J. Microbiol. Biotechnol. (2005), **15**(6), 1267–1272

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Production of Soluble Human Granulocyte Colony Stimulating Factor in *E. coli* by Molecular Chaperones

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Received: February 26, 2005
Accepted: April 27, 2005

Abstract The effects of coexpression of GroEL/ES and DnaK/DnaJ/GrpE chaperones on the productivity of the soluble form of human granulocyte colony stimulating factor (hG-CSF) in *E. coli* were examined. Recombinant hG-CSF protein was coexpressed with DnaK/DnaJ/GrpE or GroEL/ES chaperones under the control of the *araB* or *Pzt-1* promoter, respectively. The optimal concentration of L-arabinose for the expression of DnaK/DnaJ/GrpE was found to be 1 mg/ml. When L-arabinose was added at OD_{600}=0.2 (early-exponential phase), soluble hG-CSF production was greatly increased. In addition, it was observed that the DnaK/DnaJ/GrpE and GroEL/ES chaperones had no synergistic effects on preventing aggregation of hG-CSF protein. Consequently, by coexpression of the DnaK/DnaJ/GrpE chaperone, the signal intensity of the hG-CSF protein band in the soluble fraction of cell lysate was increased from 3.5% to 13.9%, and Western blot analysis also revealed about a 4–5-fold increase of production of soluble hG-CSF over the non-induction case of DnaK/DnaJ/GrpE.

Key words: Human granulocyte colony stimulating factor, DnaK/DnaJ/GrpE, GroEL/ES, molecular chaperone

Production of recombinant protein in *E. coli* often results in rapid degradation or aggregation of these foreign proteins, forming inclusion bodies that are insoluble and inactive proteins [6, 7, 10, 20]. It is widely recognized that coexpression of molecular chaperones or foldases can assist protein folding, and this leads to increased production of active protein [9, 12, 14, 24, 32]. Heat-shock protein, such as Hsp60 and Hsp70, are molecular chaperones that not only regulate the heat-shock response, but are also required for folding a newly synthesized polypeptide under normal growth condition. Hsp70 systems act by binding to hydrophobic residues and/or unstructured backbone regions of their substrate, thereby shielding the interactive surface of non-native polypeptides [24]. The DnaK/DnaJ/GrpE complex, another molecular chaperone, interacts with the nascent polypeptide chains to prevent irreversible polypeptide aggregation and to mediate partial folding [3, 25]. GroEL/ES then interacts with the partially folded proteins and completes the folding [3, 7, 27–29].

In this work, the human granulocyte colony stimulating factor (hG-CSF) was used as a target protein to investigate the effects of molecular chaperones, DnaK/DnaJ/GrpE and GroEL/ES. hG-CSF is a member of a family of glycoproteins that play an important role in stimulating proliferation, differentiation, and functional activation of blood cells [2, 8, 16, 18]. The hG-CSF has increasing clinical application on the treatment of neutropenia and has greatly reduced the infection risk associated with bone marrow transplantation by accelerating neutrophils [13]. Thus, the production of soluble hG-CSF in *E. coli* has generated great interest in supply of valuable medical protein.

Previously, hG-CSF was expressed in many other host strains, such as *E. coli*, fungi, and plant [4, 5, 8, 13, 21, 30]. However, when the hG-CSF gene (*hg-csf*) was expressed in *E. coli*, most of the protein was aggregated into the insoluble particles known as inclusion bodies [2]. Therefore, the effect of molecular chaperones, GroEL/ES and DnaK/DnaJ/GrpE, on the production of soluble hG-CSF in *E. coli* cells was investigated in the present work.

Materials and Methods

Bacterial Strain and Plasmids

E. coli BL21(DE3)[F^-, *ompT*, r_B^-, m_B^-, (DE3)] strain was used in all experiments. The plasmid pHCE-IIB-GCSF encodes the *hg-csf* gene and preS1 epitope. The transcription of the *hg-csf* gene in the plasmid is controlled by the HCE

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promoter. The HCE promoter was derived from upstream of the D-amino acid aminotransferase gene of *Geobacillus toebii* and was developed for the high-constitutive expression of foreign proteins without induction [22].

The pHCE-IIB-GCSF was constructed by BioLeaders Co. (Daejeon, Korea). A DNA fragment of the hG-CSF gene was amplified by PCR using the human breast carcinoma cDNA library (Stratagene, Inc., La Jolla, CA, U.S.A.) as the template. The forward primer: 5'-TGGCATATGACCCC-GCTGGGCCCTGCCAGCTCC-3' and reverse primer: 5'-TGTGGATCCTTATTAGGGCTGGGCAAGGTGGCGTAG-3' were designed on the basis of the hG-CSF gene. After digesting the amplified fragment with *Nde*I and *Bam*HI, it was ligated to the *Nde*I/*Bam*HI-cleaved pHCE-IIB vector (BioLeaders Co., Daejeon, Korea).

