WI, USA) according to manufacturers instructions. *E. coli* XL1 blue cells (Stratagene, La Jolla, CA, USA) were transformed with all resulting constructs. Successful cloning was verified by restriction mapping of purified plasmid DNA from several transfomants. Resulting positive clones were further verified by DNA sequencing.

2.2. Immunoblotting

Immunodetection was performed using a polyclonal rabbit antiserum, raised against purified CS. For detection, a horseradish peroxidase-linked secondary conjugate (Sigma, St. Louis, MO, USA) was used and reactive bands were visualised by Enhanced Chemiluminescense (ECL Plus) Detection Reagents (Amersham Biosciences, Uppsala, Sweden).

2.3. CS activity assay

The activity assay is based on the first step of the citric acid cycle, in which CS catalyzes the condensation of oxaloacetic acid (OAA) and acetyl-CoA to citrate and coenzyme A. The side product coenzyme A reduces stoichiometrically the Ellman's reagent dithio-1,4-nitrobenzoic acid (DTNB), a reaction that goes along with an increase in absorption

at 412 nm. For each activity assay, the reaction mixture consisted of 900 μl of TE buffer (50 m*M* Tris–HCl, 2 m*M* EDTA, pH 8.0), 10 μl of 10 m*M* OAA (in 50 m*M* Tris, pH not adjusted), 10 μl of DTNB (in TE buffer), and 30 μl of 5 m*M* acetyl-CoA (in TE buffer). This reaction mixture was incubated at 25 °C and the reaction was started by addition of 2–50 μl of *E. coli* lysate to be tested. The change in absorbance (relative activity) was monitored online over a time period of 1 min. For standardisation, commercially available CS (Roche, Basel, Switzerland) was used. The specific activity of commercially available CS is 150 U/mg, calculated as follows:

$$\text{Specific activity (U/mg)} = \Delta E/\text{min} \times V/(\varepsilon d v c)$$

where V is the test volume (ml), ε is the molar extinction coefficient of DTNB, d is the pathlength of the cell (cm), v is the sample volume (ml), and c is the enzyme concentration (mg/ml).

2.4. Culture

E. coli BL21 Codon plus cells were cultivated at 37 °C. Induction of the expression plasmids was performed at $O.D._{600} = 0.8–1.2$ with 1 m*M* IPTG and/or 0.02% arabinose. Cells were harvested at 3000 *g*, 4 °C for 10 min and washed and disrupted in 50 m*M* Tris–HCl, 2 m*M* EDTA, pH 7.0. Disruption was obtained by five times repeated sonification for 30 s using a Sonifier B-12 (Branson, Danbery, USA) or by using the Basic Z pressure disruption system (Constant Systems, Warwick, UK). Lysates were cleared by centrifugation at 40 000 *g*, 4 °C for 45 min.

2.5. Chromatography

All chromatography steps were performed on a Äkta FPLC system (Amersham Biosciences). All ion-exchange chromatography steps were performed using five column volumes (CV) for equilibration, loading of sample, 5–10 CV for washing and linear gradients of 15–25 CV. Buffer conditions, flow-rates and column type are stated in results.

2.6. Electrophoresis

SDS–PAGE was performed according to Fling and Gregerson (1986) [18] in a SE 250 Mighty Small electrophoresis system (Amersham Biosciences) at a constant current of 30 mA per gel. Coomassie and silver staining were performed as stated elsewhere [19,20].

3. Results

First we designed a regulated, recombinant expression system for CS. Therefore we cloned the CS encoding gene without mitochondrial leader sequence into pBAD (Invitrogen, Breda, The Netherlands). In this expression vector the CS gene is under control of a T7 promotor regulated by an arabinose-induced operator sequence. For expression BL21 (DE3) Codon Plus cells (Stratagene, La Jolla, USA) were transformed with the plasmid. Second, we transformed the resulting strain with a GroE-expressing plasmid (pT-GroE; [21]). This second plasmid, encodes GroE under control of a IPTG induceable lac operator and T7 promotor. Taken together, both expression vectors can be induced by different inductors. This system provided a efficient tool to study the GroE dependence of CS expression and folding in vivo and allowed the efficient expression and purification of recombinant, active CS. Furthermore, the co-expression of GroE allows the purification of GroEL and GroES in one procedure.

