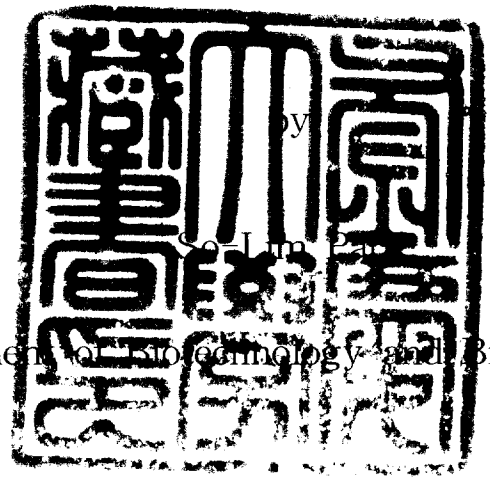
Thesis for the Degree of Master of Engineering

Soluble Production of Foreign Proteins in *E. coli* By Molecular Chaperones



by

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February 2005

*E. coli*에서 molecular chaperone을 이용한 외래 단백질의 수용성 형태 발현

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by

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A thesis submitted in partial fulfillment of the requirement
for the degree of

Master of Engineering

in the Department of Biotechnology and Bioengineering, Graduate School,
Pukyong National University

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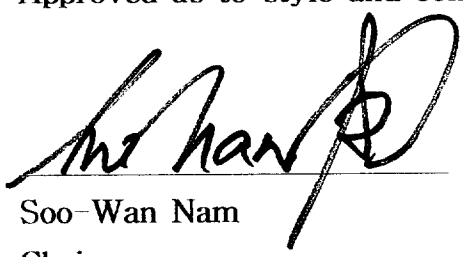
Approved as to style and content by :



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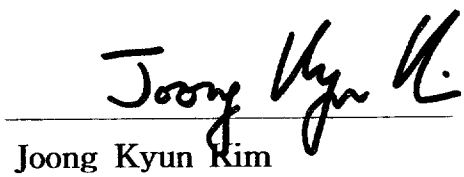

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February 25, 2005

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박소림

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요약

Molecular chaperone은 단백질이 활성을 가지는 구조를 형성하도록 접힘을 도와주는 보조 단백질로 대장균에서 재조합 외래 단백질을 발현시킬 경우 문제시 되는 불용성 복합체인 inclusion body를 활성형의 단백질로 만드는 역할을 한다. Molecular chaperone의 대표적인 예로 DnaK/DnaJ/GrpE 와 GroEL/ES가 있는데 이러한 molecular chaperone의 기능을 증명하기 위해 대장균에서 불용성복합체로 생성되는 human granulocyte colony stimulating factor (hG-CSF)와 alginate lyase를 목적 단백질로 이용하였다. 이러한 목적단백질들과 DnaK/DnaJ/GrpE, GroEL/ES을 함께 발현시킬 수 있는 공발현계 BL21/pHCE-FOREX-GCSF + pG-KJE6 와 BL21/pALP4 + pG-KJE6 가 이용되었다.

구성적 프로모터를 사용한 hG-CSF는 단독발현 시 대부분이 insoluble 형태로 발현되었으며 image analyzer로 분석한 결과 DnaK/DnaJ/GrpE와 공발현 시 유도제인 L-arabinose가 1 mg/ml 일 때 20%이상으로 가장 많은 양의 hG-CSF가 soluble form으로 전환되었다. 그리고 유도 시기는 OD_{600}= 0.2 (early- exponential growth phase)일 때가 최적인 것으로 나타났다. GroEL/ES의 공발현은 CSF 가용성 발현에는 효과가 없는 것으로 나타났으며 DnaK/DnaJ/GrpE와 GroEL/ES를 모두 사용한 경우에도 활성형 hG-CSF 생산에는 영향을 미치지 못했다.

Alginate lyase 역시 단독발현일 경우 대부분이 insoluble 형태로 발현되었으며 그 양은 hG-CSF보다 훨 씬 더 많았다. Alginate lyase의 발현 유도제인 IPTG의 최적 농도는 20 μM 이며 유도 시기는 OD_{600}=0.8 (early-exponential phase)일 때 이다. GroEL/ES와 공발현시 활성형 형태로의 전환은 나타나지 않았으며 오히려 세포성장의 저해를 가져왔다. DnaK/DnaJ/GrpE와 공발현시 유도제인 L-arabinose 0.05 mg/ml을 OD_{600}= 0.4~0.5에서 유도했을 때 단독발현에 비해 효소 활성이 0.0014 unit/mg에서 0.020 unit/mg으로 14배나 증가하는 것으로 나타났으며 image analyzer로 분석한 결과 34%이상의 alginate lyase 가 soluble form으로 전환되는 것으로 나타났다. DnaK/DnaJ/GrpE와 GroEL/ES를 모두 사용한 경우에는 활성형 alginate lyase 생산에 별다른 영향을 끼치지 못하는 것으로 나타났다.

I . Introduction

The high-level expression of recombinant gene products in the gram negative bacterium *Escherichia coli* often results in the misfolding of the protein of interest and its subsequent degradation by proteases or its deposition into biologically inactive aggregates known as inclusion bodies [2, 3, 4, 11, 19]. It is widely recognized that coexpression of molecular chaperones or foldases can assist protein folding, and this leads to increased production of active protein [5, 6, 7, 8, 9, 10, 12, 14, 18]. The most abundant and physiologically important chaperones in *E. coli* include DnaK/DnaJ/GrpE, and GroEL/ES [1, 20].

The Hsp70 chaperone machinery is one of the most conserved chaperone families across the archaeal, bacterial and eukaryotic domains. DnaK, the eubacterial Hsp70 homolog, is an ATP dependent molecular chaperone that functions together with the co-chaperones, DnaJ and GrpE, to mediate protein folding and other essential processes in the cell, under both normal and environmentally stressful growth conditions. The DnaK systems participate in folding of nascent polypeptide chains, protein transport across membranes, proteolysis, assembly of multi-domain protein structures, disassembly of protein aggregates, cell [21]. The hsp60s are large oligomeric complexes of 60 kDa subunit arranged as two stacked heptmeric rings with a central cavity. At elevated temperatures, overexpression

of both GroEL and GroES, but not of each protein separately, can protect newly synthesized proteins from aggregating in *E. coli* cells lacking a normal heat shock response. GroEL is able to prevent the thermal aggregation of a variety of enzymes. The molecular chaperone complex DnaK/DnaJ/GrpE interacts with nascent polypeptide chains to prevent irreversible polypeptide aggregation and mediate partial folding [1, 13]. GroEL/ES then interacts with the partially folded proteins and completes the folding [1, 3, 4, 15, 16, 17, 22].

In this work, the target protein was human granulocyte colony stimulating factor (hG-CSF) and alginate lyase. hG-CSF is a member of a family of glycoproteins which play an important role in stimulating proliferation, differentiation and functional activation of blood cells [23, 24, 26, 28, 29]. 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 neutrophil [27]. Previously it was reported that hG-CSF expressed in many other host strain yeast, *E. coli*, *Aspergillus niger* [23, 24, 25, 26, 27, 28, 30, 31]. But when the human granulocyte colony stimulating factor (hG-CSF) was expressed in *E. coli*, it was aggregated into the insoluble particles known as inclusion bodies [23].

Alginate lyase of *Pseudomonas elyakovii*, another target protein catalyze the depolymerization of alginates by a β-elimination mechanism with the formation of 4-deoxy-L-erythro-hex-4-ene pyranosyluronate at the nonreducing end of the resultant product.