The plasmid pG-KJE6 is a pACYC184-based chloramphenicol-resistant plasmid. The transcription of the *groEL/ES* genes and *dnaK/dnaJ/grpE* gene in the plasmid pG-KJE6 is controlled by the *Pzt-1* promoter and *araB* promoter, respectively [10]. To induce the *Pzt-1* promoter and *araB* promoter, tetracycline and L-arabinose were used. Equal amounts (1 μg) of pHCE-IIB-GCSF and pG-KJE6 were co-transformed into *E. coli* BL21(DE3), and the transformed *E. coli* cells were selected on LB agar plates containing 50 μg/ml ampicillin (selection for pHCE-IIB-GCSF) and 50 μg/ml chloramphenicol (selection for pG-KJE6).

Culture Condition

E. coli cells were grown on LB medium (1% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.5% NaCl). *E. coli* BL21(DE3) strains harboring pHCE-IIB-GCSF and pG-KJE6 were grown in the presence of 50 μg/ml ampicillin and 50 μg/ml chloramphenicol. To induce the expression of *groEL/ES* or *dnaK/dnaJ/grpE* genes, tetracycline or L-arabinose was added in the range of 0–5 ng/ml or 0–5 mg/ml, respectively. In the case of simultaneous expression of *groEL/ES* and *dnaK/dnaJ/grpE* genes, tetracycline up to 200 ng/ml was added for sufficient supply of the GroEL/ES protein over that at the individual expression of *groEL/ES* gene.

SDS-PAGE Analysis and Gel Scanning

To examine the extent of aggregation of the hG-CSF produced, *E. coli* cells were disrupted by sonication for 1 min, 70 Watt, and 7 sec cycle on ice with a sonicator (Sonoplus HD2070, Bandelin, Germany) and then centrifuged at 9,800 ×*g* for 10 min for separation into soluble and insoluble fractions. Each of the fractions obtained from 10 mg cell lysate protein/ml was analyzed by SDS-PAGE (10% gel). The GroEL/ES, DnaK/DnaJ/GrpE, and hG-CSF proteins were detected by staining the gel with Coomassie brilliant blue RT250, and the bands on the gel were scanned by an Image Analyzer (FluorChem 5500, Alpha Innotech., U.S.A.). The total intensity of protein bands in each fraction was calculated and taken as 100%. Only the intensity of the hG-CSF protein band was described as percentage.

Western Blot Analysis

E. coli cell lysates containing hG-CSF protein was blotted to PVDF membranes after SDS-PAGE (Trans-Blot SD Semi-Dry Transfer Cell, Bio-rad, Hercules, CA, U.S.A.). The membrane was blocked with blocking buffer (5% skim milk in PBS) for 1 h and then incubated overnight with primary antibody. Mouse monoclonal anti-preS1 tag (Aprogen, Korea) at 1/3,000 dilution and biotinylated anti-mouse IgG (Vector Laboratories Inc., Burlingame, CA, U.S.A.) at 1/4,000 dilution were used as primary and secondary antibodies, respectively. After washing with PBS, the membranes were incubated with horseradish peroxidase-conjugated protein A (ABC kit, Vector Laboratories, Inc., CA, U.S.A.). Protein bands were visualized by addition of 0.5 mg/ml diaminobenzidine (DAB substrate kit for peroxidase) and 0.05% H_2O_2 in PBS.

Results and Discussion

Effect of GroEL/ES on Production of Soluble hG-CSF

The effect of GroEL/ES on the soluble hG-CSF expression was investigated at tetracycline concentrations ranging from 0–5 ng/ml. Thus, *E. coli* BL21 cells harboring pHCE-IIB-GCSF and pG-KJE6 plasmids were cultivated on LB medium at 37°C, and various concentrations of tetracycline were added at the early-exponential phase (OD_{600}=0.2–0.3). As shown in Fig. 1, most of the hG-CSF (18.8 kDa) was produced as an insoluble form. This result is similar to that previously reported for the hG-CSF expression in *E. coli* [2, 16, 30]. Most of the GroEL

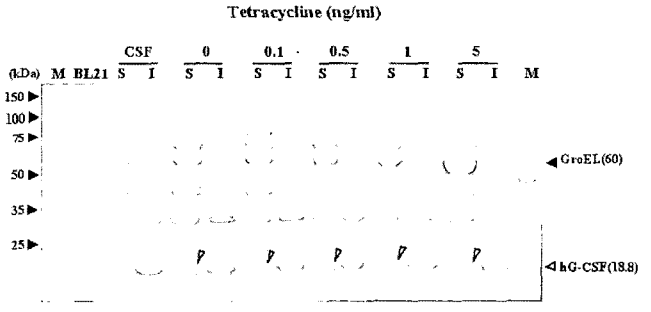

Fig. 1. Effect of GroEL/ES chaperones on the hG-CSF production in *E. coli* BL21 cell harboring pHCE-IIB-GCSF and pG-KJE6 plasmids.