In Fig. 1 the kinetics of the CS expression without GroE co-expression is shown. CS expression was induced when the *E. coli* cultures reached an $O.D._{600} = 0.8$ with 40 m*M* arabinose. As monitored CS expression reaches its maximum after 4 h and stays constant for up to 17 h (Fig. 1A). But as revealed by immunoblot analysis (Fig. 1B), after 17 h a huge amount of CS is already degraded. To test whether the CS expressed in soluble or insoluble form we lysed cell samples after 4 h of induction by sonification and separated insoluble material from soluble by centrifugation at 40 000 *g* for 15 min at 4 °C (Fig. 1C). This analysis revealed that only 50% of the expressed CS was soluble. Because folding of CS is extremely temperature dependent in vitro, we

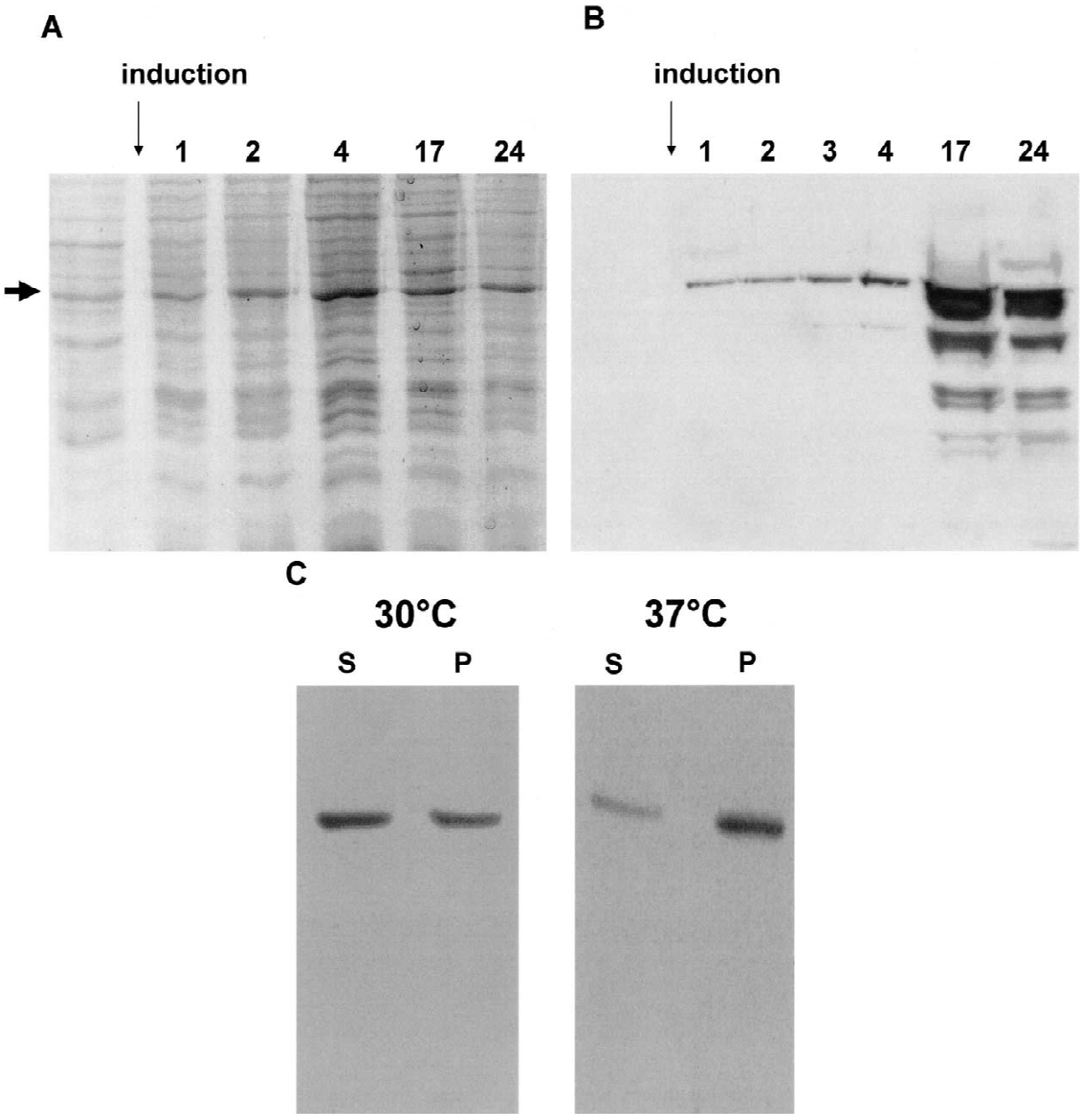


Fig. 1. Kinetic analysis of CS expression without GroE co-expression. (A) Coomassie-stained SDS–PAGE of cell samples taken at indicated timepoints after induction. Spark, timepoint of expression induction with 40 m*M* arabinose. First lane, uninduced control. A total of 1×10^4 cells were lysed and loaded per lane. (B) Immunoblot performed with antisera against CS raised in rabbit. Spark, timepoint of expression induction. First lane, uninduced sample. A total of 5×10^3 cells were lysed and loaded per lane. Samples were taken after induction at timepoints indicated. (C) A total amount of 1×10^9 cells was lysed 4 h after induction with 40 m*M* arabinose. Cells were lysed by sonification and the insoluble content was pelleted by centrifugation. Samples were analyzed by immunoblotting. Left, samples taken from an expression culture at 30 °C. Right, samples taken from an expression culture shifted to 37 °C after induction. S, soluble fraction; P, pelleted, insoluble fraction.

lowered the cultivation temperature to 30 °C after induction of CS expression. As monitored in Fig. 1C this lowering of the temperature resulted in an increase of the soluble amount of the CS. According to this result, in all following experiments the expression cultures were shifted to 30 °C after induction. Having established this expression system we set out to analyze the activity of the expressed CS and the influence of GroE co-expression on CS expression.