Alginates are synthesized as cell wall components by brown

seaweeds and linear (1-4)-linked glycuronans comprised of residues of β-D-mannosyluronic acid (M) and its C-5 epimer α-L-gulosyluronic acid (G) [32, 33, 34, 35].

Alginate is widely used in food and pharmaceutical industries due to its ability to chelate metal ions and form a highly viscous solution [36], recently, polymers and oligosaccharides with novel physicochemical and physiological functions are sought by biopolymer-based industries in order to expand the application areas of polysaccharides [37]. However, it is not easy to obtain novel polysaccharides with more excellent and safe properties than existing ones through screening of microorganisms. In cystic fibrosis patients, *Pseudomonas aeruginosa* produces alginate, which facilitates the attachment of the bacterium to tracheal mucins. The exopolysaccharide protects the microorganism from phagocytes and prevents antibiotic uptake. Consequently, it is a major pathogenic factor in these patients [38]. It is necessary to look inhibitors of alginate synthesis with potential use as therapeutic agents in cystic fibrosis patients [35].

In this paper, we described the soluble production of foreign proteins in *E. coli* by molecular chaperones. The effect of molecular chaperones DnaK/DnaJ/GrpE and GroEL/ES on the production of soluble hG-CSF and soluble alginate lyase in recombinant *E. coli* was investigated.

Table 1. Characteristics and functions of chaperones used in this work

Gene	Protein	Subunit size (kDa)	Function
groEL	GroEL	60	Promotes protein folding; required for phage assembly
groES	GroES	10	mRNA stabilization
dnaK	DnaK	70	Stabilizes newly made proteins *in vivo*; preserves folding competence of polypeptides; promotes oligomer assembly /disassembly
dnaJ	DnaJ	40	
grpE	GrpE	26	

Fig. 1. The functional expression mechanism of the molecular chaperone GroEL/ES.

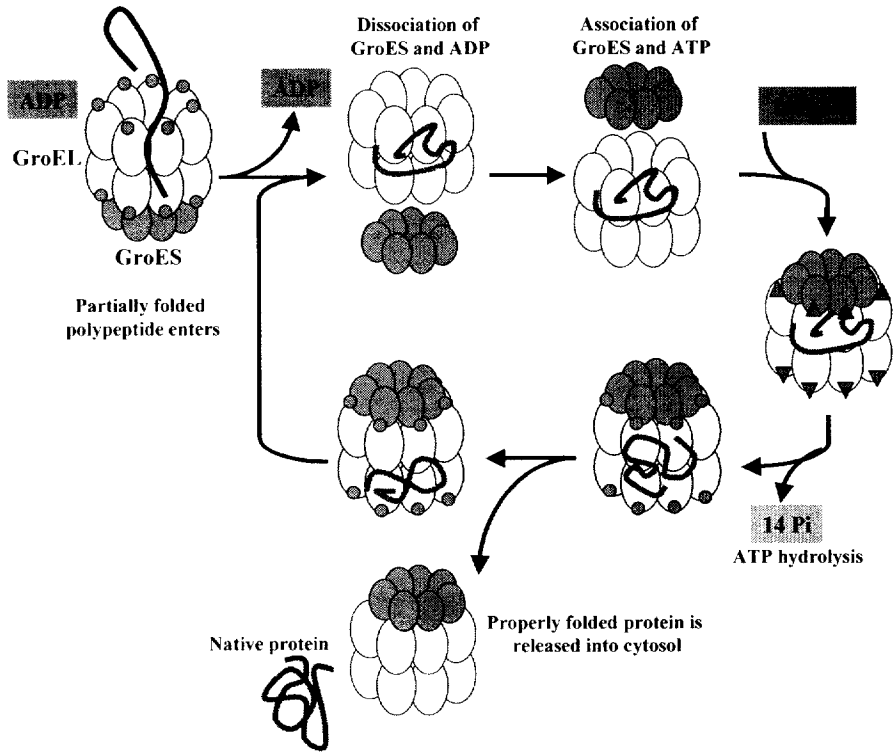
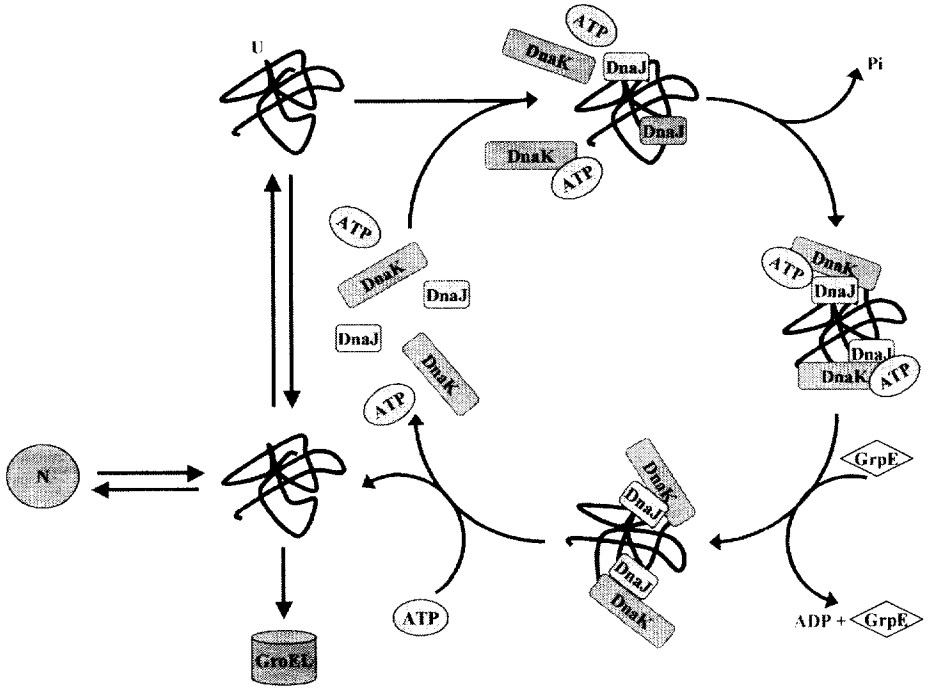


Fig. 2. The functional expression mechanism of the molecular chaperone DnaK/DnaJ/GrpE.



II. MATERIALS AND METHODS

1. Bacterial Strain, Plasmids and media

E. coli BL21(DE3)[F , *ompT*, r_B , m_B ,(DE3)] strain was used in all experiments. The plasmid pHCE-FOREX-GCSF was encoded *hg-csf* gene. The transcription of *hg-csf* gene in the plasmid pHCE-FOREX-GCSF is controlled by HCE promoter. HCE promoter derived up stream from the D-amino acid aminotransferase gene of *geobacillus toebii* was developed for the high constitutive expression of foreign proteins without induction [39] and pALP4, alginate lyase (*aly*) gene were constructed under the control of T7 promoter. The plasmid pG-KJE6 is a pACYC184-based chloramphenicol-resistant plasmid. The transcription of *groEL/ES* genes and *dnaK/dnaK/grpE* gene in the plasmid pG-KJE6 is controlled by *Pzt*-1 promoter and *araB* promoter [20]. The transformed *E. coli* cells were selected on LB agar plates containing 50 ㎍/ml ampicillin (selection for pHCE-FOREX-GCSF and pALP4) and 50 ㎍/ml chloramphenicol (selection for pG-KJE6).

