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Construction of *Escherichia coli dnaK*-deletion mutant infected by λDE3 for overexpression and purification of recombinant GrpE proteins

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ABSTRACT

Escherichia coli is widely employed to produce recombinant proteins because this microorganism is simple to manipulate, inexpensive to culture, and of short duration to produce a recombinant protein. However, contamination of molecular chaperone DnaK during purification of the recombinant protein is sometimes a problem, since DnaK sometimes has a negative effect on subsequent experiments. Previously, several efforts have been done to remove the DnaK contaminants by several sequential chromatography or washing with some expensive chemicals such as ATP. Here, we developed a simple and inexpensive method to express and purify recombinant proteins based on an *E. coli dnaK*-deletion mutant. The *E. coli ΔdnaK52* mutant was infected by λDE3 phage to overexpress desired recombinant proteins under the control of T7 promoter. Using this host cell, recombinant hexa histidine-tag fused GrpE, which is well known as a co-chaperone for DnaK and to strongly interact with DnaK, was overexpressed and purified by one-step nickel affinity chromatography. As a result, highly purified recombinant GrpE was obtained without washing with ATP. The purified recombinant GrpE showed a folded secondary structure and a dimeric structure as previous findings. *In vitro* ATPase activity assay and luciferase-refolding activity assay demonstrated that the recombinant GrpE worked together with DnaK. Thus, this developed method would be rapid and useful for expression and purification of recombinant proteins which is difficult to remove DnaK contaminants.

Recently, a lot of information about genome has become available by achievement of the genome projects carried out all over the world. In order to examine the functions and structures of interesting proteins, expression of recombinant proteins in several host cells [1] or in cell-free expression system [2] is attempted based on the genome sequence, which is widely used for protein research as well as commercial purpose. The most popular expression system is *Escherichia coli* (*E. coli*)[1] with expression vector system. Benefits of using it are as follows: (i) This microorganism is very simple to manipulate, (ii) it is inexpensive to culture, and (iii) the amount of time necessary to produce a recombinant protein is short. In many cases, peptide- or protein-tags are fused to N-terminus or C-terminus of the desired protein, which will provide us some advantages: (i) Purification will become easy using a specific affinity chromatography. (ii) Expression level will be increased. (iii) Solubility of the protein will be enhanced [1].

After expression and extraction of recombinant proteins, affinity chromatography is usually performed to purify them. However, contaminations of molecular chaperones often occur, which sometimes affect the subsequent experiments including folding and unfolding of the proteins, production of antibody, and so on [3–6]. One of the major molecular chaperones is DnaK, the bacterial heat shock protein 70 homolog, which strongly binds unfolded proteins or peptide with specific recognition sequences [7]. In order to separate the desired recombinant proteins and DnaK contaminants, further complicated purification steps are needed. Recently, to remove the DnaK contaminants, several efforts have been done based on the feature of DnaK. DnaK binds to the substrate proteins or peptides in the ADP-bound state or nucleotide-free state and releases them in the ATP-bound state [7]. These states of DnaK are tightly regulated by the co-chaperones DnaJ and GrpE. DnaJ stimulates the low intrinsic ATPase activity of DnaK [8], while GrpE functions as a nucleotide-exchange factor that promotes ADP-dissociation from DnaK [9]. Therefore, it was attempted to separate the DnaK contaminants were tried to separate from the

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[1] *Abbreviations used: E. coli*, *Escherichia coli*; His-tag, hexa histidine-tag; PCR, polymerase-chain reaction; LB, luria-bertani; IPTG, isopropyl-β-D-thiogalactoside; PMSF, phenylmethylsulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CD, circular dichroism.

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desired proteins by washing with ATP in the affinity column or gel filtration column [3,10]. Furthermore, other methods were also developed (e.g., washing with glycerol instead of ATP) [4]. However, there were difficulties to remove DnaK contaminants from some proteins by the above-mentioned methods due to their strong affinity with DnaK. In addition, use of ATP or glycerol is expensive and thus is not suitable for commercial purpose.

In this study, we developed a rapid and simple method to purify recombinant proteins. Our target protein in this study was a co-chaperone GrpE which is well known to strongly bind to nucleotide-free or ATP-bound state of DnaK [10]. We used an *E. coli dnaK*-deletion mutant and modified it to overexpress desired recombinant proteins by T7 promoter-based expression system. The resulting strain could successfully overexpress recombinant GrpE fused with a hexa histidine-tag (His-tag) at the N-terminus, and active recombinant GrpE was obtained by one-step purification with nickel affinity chromatography.

Materials and methods

Bacterial strains

Bacterial strains used in this study are listed in Table 1. They were grown as previously described [11–13]. Experimental procedures for construction of *E. coli* BM271 (DE3) are summarized in Fig. 1.

Plasmid construction

To complement the *E. coli ΔdnaK52* mutation, a plasmid encoding an intact DnaK gene was constructed. An *E. coli dnaK* gene with its original promoter and ribosome-binding site was polymerase-chain reaction (PCR)-amplified using KOD-plus DNA polymerase (Toyobo, Tsuruga, Japan), a primer set DNAK-pro-S1/DNAK-A1 (5′-TTT GCA TCT CCC CCT TGA TGA TGA CGT GGT-3′/5′-ACC CTG GTT AGG ATC CGG GTG GTA TTT-3′), and *E. coli* genomic DNA as a template. The amplified fragment contained a ribosome-binding sites of the *dnaK* gene; using *Taq*™ DNA polymerase (Promega, Madison, WI, USA) and dATP, an adenine base was added to the 3′-terminal of each amplified fragment. The modified DNA fragment was ligated into the pGEM-T cloning vector (Promega), and the resulting plasmid was termed pEcoDNAK (Fig. 2 and Table 1).