In a first approach we tested whether the co-expression of GroE might influence the solubility of the expressed CS. Therefore we took cell samples at different time points after induction of the expression. We lysed the cells by sonification and separated the soluble from the unsoluble fraction by centrifugation. Samples of these fractions were analyzed by immunoblotting with antisera against CS. As shown in Fig. 2, the co-expression of GroE shifts part of the CS molecules from the insoluble fraction to the soluble. Furthermore, the general amount of expressed CS also rises significantly compared to the expression without GroE induction (Fig. 2).

In a further experiment we determined the activity of the expressed CS. Therefore, we induced GroE expression by addition of 1 m*M* IPTG and CS expression by addition of 40 m*M* arabinose at the same timepoint. Fig. 3 compares the amount of active CS in cell lysates with and without co-expression of GroE. Without GroE co-expression CS activity reaches a maximum at 2–4 h after induction with 40 m*M* arabinose. Interestingly, further cultivation of the cells results in a decrease of CS activity. However, the co-expression of GroE increases the amount of active CS after 2–4 h and, keeps the expressed CS in active state, during further cultivation. Nevertheless a clear maximum of activity here also is reached after 2–4 h after induction.

To analyze whether the rise of activity of CS is due to co- or post-translational folding reactions supported by GroE, we first induced only the expression of CS. After 4 h of induction we lysed the cells by sonification and added purified GroEL/GroES to a final content of 1 μ*M* to the cell lysates (Fig. 4A). Surprisingly, this post-translational addition of GroE resulted in a rise of CS activity (Fig. 4B). Thus, while the general rise of CS expression might be due to a co-translational influence of GroE, the increase of solubility seems to be due to post-translational folding reactions chaperoned by GroE.