2. Culture media and culture conditions

E. coli cells were grown on LB medium (1% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.5% NaCl). *E. coli* BL21(DE3) strains

Table 2. Bacterial strains and expression plasmids used in this work

harboring pHCE-FOREX-GCSF and pALP4 were grown in the presence of 10 ml LB + 50 ㎍/ml ampicillin, pHCE-FOREX-GCSF +

Table 2. Bacterial strain and expression plasmids used in this work

Strains	Genetic markers	Sources
Host Strains		
E. coli DH5α	*sup44 Δlac U169(ΦlacZΔM15) hsdR17 recA1 endA1 gyrA96 Tri-1 relA1*	KRIBB
BL21	F-, *ompT*, rB-, mB-, (DE3)	KRIBB
Plasmids	**Characteristics**	**Sources**
pHCE-FOREX-GCSF	pHCE-FOREX-*hg-csf* from human	BioLeaders
pALP4	pET25-*alg* from *P. elyakovii*	Ref. 32
pG-KJE6	*pzt-1*p-*groEL/ES* + *araB*p-*dnaK/dnaJ/grpE*	Ref. 28

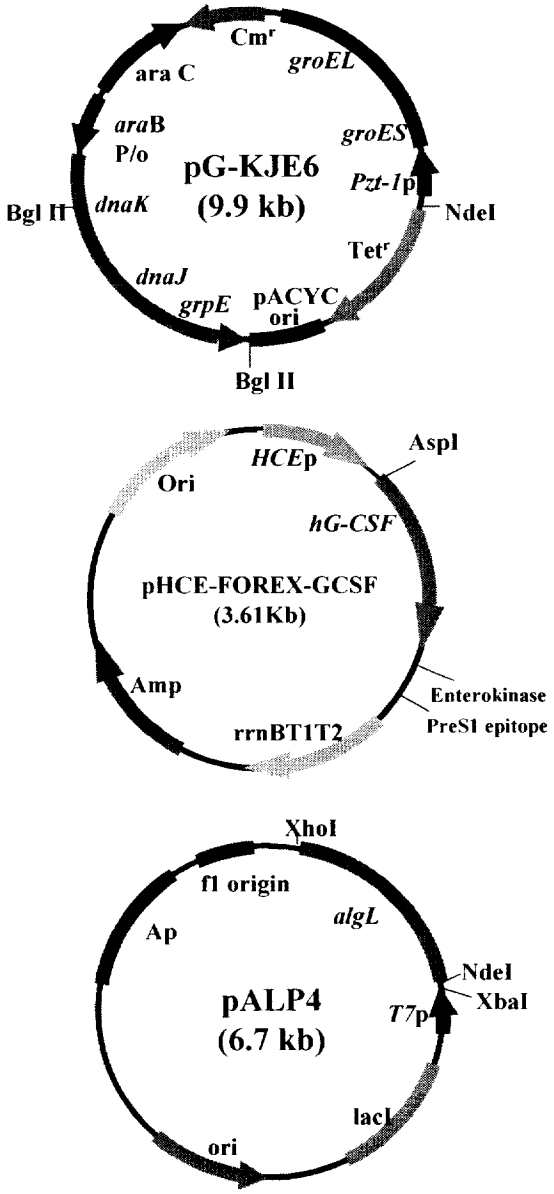


Fig. 3. Schematic diagram of recombinant plasmids used in this work.

pG-KJE6 and pALP4 pG-KJE6 were grown in the presence of 10 ml LB + 50 ㎍/ml and ampicillin and 50 ㎍/ml chloramphenicol. To induce the expression of *groEL/ES* and *dnaK/dnaK/grpE* genes, tetracycline and L-arabinose, were used.

3. Measurement of protein concentration

cells were disrupted by sonication (Sonoplus HD2070, Bandelin., Germany) for 1 min on ice, and then centrifuged at 9800×*g* for 10 min for the separation into of the soluble and insoluble fractions. Each of the fractions were analyzed by SDS-PAGE. For the determination of protein concentration, modified micromethod of Lowry was used.

4. SDS-PAGE analysis

10% SDS-PAGE take advantage of protein separating. The GroEL/ES, DnaK/DnaJ/GrpE and hG-CSF, alginate lyase proteins were detected by staining the gel with Coomassie brilliant blue RT250. The gel was scanned by an Image Analyzer (FluorChem 5500, Alpha Innotech., USA)

5. Alginate lyase enzyme activity

Alginate lyase assayed in a mixture containing 0.2% alginate · Na (Sigma, USA) 1M Tris-HCl (pH 7.5), 0.3M NaCl [32]. The reaction was monitored at 37℃ for 5 min, depending on the increase in absorbance at 235 nm in comparison without enzyme. One unit of alginate lyase was defined as the amount of enzyme required to increase the absorbance by 1.0 at 235 nm per min [40].

6. Western blot analysis

hG-CSF were blotted to PVDF membranes after SDS-PAGE (Trans Blot SD Semi-Dry Transfer Cell, BIO-RAD., USA). The membrane were blocked with blocking buffer (5% skim milk) for 1 hr, then incubated for O/N with primary antibody. mouse monoclonal anti-preS1 tag (APROGEN, Korea) at a 1/3000 dilution, and biotinylated anti-mouse IgG (VECTORLAB, USA), at a 1/4000 dilution were used as a primary and secondary antibodies. After washing, the membrane were incubated with ABC kit (VECTORLAB, USA). Band were visualized by addition of diaminbenzidine (DAB substrate kit for peroxidase, VECTORLAB, USA) and H_2O_2 in PBS.

III. RESULTS AND DISCUSSION

1. Effect of coexpression with chaperone on the soluble hG-CSF production

1-1. Effect of GroEL/ES chaperones on the soluble hG-CSF production

Equal amounts (1 ㎍) of pHCE-FOREX-GCSF and pG-KJE6 were co-transformed into *E. coli* BL21(DE3). The transformed *E. coli* cells were selected on LB agar plates containing 50 ㎍/ml ampicillin (selection for pHCE-FOREX-GCSF) and 50 ㎍/ml chloramphenicol (selection for pG-KJE6). To induce the expression of *groEL/ES* genes, tetracycline were used. The effect of GroEL/ES on the hG-CSF expression was investigated by various tetracycline concentrations from 0~5 ng/ml. The recombinant *E. coli* cell, BL21/pHCE-FOREX GCSF + pG-KJE6, was cultivated on LB medium at 37℃ and tetracycline was added at OD_{600}= 0.2~0.3. After 6 hr induction, cells were harvested, determined the protein concentration, and separated as soluble (S) and insoluble (I) fractions. As shown in Fig. 4, most of hG-CSF (18.8 kDa) was expressed as insoluble form. This result was also observed in the hG-CSF expression in *E. coli* [23, 31]. The hG-CSF decreased with increasing tetracycline concentration and on SDS-PAGE analysis with soluble and insoluble fractions, GroEL (60 kDa) was found out to express but hG CSF was not found in the soluble fraction. Furthermore when the tetracycline concentration 5 ng/ml over, cell growth was

considerably decreased and for that reason, total amount of hG-CSF protein was reduced. Since the molecular weight of GroES protein is too small (10 kDa), GroES protein was clearly not shown in the gel. The hG-CSF protein was accumulated within the cell as inclusion bodies (Fig. 4) and it appear that GroEL/ES chaperone was not effect on soluble hG-CSF production.

1 2. Effect of DnaK/DnaJ/GrpE chaperones on the soluble hG-CSF production

The effects of coexpression of DnaK/DnaJ/GrpE chaperone on the synthesis and solubilization of hG-CSF was tested. To induce the expression of *dnaK/dnaJ/grpE* genes, L-arabinose were used. The recombinant *E. coli* cell, BL21/pHCE-FOREX-GCSF + pG-KJE6, was cultivated on LB medium at 37℃ and tetracycline was added at OD_{600}= 0.2~0.3. After 6 hr induction, cells were harvested, determined the protein concentration, and separated as soluble (S) and insoluble (I) fractions. The result that various L-arabinose concentration from 0~10 mg/ml examined, the greatest amount of soluble hG-CSF protein was detected at 1 mg/ml L-arabinose (Fig. 5).