Cells were grown on 10 ml of LB with tetracycline (0–5 ng/ml), which was added at the early-exponential phase. After 6 h of induction, cells were harvested, protein concentration determined, and separated as soluble (S) and insoluble (I) fractions. Lane M, protein marker; BL21, host cell lysate; CSF, only expression of CSF. The fractions were resolved by SDS-PAGE (10% gel).

(60 kDa) was detected in the soluble fraction, whereas GroES protein was clearly not shown in the gel since the molecular weight of GroES protein is too small (10 kDa). When the tetracycline concentration was added at more than 5 ng/ml, cell growth was considerably inhibited (less than 1.0 of OD_{600}), and therefore the total amount of hG-CSF protein was decreased. As a result, it appears that the GroEL/ES chaperone had no effect on the production of soluble hG-CSF.

Effect of DnaK/DnaJ/GrpE on Soluble Production of hG-CSF

The effect of DnaK/DnaJ/GrpE chaperone on the soluble production of hG-CSF was examined. With L-arabinose concentrations of 0–5 mg/ml, the largest amount of soluble hG-CSF protein was detected at 1 mg/ml L-arabinose (Fig. 2). SDS-PAGE analysis showed that the DnaK (70 kDa), DnaJ (40 kDa), and GrpE (26 kDa) proteins were overexpressed and found in the soluble fraction. Since the cell concentration (OD_{600}) was maintained above 3.0 even at high concentration of L-arabinose, the growth of *E. coli* was not inhibited. To quantitatively analyze the hG-CSF expression in *E. coli* cell, the SDS-PAGE gel was scanned: The total intensity of protein bands in each fraction was calculated and taken as 100%, and only the intensity of the hG-CSF protein band was described as a percentage. The signal intensity of the hG-CSF protein band in the soluble fraction was increased from 3.5% at only hG-CSF expression to 13.9% at the L-arabinose concentration of 1 mg/ml (Fig. 2, Table 1). However, the signal intensity of the hG-CSF band in the insoluble fraction was decreased from 73.9% to 66.1%. Considering the protein bands of DnaK/DnaJ/GrpE chaperone in the soluble fraction, the increase of hG-CSF production would be much higher than 13.9%. Thus, it appears that the enhanced production of soluble hG-CSF protein was totally contributed by the DnaK/DnaJ/GrpE chaperone.

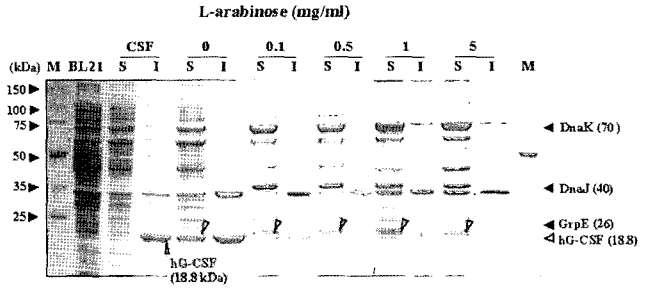

Fig. 2. Effect of the DnaK/DnaJ/GrpE chaperones on hG-CSF production in *E. coli* BL21 cells harboring pHCE-IIB-GCSF and pG-KJE6 plasmids.

Cells were grown on 10 ml of LB with L-arabinose (0–5 mg/ml), which was added at the early-exponential phase. After 6 h of induction, cells were harvested, the protein concentration determined, and separated as soluble (S) and insoluble (I) fractions. Lanes M, BL21, and CSF are the same as in Fig. 1.

Table 1. Ratio of hG-CSF protein in the soluble and insoluble fractions of cell lysates of *E. coli* BL21 harboring pHCE-IIB-GCSF or [pHCE-IIB-GCSF+pG-KJE6] plasmids. Withthe pG-KJE6 plasmid, only DnaK/DnaJ/GrpE was produced. Each fraction was separated on 10% SDS-PAGE, followed by Coomassie staining, and scanning by Image Analyzer. The total intensity of protein bands in each fraction was taken as 100%, and only the intensity of the hG-CSF protein band was represented as a percentage.