Having established a co-expression system providing CS as 20% of total cellular protein (Figs. 1 and 5), we set out to purify active CS. Therefore, the cells were cultivated at 37 °C to an $O.D._{600} = 1.2$ and then induced by addition of 1 m*M* IPTG and 0.02% arabinose. At the time point of induction the cells were shifted to 30 °C and cultivated for additional 4 h, harvested and disrupted as described in Section 2. For the first chromatographic purification step, cleared lysate was loaded on a XK 26/20 DEAE-Sephacel (Amersham Biosciences) anion-exchange column equilibrated in 50 m*M* Tris–HCl, 2 m*M*

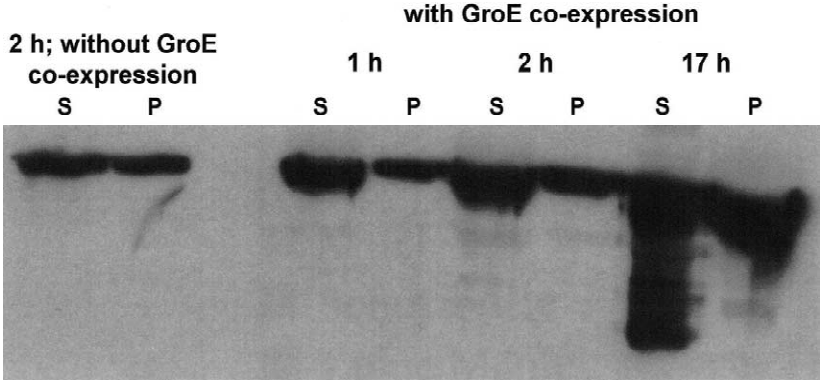


Fig. 2. Kinetic analysis of CS activity. Samples were taken at indicated timepoints after induction of CS expression with and without co-expression of GroE. A total of 1×10^9 cells were lysed by sonification and CS activity in the cell lysates was measured as described Section 2.