An SDS-PAGE analysis showed that the DnaK (70 kDa), DnaJ (40 kDa), GrpE (26 kDa) protein was overexpressed and found in the soluble fraction. In spite of high concentration of L-arabinose, cell growth obstruction was less than tetracycline concentration.

1-3. Effect of L-arabinose induction time

To monitor effect of L-arabinose induction time, the cell was grown on LB with L-arabinose 1 mg/ml, which was added at the

OD_{600}= 0, 0.2, 0.5 and 0.8. When induction of DnaK/DnaJ/GrpE at OD_{600}= 0, cell growth was significantly block (Fig. 6). This decrease of cell growth and hG-CSF protein in absence L-arabinose at OD_{600}=0 were most likely due to the metabolic stress cause by replication and maintenances of the two plasmid. When L-arabinose were added at OD_{600}=0.2, active hG-CSF production was significantly increased (Fig. 6).

1-4. Synergic effect of GroEL/ES and DnaK/DnaJ/GrpE chaperones on the soluble hG-CSF production

The synergic effect of GroEL/ES and DnaK/DnaJ/GrpE chaperones on the soluble hG-CSF production was tested. For the DnaK/DnaJ/GrpE expression, L-arabinose 1 mg/ml with tetracycline 0~300 ng/ml which was added at OD_{600}= 0.2~0.3. After 6 hr induction, cells were harvested, determined the protein concentration, and separated as soluble (S) and insoluble (I) fractions. DnaK/DnaJ/GrpE decreased with increasing tetracycline concentration, what is worse amount of insoluble hG-CSF protein was decreased too (Fig. 7).

1-5. Image analysis of SDS- PAGE and western blot analysis

The gel (Fig. 5) was scanned by an Image Analyzer (FluorChem 5500, Alpha Innotech., USA). When scanned the protein bands on the gel, the percentage of hG-CSF band in the soluble fractions was increased from 2% to 22% by increasing the L-arabinose concentration from 0 mg/ml to 5 mg/ml (Fig. 5, Table. 3). It were increased 20% compare with non-induction sample. The

soluble and insoluble fraction from culture samples of the same amount (10μg) were analyzed by SDS-PAGE using 10% gel. And then, hG-CSF confirmed by Western blotting analysis with mouse monoclonal anti-preS1 tag (APROGEN, KOREA), and biotinylated anti-mouse IgG (VECTORLAB, USA) used as a primary and secondary antibodies. Band were visualized by addition of ABC kit (VECTORLAB, USA), diaminbenzidine (DAB substrate kit for peroxidase, VECTORLAB, USA) and H_2O_2 in PBS. When DnaK/DnaJ/GrpE chaperones were induced 1mg/ml L-arabinose the amount of hG-CSF in the soluble fraction was increased 3-fold significantly (Fig. 8). This result was proved that result of Fig. 5.

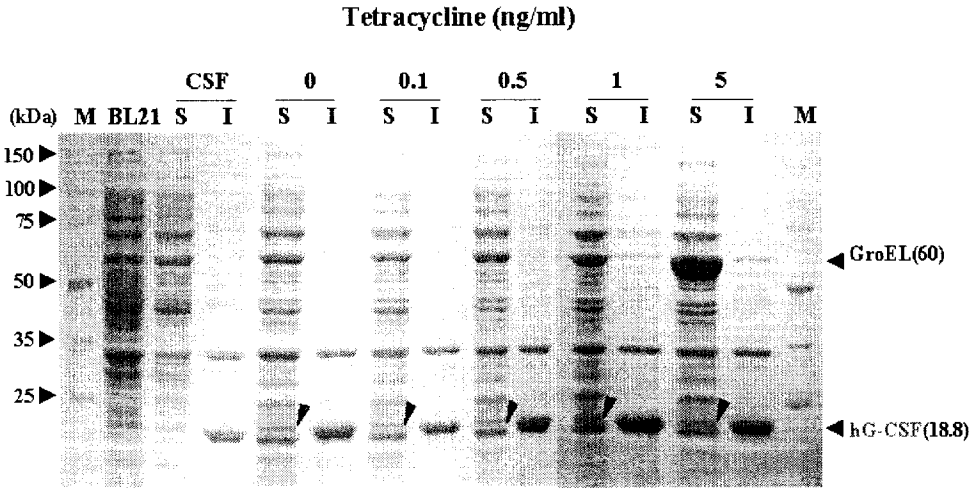


Fig. 4. Effect of GroEL/ES chaperones on the hG-CSF production in the recombinant *E. coli* BL21/pHCE-FOREX-GCSF + pG-KJE6. The cell was grown on 10 ml LB with tetracycline (0~5 ng), which was added at the early-exponential phase. After 6 hr induction, cells were harvested, determined the protein concentration, and separated as soluble (S) and insoluble (I) fractions. The fractions were analyzed by SDS-PAGE (10% gel).

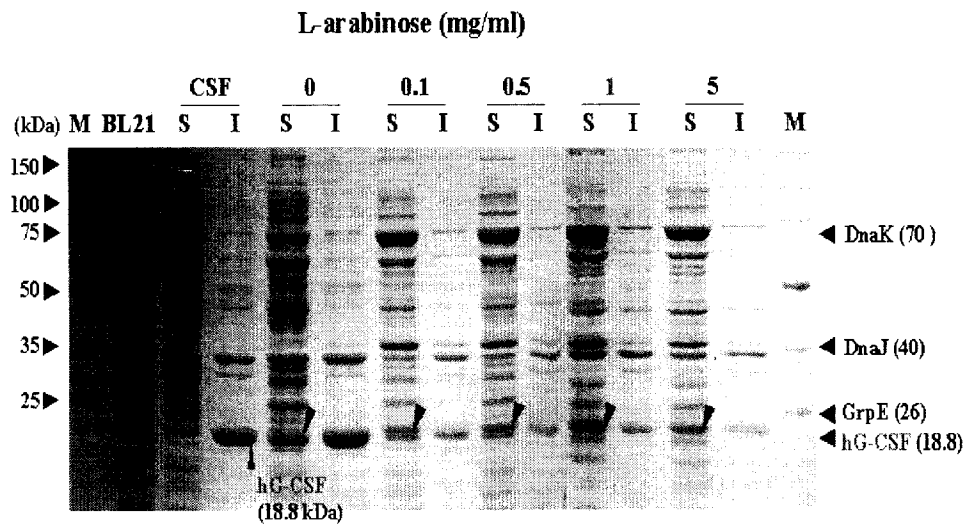


Fig. 5. Effect of DnaK/DnaJ/GrpE chaperones on the hG-CSF production in the recombinant *E. coli* BL21/pHCE-FOREX-GCSF + pG-KJE6. The cell was grown on 10 ml LB with L arabinose (0~5 mg), which was added at the early-exponential phase. After 6 hr induction, cells were harvested, determined the protein concentration, and separated as soluble (S) and insoluble (I) fractions. The fractions were analyzed by SDS-PAGE (10% gel).