A vector for overexpression of N-terminal His-tag fused GrpE of *E. coli* was also constructed as follows: the gene encoding GrpE was PCR-amplified using KOD-plus and a primer set TOPO-GRPE-S1 and TOPO-GRPE-A1 (5′-CAC CAT GAG TAG TAA AGA ACA GAA AAC GCC TG-3′/5′-TTT CCT GTG AAA CCG CTG CGC GAG AGT-3′). The PCR-amplified fragment encoding GrpE was cloned into the pET-100/D-TOPO (Invitrogen, San Diego, CA, USA) vector according to the manufacturer's procedure. The resulting plasmid was termed pEcoGRPE-His (Fig. 2 and Table 1).

Table 1
Bacterial strains and plasmids used in this study

Strains and plasmids	Description[a]	Source/ reference
E. coli strains		
JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* (*lac-proAB*) [F′ *traD36 proAB*⁺ *LacI*ᵠ *lacZ* M15]	Invitrogen
BL21 (DE3)	F^- *ompT* $hsdS_B(r_B^-m_B^-)$*gal dcm* (DE3)	Invitrogen
BL21 (DE3) pEcoGRPE-His	BL21 (DE3) pEcoGRPE-His	This study
MC4100	*F– araDl39 Δ*(*argF*⁻*lac*)*U169 rpsL150 relA1 deoCl ptsF25 rpsR flbB301*	Tomoyasu
MC4100 pGEM	MC4100 transformed pGEM	This study
BM271	MC4100*ΔdnaK*52::Cm^R	Wada/13
BM271 pGEM	BM271 harboring pGEM	This study
BM271 pEcoDNAK	BM271 harboring pEcoDNAK	This study
BM271 (DE3)	BM271 infected by λDE3	This study
BM271 (DE3) pEcoDNAK	BM271 (DE3) harboring pEcoDNAK	This study
BM271 (DE3) pEcoGRPE-His	BM271 (DE3) harboring pEcoGRPE	This study
Plasmids		
pGEM-T	M13 *ori* pBR322*ori*, linear T overhangs vector; Amp^R	Promega
pGEM	Self-ligated pGEM-T; Amp^R	This study
pET-100/D-TOPO	Directional TOPO expression vector; Amp^R	Invitrogen
pEcoDNAK	*E. coli dnaK* gene cloned in pGEM-T; Amp^R	This study
pEcoGRPE-His	*E. coli grpE* cloned in pET100-D/TOPO; Amp^R	This study

[a] Cm^R, chloramphenicol resistance gene; Amp^R, ampicillin resistance gene.

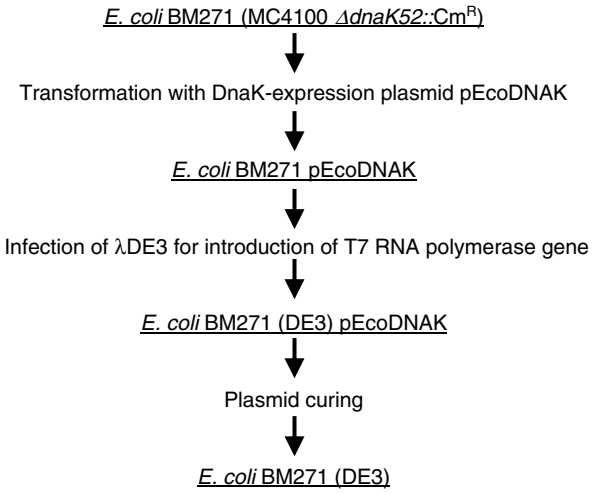

Fig. 1. Diagram showing the steps used in construction and lysogenization of *E. coli dnaK*-deletion mutant. *E. coli dnaK*-deletion mutant BM271 (Table 1) was transformed with pEcoDNAK (Table 1), and the resulting transformant was termed *E. coli* BM271 pEcoDNAK (Table 1). The BM271 pEcoDNAK was infected by λDE3 for introduction of T7 RNA polymerase gene, and the resulting lysogen was termed *E. coli* BM271 (DE3) pEcoDNAK. Finally, *E. coli* BM271 (DE3) was constructed by curing the pEcoDNAK from the *E. coli* BM271 (DE3) pEcoDNAK. *E. coli* strains are indicated with underlines.

Complementation of ΔdnaK52 mutation by the cloned dnaK gene in plasmid DNA

Since *E. coli* BM271, *ΔdnaK52* mutant, lacks functional DnaK, it is unable to be infected by λDE3. Therefore, a plasmid encoding DnaK, pEcoDNAK, was transformed into thermosensitive *E. coli* BM271 (Table 1) to complement its defect in lysogenization of λDE3. The transformant was subjected to thermosensitive test as previously described [11]. *E. coli* MC4100 and BM271 were transformed with a self-ligated pGEM-T vector, pGEM. The resulting transformants, MC4100 pGEM and BM271 pGEM (Table 1), were also used as a positive control and a negative control, respectively.

Infection of λDE3 for introduction of T7 RNA polymerase gene

The *E. coli* transformant BM271 pEcoDNAK (Table 1) was infected by λDE3 using a λDE3 Lysogenization Kit (Novagen, Madison, WI, USA) according to the manufacturer's instruction [11]. λDE3 lysogen candidates were evaluated by their ability to support the growth of the T7 Tester Phage. T7 Tester Phage (1×10^3 plaque forming unit) in phage dilution buffer composed of 20 mM Tri–HCl (pH 7.4), 100 mM NaCl, and 10 mM $MgCl_2$ was spotted on Luria–Bertani (LB) agar plate with or without isopropyl-β-D-thiogalacto-