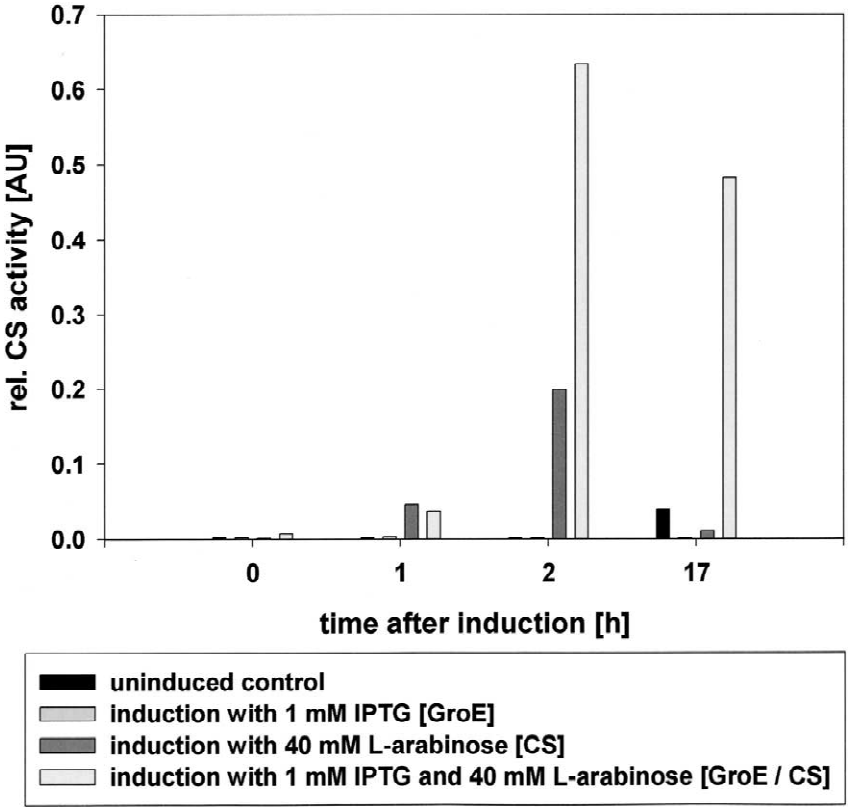


Fig. 3. Analysis of solubility of expressed CS with and without GroE co-expression. Samples of 1×10^9 cells were taken at indicated timepoints after induction. Cells were lysed by sonification and soluble and insoluble fractions were separated by centrifugation at 40 000 *g* for 15 min at 4 °C. CS was detected by immunoblotting. Left site, without GroE co-expression. Right site, with GroE co-expression. S, soluble fraction; P, pelleted, insoluble fraction.

EDTA, pH 7.0 (TE-buffer) with a constant flow-rate of 1 ml/min at 4 °C. Bound proteins were eluted in a gradient of 0–400 m*M* NaCl (Fig. 5A). In this gradient, CS eluted very early at a NaCl concentration of 15–25 m*M* (Fig. 5A). The CS containing fractions were pooled and dialysed against buffer containing 20 m*M* Tris–HCl, 2 m*M* EDTA at pH 8.0. In a second step the protein was purified on a XK 26/10 Q-Sepharose fast flow column (Amersham Biosciences) pre-equilibrated with the identical buffer under a constant flow-rate of 1 ml/min at 4 °C (Fig. 5B). Bound proteins were eluted in a gradient from 0 to 250 m*M* NaCl. Similar to the first purification step CS eluted very early at salt concentrations around 20–50 m*M* (Fig. 5B). In a final purification step, CS was separated on a 16/60 Superdex 75 pg column (Amersham Biosciences) equilibrated in 20 m*M* Tris–HCl, 2 m*M* EDTA, 400 m*M* NaCl, pH 7.0, under a constant flow-rate of 1 ml/min at 4 °C (Fig. 5C). The purified CS was pooled and for further storage at −80 °C dialysed against TE-buffer. Following this procedure, 1 mg CS per g *E. coli* wet weight, with a purity of 99% was obtained. Fig. 5C shows a comparison between commercially available CS and purified CS as described above.

Further we purified the co-expressed chaperone GroEL and its co-chaperone GroES from the same expression system. According to the elution profile of the DEAE-Sephacel purification step (Fig. 5A), GroEL eluted at 400–600 m*M* NaCl. Those GroEL containing fractions were pooled, dialysed against TE-buffer and loaded on a XK 16/10 (Amersham Biosciences) Affigel Blue-Sepharose (BioRad,

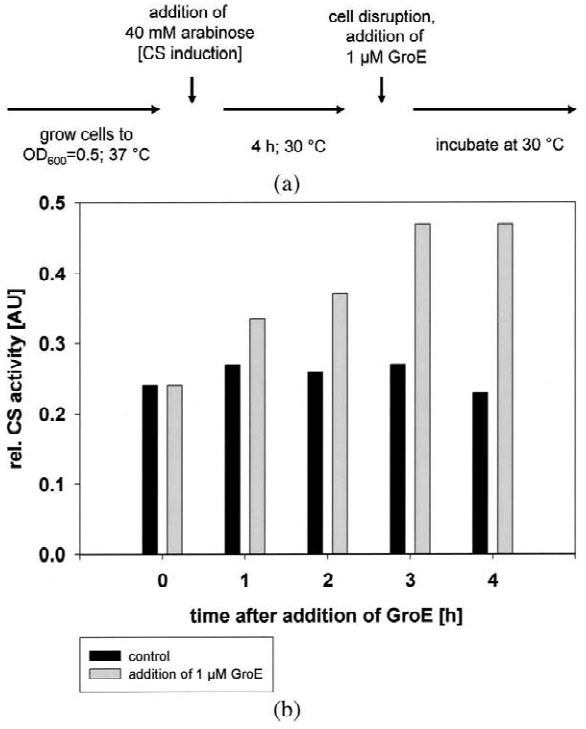


Fig. 4. Activation of insoluble CS by subsequent addition of GroE. After 4 h of CS expression without GroE expression, a total of 1×10^9 cells was were lysed by sonification. A total amount of 1 µ*M* GroE was added to the cell lysates. (A) Experimental setting. (B) CS activity was determined at indicated timepoints.