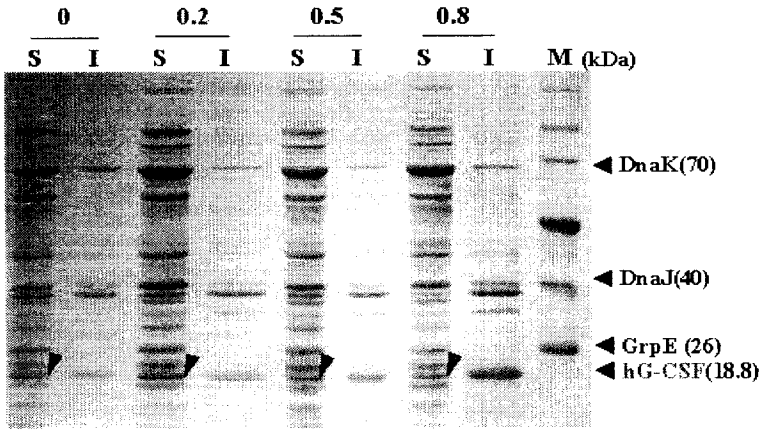


Fig. 6. Effect of L-arabinose induction time in BL21/pHCE-FOREX-GCSF + pG-KJE6. The cell was grown on 10 ml LB with L-arabinose (1 mg), which was added at the OD_{600}= 0, 0.2, 0.5 and 0.8. After 4 hr induction, cells were harvested, determined the protein concentration, and separated as soluble (S) and insoluble (I) fractions.

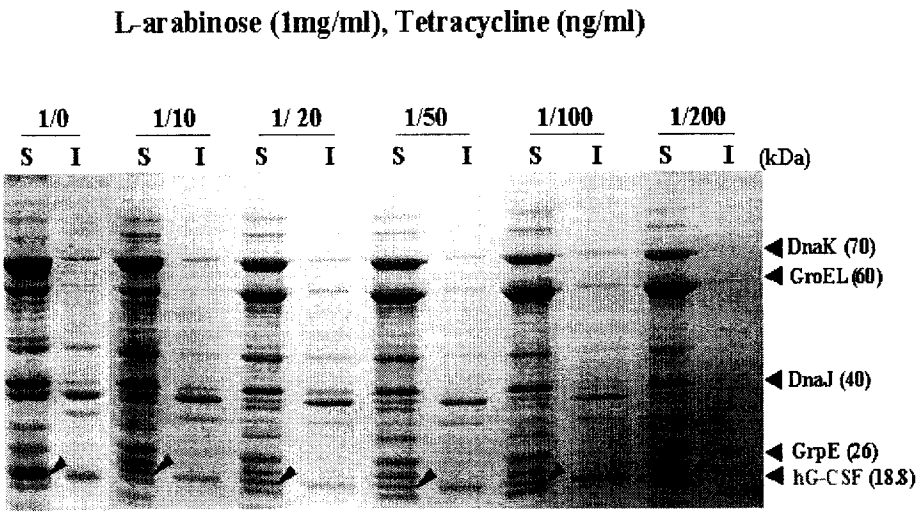


Fig. 7. Synergic effect of GroEL/ES and DnaK/DnaJ/GrpE chaperones on the hG-CSF production in the recombinant BL21/pHCE-FOREX-GCSF + pG-KJE6. The cell was grown on 10 ml LB with L-arabinose (1 mg), tetracycline (0~200 ng) which was added at the early-exponential phase. After 6 hr induction, cells were harvested, determined the protein concentration, and separated as soluble (S) and insoluble (I) fractions. The fractions were analyzed by SDS-PAGE (10% gel).

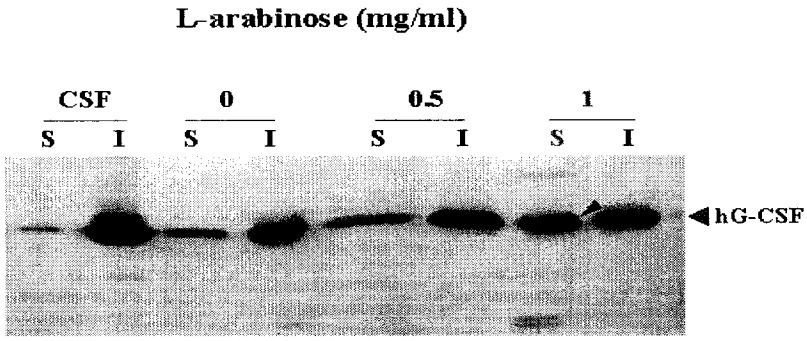


Fig. 8. Western blot analysis of hG-CSF production in the recombinant *E. coli* BL21/pHCE- FOREX-GCSF + pG-KJE6. Soluble form (S) and insoluble form (I). Arrows indicate the soluble form of hG-CSF.

Table 3. Ratio of hG-CSF protein in the soluble and insoluble forms. Only DnaK/DnaJ/GrpE was produced. Each fraction was separated on 10% SDS-PAGE, followed by Coomassie staining, and scanned by the Image Analyzer. The total amount of hG-CSF produced was taken as 100%.

Condition	hG-CSF	
	Soluble (%)	Insoluble (%)
pHCE-FOREX-GCSF	2	98
pHCE-FOREX-GCSF + pG-KJE6 (DnaK/DnaJ/GrpE)	22	50

2. Effect of coexpression with chaperones on the soluble alginate lyase production

2-1. Effect of IPTG concentration on the alginate lyase

1 ㎍ of pALP4 was into *E. coli* BL21(DE3). The transformed *E. coli* cells were selected on LB agar plates containing 50 ㎍/ml ampicillin. *E. coli* cells were grown on LB medium (1% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.5% NaCl) + 50 ㎍/ml ampicillin. To induce the expression of *aly* gene, IPTG were used. The optimal IPTG concentration for the expression of alginate lyase was 20 μM, and alginate lyse activity was 0.0014 unit/㎎ (Fig. 9). Because almost of alginate lyase was expressed as inactive inclusion body, enzyme activity was very lower. This is probably because a high expression rate causes the protein to accumulate with an abnormal conformation, leading to the creation of inclusion bodies, or that it was degraded by the proteolytic machinery of the cells.

2-2. Effect of GroEL/ES chaperones on the soluble alginate lyase production

Equal amounts (1 ㎍) of pALP4 and pG-KJE6 were co-transformed into *E. coli* BL21(DE3). The transformed *E. coli* cells were selected on LB agar plates containing 50 ㎍/ml ampicillin (selection for pALP4) and 50 ㎍/ml chloramphenicol (selection for pG-KJE6). The effect of GroEL/ES on the alginate lyase expression was investigated by various tetracycline concentrations from 0~100 ng/ml. The recombinant *E. coli* cell, BL21/pALP4 + pG-KJE6, was

cultivated on LB medium at 37℃ and tetracycline was added at OD_{600}= 0.4~0.5 then IPTG 20 μM was added at OD_{600}= 0.8. After 5 hr induction, cells were harvested, determined the protein concentration, and separated as soluble (S) and insoluble (I) fractions. Most of alginate lyase (51 kDa) was expressed as insoluble form (Fig. 10). The alginate lyase decreased with increasing tetracycline concentration. When tetracycline concentration overhead 20 ng/ml, cell growth and alginate lyase enzyme activity was reduced as a half. It appear that GroEL/ES chaperone was not effective on the soluble alginate lyase production.