Condition	hG-CSF	
	Soluble (%)	Insoluble (%)
pHCE-IIB-GCSF	3.5	73.9
pHCE-IIB-GCSF+pG-KJE6 (DnaK/DnaJ/GrpE)	13.9	66.1

Effect of Induction Time

To monitor the effect of L-arabinose induction time on the production of soluble hG-CSF, *E. coli* BL21 cells harboring pHCE-IIB-GCSF and pG-KJE6 plasmids were grown on LB medium, in which L-arabinose (final concentration of 1 mg/ml) was added at different growth phases, such as OD_{600}=0, 0.2, 0.5, and 0.8. When induction of DnaK/DnaJ/GrpE was achieved at OD_{600}=0, cell growth was significantly inhibited, since OD_{600} less than 1.7 was obtained. This inhibition of cell growth was most likely due to the metabolic burden at low cell concentration caused by replication and maintenances of the two plasmids, and/or by oversynthesis of chaperone protein. When L-arabinose was added at OD_{600}=0.2 (early-exponential phase) rather than at OD_{600}=0.5 (mid-exponential phase) or OD_{600}= 0.8 (late-exponential phase), the active and soluble hG-CSF production was significantly increased (Fig. 3). Such growth phase-dependency in the soluble production of

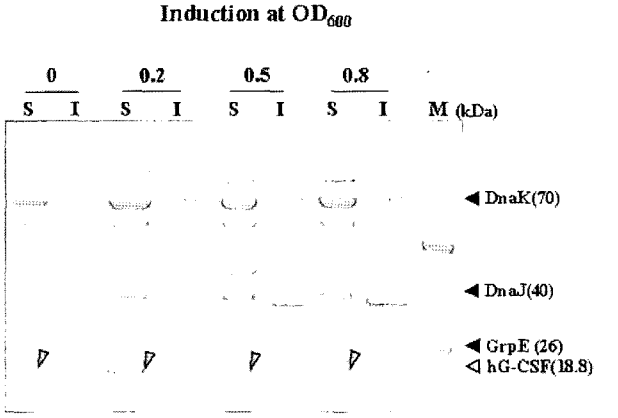

Fig. 3. Effect of L-arabinose induction time on the hG-CSF production in *E. coli* BL21 cells harboring pHCE-IIB-GCSF and pG-KJE6 plasmids.

The cell was grown in 10 ml of LB with L-arabinose (1 mg/ml), which was added at the OD_{600}=0, 0.2, 0.5, and 0.8. After 4 h induction, cells were harvested, the protein concentration determined, and separated as soluble (S) and insoluble (I) fractions. Lanes M, BL21, and CSF are the same as in Fig. 1.

foreign proteins in *E. coli* was also observed in the expression of cyclodextrin glucanotransferase [12].

Synergistic Effect of DnaK/DnaJ/GrpE and GroEL/ES

It has been reported that GroEL/ES and DnaK/DnaJ at physiological concentrations act synergistically to ensure proper folding and/or assembly of proteins [3], and coexpression of GroEL/ES together with DnaK/DnaJ/GrpE is more effective in the production of some heterogeneous proteins [15, 19]. On the basis of these findings, the synergistic effect of GroEL/ES and DnaK/DnaJ/GrpE chaperones on the soluble production of hG-CSF was investigated. The expression of the DnaK/DnaJ/GrpE complex was induced by the addition of L-arabinose (final concentration of 1 mg/ml), and the expression of the GroEL/ES chaperone was quantitatively manipulated by adding tetracycline (0–200 ng/ml). In this experiment, L-arabinose and tetracycline were added at the same culture time. In addition, to increase the expression of the *groEL/ES* gene, the tetracycline concentration upto 200 ng/ml was used.

As shown in Fig. 4, the production of DnaK/DnaJ/GrpE was decreased with increasing tetracycline concentration or GroEL/ES expression, and the amount of insoluble hG-CSF protein was also decreased: That is, a greater production of GroEL/ES protein in cells resulted in a reverse effect on the coexpression partners such as DnaK/DnaJ/GrpE and hG-CSF. Comparing Fig. 1 and Fig. 4, the *groEL/ES* gene is seen to be highly expressed by a greater concentration of tetracycline, but the formation of soluble hG-CSF was gradually reduced. This result indicates that the GroEL/ES chaperone, even at high concentration, was not appropriate for the production of soluble hG-CSF.