Munich, Germany) column preequilibrated in TE-buffer under a constant flow-rate of 3 ml/min at 4 °C. While most of the sample got bound to the column, GroEL did not bind and was collected in the flow through (Fig. 6A). To the GroEL containing flow through fraction, ammonium sulfate was added to a final concentration of 1.5 *M*. The so prepared sample was loaded on a XK 16/10 Butyl-Sepharose fast flow column (Amersham Biosciences), pre-equilibrated in 20 m*M* Tris–HCl, 1 m*M* EDTA, 1.5 *M* $[NH_4]_2SO_4$, pH 7.5, under a constant flow-rate of 1 ml/min at room temperature (Fig. 6B). Elution of bound proteins, was performed running a gradient to a 20 m*M* Tris–Cl, 1 m*M* EDTA, pH 7.5, buffer without ammonium sulfate. As shown in Fig. 6B, GroEL eluted in the gradient segment from 800 to 300 m*M* ammonium sulfate. Purified GroEL was pooled, dialysed against TE-buffer and stored at −20 °C.

To purify GroES we pooled the GroES-containing fractions from the 150 to 300 m*M* NaCl section of the DEAE-Sephacel elution (Fig. 5A). Those fractions were further purified as described by Schmidt et al. [22].

The identity of all three proteins was verified by immunoblotting. For further purity control and concentration determination all three proteins were analyzed by UV–Vis and fluorescence spectroscopy (data not shown).

4. Discussion

Under heat shock or other stress conditions many proteins loose their native conformation and denature rapidly, resulting in the population of irreversibly unfolded polypeptides and aggregates [23–25]. To prevent such irreversible reactions and to maintain viability, the production of heat shock proteins is induced rapidly. It is known, the GroE system promotes protein folding under conditions under which no spontaneous folding occurs by preventing aggregation [15,26,27]. In vivo the co-expression of GroE was shown to make the recombinant expression of bacterial RuBisCO [28] and adrenodoxin-reductase [29] possible. As demonstrated here, GroE also supports the recombinant expression of mitochondrial CS. In this context, the co-expression of GroE seems to influence co-translational processes as well as post-translational folding of CS. The observation that GroE co-expression enhances the amount of expressed CS (Fig. 2) indicates, that co-translational processes might be enhanced. But further investigation is necessary to distinguish if GroE itself supports co-translational folding of CS or if GroE stabilizes components of the protein translation machinery. This would be in agreement with previous reports demonstrating that several ribosomal proteins, elongation factor Tu and other proteins engaged in transcription and translation, are GroE substrates in vivo [30]. Concerning the results of the subsequent activation of previously expressed CS by GroE addition (Fig. 4), GroE is surely involved in post-translational folding of CS. Furthermore, these results indicate that the expressed CS is partly in a soluble but inactive form, which can be activated by GroE. Concerning a model of CS folding in vitro