2-3. Effect of DnaK/DnaJ/GrpE chaperones on the soluble alginate lyase production

The effects of coexpression of DnaK/DnaJ/GrpE chaperone on the synthesis and solubilization of alginate lyase was tested. To induce the expression of *dnaK/dnaJ/grpE* genes, L-arabinose were used. The recombinant *E. coli* cell, BL21/pHCE-FOREX-GCSF + pG-KJE6, was cultivated on LB medium at 37℃ and L-arabinose was added at OD_{600}= 0.4~0.5 and then IPTG 20 μM was added at OD_{600}= 0.8. After 5 hr induction, cells were harvested, determined the protein concentration, and separated as soluble (S) and insoluble (I) fractions. The result that various L-arabinose concentration from 0~1 mg/ml examined, the greatest amount of soluble alginate lyase protein was detected at 0.05 mg/ml L-arabinose (Fig. 11).An SDS PAGE analysis showed that the DnaK (70 kDa), DnaJ (40 kDa), GrpE (26 kDa) protein was overexpressed and found in the soluble fraction. DnaJ was significantly increased at 0.05 mg/ml L-arabinose

concentration. Enzyme activity of alginate lyase in the soluble fraction was increased from 0.0014 unit/ml to 0.0200 unit/ml by increasing the L-arabinose concentration from 0 mg/ml to 0.05 mg/ml. It were increased 14-fold compare with non-induction sample. And after this, expression of alginate lyase in both of soluble and insoluble fraction and cell growth was decreased. This result seems due to an metabolite burden by overexpression of DnaK/DnaJ/GrpE chaperones have an effect on cell growth.

2 4. Effect of L-arabinose induction time

To monitor effect of L-arabinose induction time, the cell was grown on LB with L-arabinose 0.05 mg/ml, which was added at the OD_{600}= 0, 0.2, 0.4, 0.6 and 0.8. When induction of DnaK/DnaJ/GrpE at OD_{600}= 0, cell growth was significantly block (Fig. 12). This decrease of cell growth and alginate lyase protein in absence L-arabinose at OD_{600}=0 were most likely due to the metabolic stress cause by replication and maintenances of the two plasmid. When L-arabinose was added at OD_{600}=0.4(early-exponential phase), active alginate lyase production was significantly increased (Fig. 12).

2-5. Synergic effect of GroEL/ES and DnaK/DnaJ/GrpE chaperones on the soluble alginate lyase production

The synergic effect of GroEL/ES and DnaK/DnaJ/GrpE chaperones on the soluble alginate lyase production was tested. For the DnaK/DnaJ/GrpE expression, L-arabinose 0.05 mg/ml with tetracycline 0~50 pg/ml which was added at OD_{600}= 0.4~0.5 then IPTG 20 μM was added at OD_{600}= 0.8. After 5 hr induction, cells

were harvested, determined the protein concentration, and separated as soluble (S) and insoluble (I) fractions. DnaK/DnaJ/GrpE and soluble alginate lyase decreased with increasing tetracycline concentration, and hence amount of insoluble alginate lyase protein was decreased, too (Fig. 13). The amount of active alginate lyase produced differed considerably depending on the molecular chaperone coexpressed. Coexpression of GroEL/ES did not improve the productivity of correctly folded active alginate lyase. This is probably because correct disulfide bond formation is critical for correct folding of alginate lyase. When GroEL/ES is overexpressed, partially folded proteins interacting with GroEL/ES are accumulated and are most likely degraded by proteases.

2-6. Image analysis of SDS- PAGE

The gel (Fig. 11) was scanned by an Image Analyzer (FluorChem 5500, Alpha Innotech., USA). Each fractions were separated on 10% SDS-PAGE, followed by Coomassie staining, and scanned by the Image Analyzer. The total amount of alginate lyase(pALP4) produced was taken as 100%. When scanned the protein bands on the gel, the percentage of alginate lyase band in the soluble fractions was increased from 3% to 37% by increasing the L-arabinose concentration from 0 mg/ml to 0.05 mg/ml (Fig. 11, Table. 4). It was increased by 34% compared with non-induction sample.

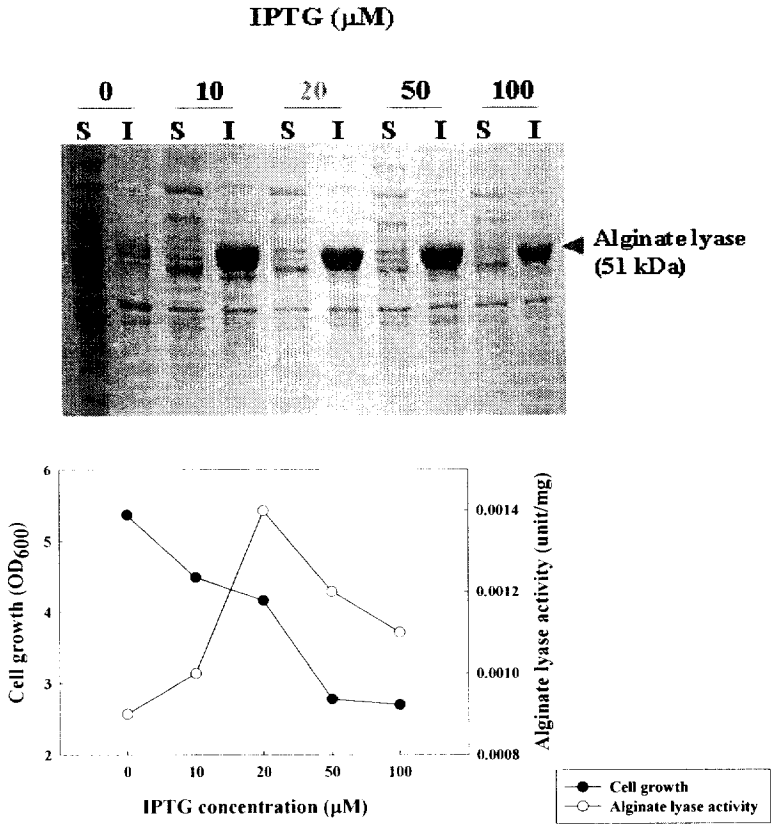


Fig. 9. Effect of IPTG concentration on the alginate lyase production in the recombinant *E. coli* BL21/pALP4. The cell was grown on 10 ml LB with IPTG (0 ~ 100 μM), which was added at the mid-exponential phase. After 5 hr induction, the cells were harvested, disrupted with sonication, and separated as soluble (S) and insoluble (I) fractions. Each fractions were analyzed by SDS-PAGE (10% gel), and soluble fraction was assayed for alginate lyase activity.

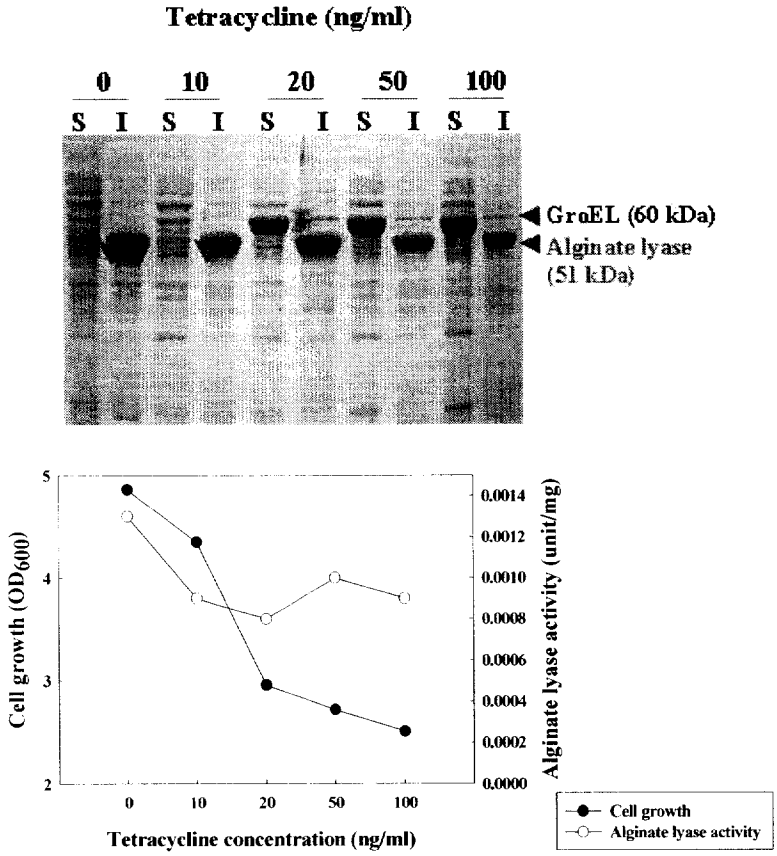


Fig. 10. Effect of GroEL/ES chaperones on the alginate lyase production in the recombinant *E. coli* BL21/pALP4 + pG-KJE6. The cell was grown on 10 ml LB with tetracycline (0 ~ 100 ng), which was added at the early-exponential phase. After 5 hr induction, the cells were harvested, disrupted with sonication, and separated as soluble (S) and insoluble (I) fractions. Each fractions were analyzed by SDS PAGE(10% gel), and soluble fraction was assayed for alginate lyase activity.