In order to examine the promoter effect on the soluble production of hG-CSF, *E. coli* BL21 cells harboring pGro7

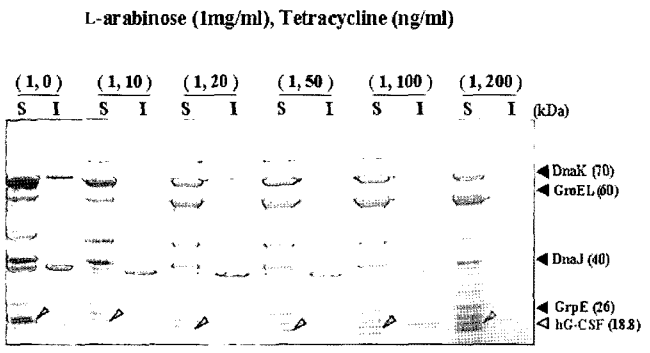

Fig. 4. Synergistic effect of GroEL/ES and DnaK/DnaJ/GrpE chaperones on the hG-CSF production in *E. coli* BL21 cells harboring pHCE-IIB-GCSF and pG-KJE6 plasmids.

Cells were grown on 10 ml of LB with L-arabinose (1 mg/ml) and tetracycline (0–200 ng/ml), added at the early-exponential phase. After 6 h of induction, cells were harvested, the protein concentration determined, and separated as soluble (S) and insoluble (I) fractions. Lanes M, BL21, and CSF are the same as in Fig. 1.

plasmid, in which the *groEL/ES* gene is under the *araB* promoter [19], was tested by varying the L-arabinose concentration. However, at any concentration of L-arabinose, hG-CSF protein was detected significantly in the soluble fraction of cell lysate (data not shown). Therefore, it could be concluded that the large production of soluble hG-CSF was caused by chaperone DnaK/DnaJ/GrpE, not by the *araB* promoter.

The DnaK/DnaJ/GrpE chaperone had an essential effect on the production of soluble hG-CSF. DnaK/DnaJ/GrpE binds to hydrophobic segments of the unfolding polypeptide in order to maintain solubility and prevent aggregation [1]. In contrast, the GroEL/ES chaperone system binds to misfolded polypeptide and allows it to refold when released [11, 18, 28]. Therefore, it seems from this work that the GroEL/ES chaperone interacts with target protein (hG-CSF) after an interaction with the DnaK/DnaJ/GrpE chaperone. Previously, it was reported that the limiting step in hG-CSF periplasmic production in *E. coli* was the cytoplasmic maintenance of a structure component for translocation [21]. In addition, DnaK/DnaJ have eukaryotic counterparts that are required for efficient translocation or secretion of several proteins in yeasts as well as mammalian cells [25]. If hG-CSF has structural features specifically recognized by the DnaK/DnaJ family chaperones, then the DnaK/DnaJ/GrpE chaperone encoded by plasmid pG-KJE6 could improve the production of soluble hG-CSF, and the misfolded hG-CSF is not likely to be restored by the GroEL/ES chaperone. Because of these possibilities of cooperation, the signal intensity of the hG-CSF protein band in the soluble fractions was likely increased from 3.5% to 13.9%, and Western blot analysis also represented about a 4–5-fold increase in the production of soluble hG-CSF over the non-induction case of DnaK/DnaJ/GrpE (Fig. 5).

In conclusion, optimal production of DnaK/DnaJ/GrpE rather than the GroEL/ES chaperone could effectively improve the formation of active and soluble hG-CSF. The concept contained in the present results would be useful in the commercial production of recombinant medical proteins in *E. coli* as active and soluble forms.

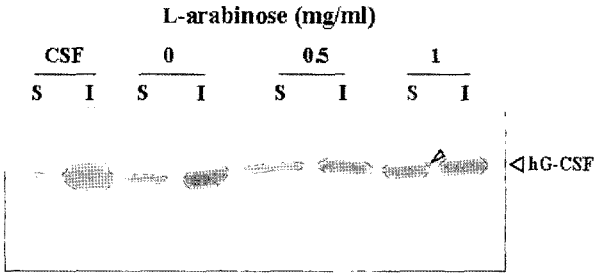

Fig. 5. Western blot analysis of hG-CSF production in *E. coli* BL21 cells harboring pHCE-IIB-GCSF and pG-KJE6 plasmids.

Soluble fraction (S) and insoluble fraction (I). Arrows indicate the soluble form of hG-CSF.

Acknowledgments

This research was supported by a grant (B-2004-04) from Marine Bioprocess Research Center of the Marine Bio 21 Center funded by the Ministry of Maritime Affairs & Fisheries, Republic of Korea. S. L. Park is the recipient of graduate fellowships from the Ministry of Education through the Brain Korea 21 Project.

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