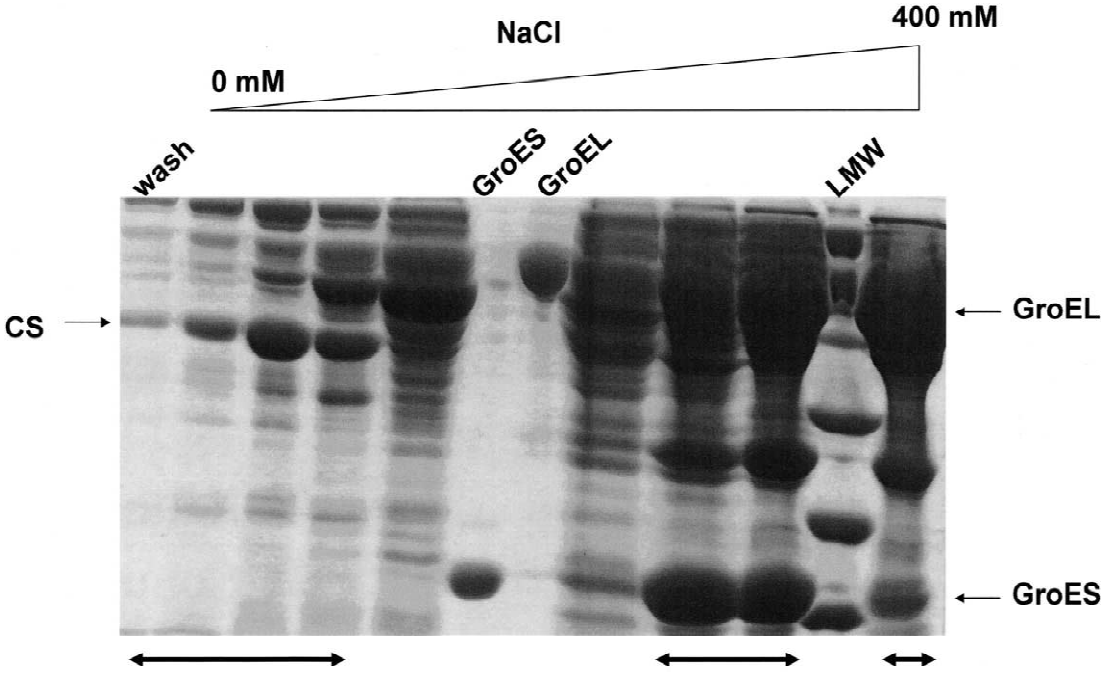
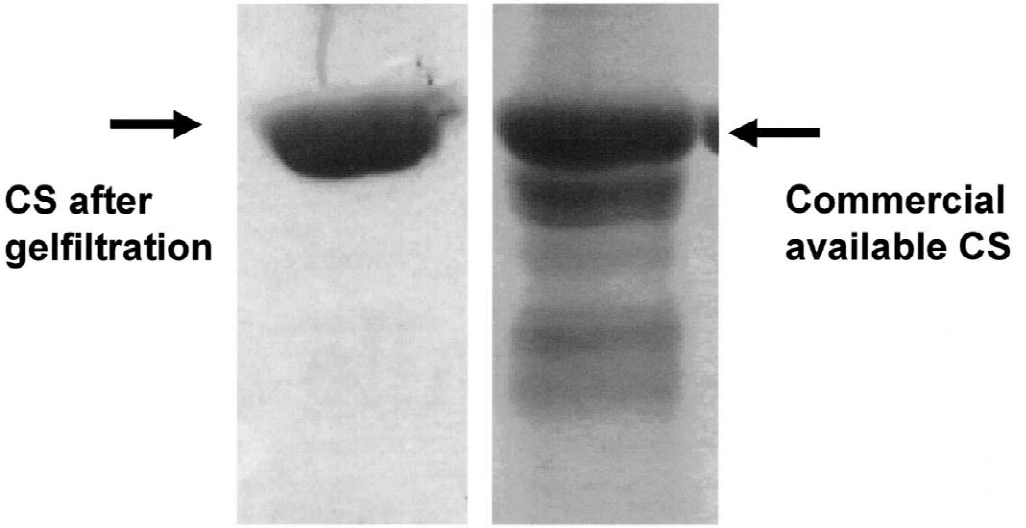


Fig. 5. Purification of CS. (A) Samples of DEAE-Sephacel separation were analyzed by SDS–PAGE. Analyzed segment of elution gradient as indicated. Double sparks, pooled fractions of CS. (B) Samples of Q-Sepharose separation analyzed by SDS–PAGE. (C) Left, pure CS after separation on a 16/60 Superdex 75 pg gel permeation column. Right, commercially available CS (Roche, Basel, Switzerland). LMW, low-molecular mass marker (BioRad, Munich, Germany). Further description and detailed experimental settings as described in the text.