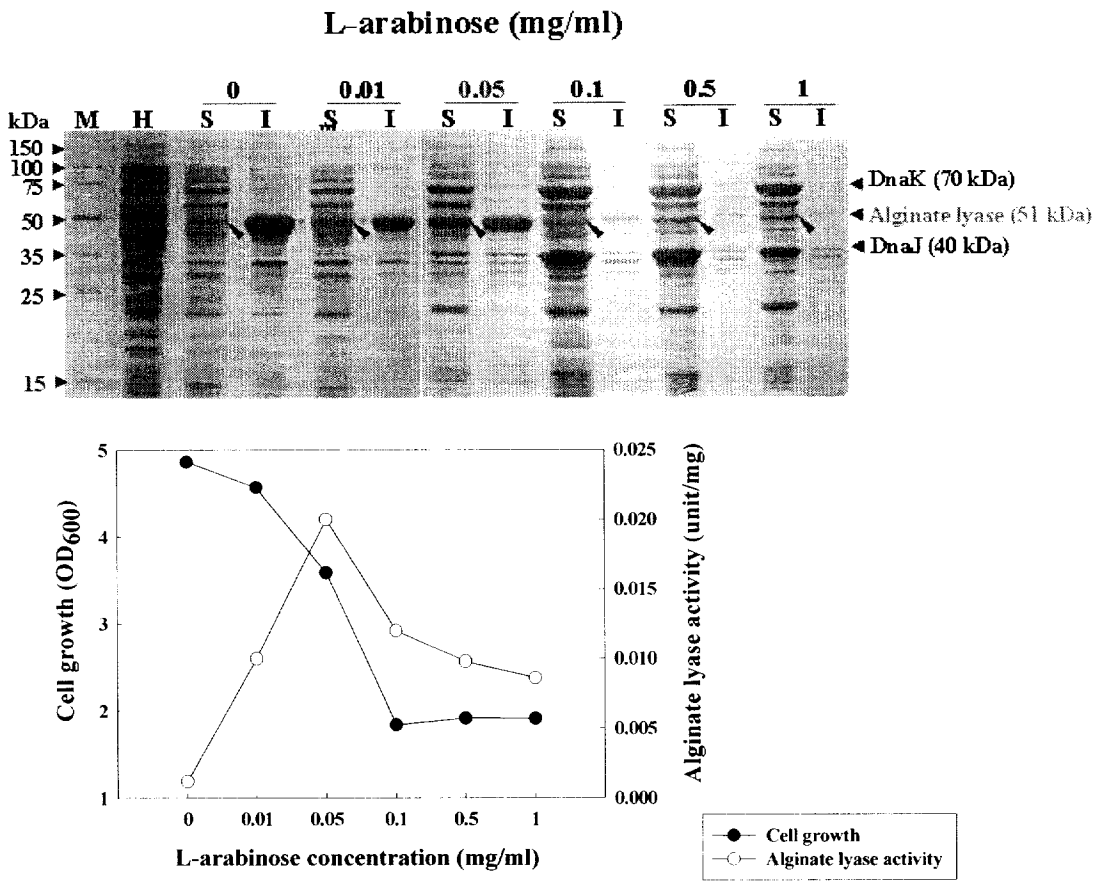


Fig. 11. Effect of DnaK/DnaJ/GrpE chaperones on the alginate lyase production in the recombinant *E. coli* BL21/pALP4 + pG-KJE6. The cell was grown on 10 ml LB with L-arabinose (0~1 mg), which was added at the early-exponential phase. After 5 hr induction, the cells were harvested, disrupted with sonication, and separated as soluble (S) and insoluble (I) fractions. Each fractions were analyzed by SDS-PAGE (10% gel), and soluble fraction was assayed for alginate lyase activity.

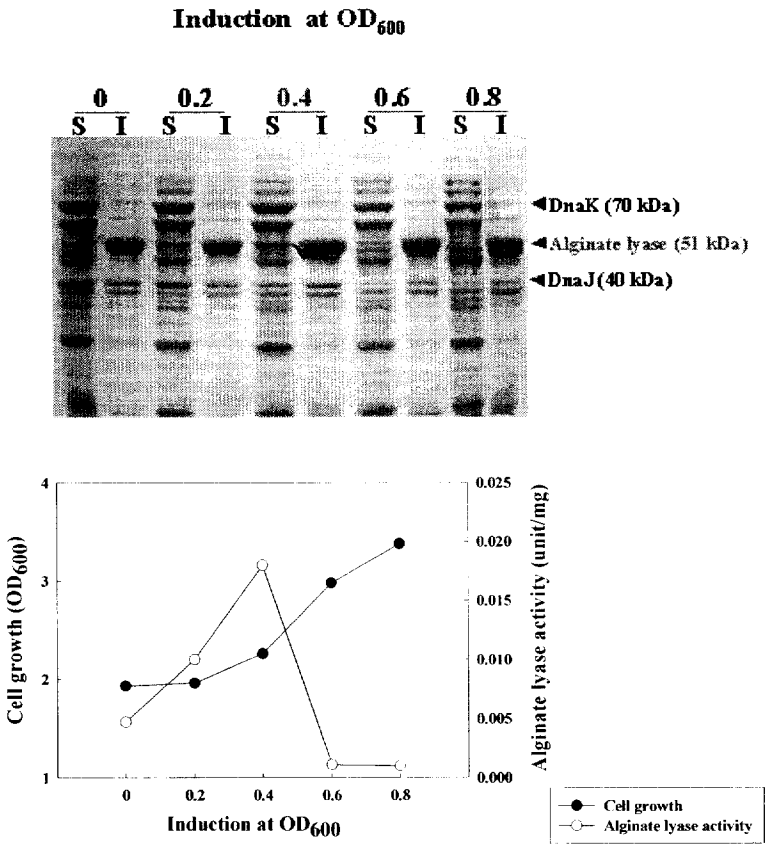


Fig. 12. Effect of L-arabinose induction time on the alginate lyase production in *E. coli* BL21/pALP4 + pG-KJE6. The cell was grown on 10 ml LB with L-arabinose (0.05 mg), which was added at the OD_{600}=0, 0.2, 0.4, 0.6 and 0.8. After 5 hr induction, the cells were harvested, disrupted with sonication, and separated as soluble (S) and insoluble (I) fractions. Each fractions were analyzed by SDS-PAGE (10% gel), and soluble fraction was assayed for alginate lyase activity.