(c)

Fig. 5. (*continued*)

[9–11] where a soluble but inactive dimeric form of CS and a inactive but soluble monomeric form is proposed, GroE shifts the equilibrium between unproductive and productive folding pathways towards the active native state.

The enhancement of CS expression and folding by GroE revealed the ability to purify the recombinant expressed CS (Fig. 5). Recombinant expression of CS in *E. coli* was already demonstrated by Evans et al. [17] but no recombinant purification strategy was described yet. The purified CS revealed an increased purity compared to commercially available CS (Fig.

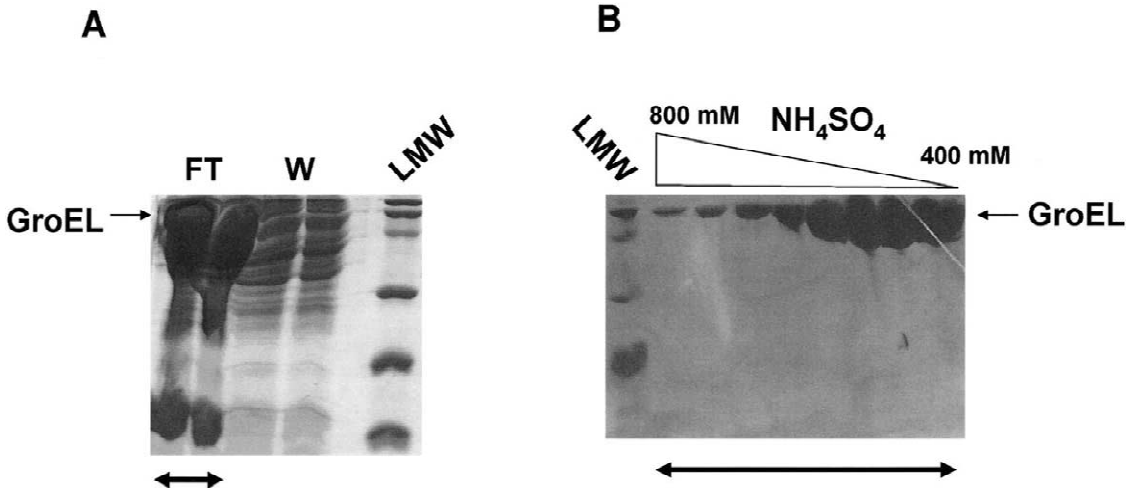


Fig. 6. Purification of GroEL. (A) Samples of Affigel Blue-Sepharose separation were analyzed by SDS–PAGE. FT, flowthrough while sample loading. W, washing fractions. Double spark, pooled fractions of GroEL. (B) Samples of Butyl-Sepharose separation analyzed by silver stained SDS–PAGE. Analyzed segment of elution gradient as indicated. LMW, low-molecular mass marker (BioRad). Further description and detailed experimental settings as described in the text.

5C). This CS is therefore very well suited to study chaperone function in vitro. In this area of research, active and highly pure CS is a necessary and powerful tool. Furthermore, the co-expression strategy allowed the co-purification of GroEL and GroES (Fig. 6). Thus, all three proteins could be purified from the same expression culture. And as already discussed, this co-expression system also might represent a helpful tool in understanding GroE function in vivo as well.

5. Conclusions

Taken together the here-described CS expression and purification system provides a promiscuous tool to study the chaperone function of GroE. The here-described and previously reported enhancement of protein expression and folding by chaperones demonstrates the biotechnological properties of these protein family. Furthermore, the purification of three proteins from the same expression culture demonstrates a possibility of biotechnological process optimisation.

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