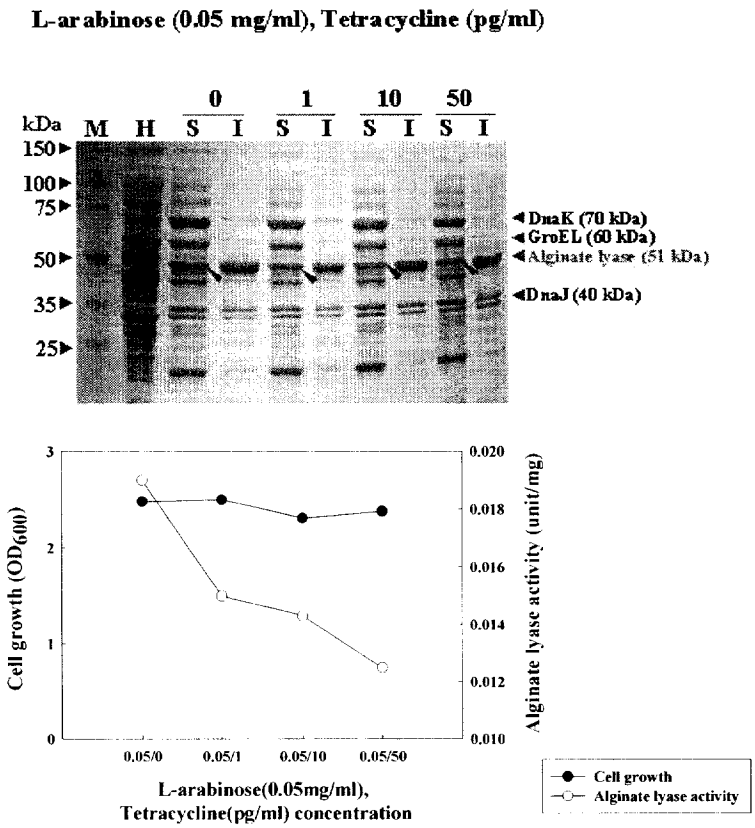


Fig. 13. Synergic effect of DnaK/DnaJ/GrpE and GroEL/ES chaperones on the alginate lyase production in the recombinant *E. coli* BL21/pALP4 + pG-KJE6. The cell was grown on 10 ml LB with L-arabinose (0.05 mg) and tetracycline (0~50 pg), which were added at the early-exponential phase. After 5 hr induction, the cells were harvested, disrupted with sonication, and separated as soluble (S) and insoluble (I) fractions. The fractions were analyzed by SDS-PAGE (10% gel), and soluble fraction was assayed for alginate lyase activity.

Table 4. Ratio of alginate lyase protein in the soluble and insoluble forms when only DnaK/DnaJ/GrpE was produced. Each fractions were separated on 10% SDS-PAGE, followed by Coomassie staining, and scanned by the Image Analyzer. The total amount of alginate lyase(pALP4) produced was taken as 100%.

Condition	Alginate lyase		
	Soluble (%)	Insoluble (%)	Activity(unit/mg)
pALP4	3	97	0.0014
pALP4 + pG-KJE6 (DnaK/DnaJ/GrpE)	37	50	0.0200

Ⅳ. CONCLUSION

When overexpression of recombinant human granulocyte colony stimulating factor (hG-CSF) gene and alginate lyase (aly) gene in the gram negative bacterium *Escherichia coli* results in inactive aggregates known as inclusion bodies. To improved that soluble expression of this proteins, molecular chaperones DnaK/DnaJ/GrpE, and GroEL/ES were coexpressed with target proteins.

GroEL/ES was not effect on soluble production of hG-CSF. The greatest amount of soluble hG-CSF protein was detected at 1 mg/ml L-arabinose for induced the DnaK/DnaJ/GrpE chaperone. When L-arabinose were added at OD_{600}-0.2, active hG-CSF production was significantly increased. Synergic effect of GroEL/ES and DnaK/DnaJ/GrpE on the soluble hG-CSF production was not observed. Result of Image Analysis, the percentage of hG-CSF band in the soluble fractions was increased from 2% to 22%. It were increased 20% compare with non-induction sample. And western blot analysis was correspond to image analysis result.

On soluble expression of alginate lyase, GroEL/ES also was not effective. The greatest amount of soluble alginate lyase protein was detected at 0.05 mg/ml L-arabinose for induced the DnaK/DnaJ/GrpE at OD_{600}= 0.4~0.5. Enzyme activity of alginate lyase in the soluble fraction was increased from 0.0014 unit/ml to 0.0200 unit/ml by increasing the L-arabinose concentration from 0 mg/ml to 0.05 mg/ml. It were increased 14-fold compare with non-induction sample.

Result of Image Analysis, percentage of alginate lyase band in the soluble fractions was increased from 3% to 37% by increasing the L-arabinose concentration from 0 mg/ml to 0.05 mg/ml. It were increased 34% compare with non-induction sample. Synergic effect of GroEL/ES and DnaK/DnaJ/GrpE on the soluble alginate lyase production was not observed. In the overexpression of proteins that have several cystein thiol groups and no disulfide bond, like glutamate racemase, it appears that GroEL/ES are probably most effective for improving the formation of correctly folded and active proteins[41]. Previously reports, CGTase that have no disulfide bond was coincide with this fact. On the soluble production of CGTase was more effective GroEL/ES chaperone [6, 7]. Since hG-CSF and alginate lyase have 5 and 2 cystein, coexpression of DnaK/DnaJ/GrpE significantly increased the formation of active hG-CSF and alginate lyase, while GroEL/ES reduced it.

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Ⅵ. ACKNOWLEDGEMEN

높아만 보이던 시작이 어느새 끝나버린 지금 더 열심히 하지 못했던 것에 대해 후회가 듭니다. 하지만 지금의 지식을 배울 수 있게 학부 때부터 5년이라는 시간동안 후원해주시고 지도해주신 남수완 교수님께 감사의 마음을 전하고 싶습니다. 그리고 김성구 교수님과 김중균 교수님께서 주셨던 애정 어린 관심과 격려 또한 감사 할 따름입니다. 언제나 밝은 얼굴로 인사해 주셨던 최우봉 교수님과 김동은 교수님 그리고 전승종교수님께도 고마운 마음을 전하고 싶습니다.

실험실의 큰 버팀목이 되어주는 종현선배와 연희언니, 언제든 따뜻하게 맞아준 영원한 사수 미정이언니와 재범선배 그리고 동하선배 부부에게도 고마움을 전하고 싶습니다. 괴롭고 힘들 때마다 술과 함께 값진 충고가 되어준 선연언니와 그 자리에 언제나 함께 했던 잊을 수 없는 나의 동기들 정하와 주희 그리고 낙천적인 성격으로 나를 편하게 이끌었던 후배 연화와 책임감 있게 뒷받침해 주었던 믿음직한 현철선배 언제까지나 잊을 수 없을 겁니다. 언니가 졸업할 수 있도록 많은 실험을 거들어준 마음 착한 부사수 은정이와 은혜, 그리고 예쁜 쌍둥이 미진이와 현진, 발랄한 수경이와 정현 그리고 믿음직한 승희와 동형이도 너무나 고마웠단다.

많은 도움을 준 부경대 손정화 박사님, 효진언니 그리고 생물고분자 공학실험실 가족에게도 앞으로 좋은 일만 일어나길 바랍니다.

그리고 학부 때부터 나와 함께 많은 고통을 감내한 나의 졸업동기 소진이의 앞길에 축복이 가득하길 기원합니다. 언제나 마음속에 있어 위안이 되고 힘이 되어준 나의 친구들 혜경이와 소정이 마음의 평안을 준 건태에게도 마음을 전하고 싶으며 제가 여기까지 올 수 있도록 믿고 밀어주신 부모님과 언니, 동생들에게 이 논문을 바칩니다.

석사생활 동안 만나 저를 도와주시며 생각해 주셨던 모든 분들에게 감사드립니다.

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M.S. Thesis : Soluble Production of Foreign Proteins in *E. coli* By Molecular Chaperones

Presentation :

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