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Effect of Molecular Chaperones on the Soluble Expression of Alginate Lyase in *E. coli*

Eun-Jung Shin[1], So-Lim Park[2], Sung-Jong Jeon[1,3], Jin-Woo Lee[4], Young-Tae Kim[5], Yeon-Hee Kim[6], and Soo-Wan Nam[1,3]*

[1] Department of Biomaterial Control, Dong-Eui University, Busan 614-714, Korea
[2] BioLeaders Corp., Daejeon 301-212, Korea
[3] Department of Biotechnology and Bioengineering, Dong-Eui University, Busan 614-714, Korea
[4] Department of Biotechnology, Dong-A University, Busan 604-714, Korea
[5] Department of Microbiology, Pukyong National University, Busan 608-737, Korea
[6] Department of Biotechnology, Osaka University, Osaka 565-0871, Japan

Abstract When the alginate lyase gene (*aly*) from *Pseudoalteromonas elyakovii* was expressed in *E. coli*, most of the gene product was organized as aggregated insoluble particles known as inclusion bodies. To examine the effects of chaperones on soluble and nonaggregated form of alginate lyase in *E. coli*, we constructed plasmids designed to permit the coexpression of *aly* and the DnaK/DnaJ/GrpE or GroEL/ES chaperones. The results indicate that coexpression of *aly* with the DnaK/DnaJ/GrpE chaperone together had a marked effect on the yield alginate lyase as a soluble and active form of the enzyme. It is speculated this result occurs through facilitation of the correct folding of the protein. The optimal concentration of L-arabinose required for the induction of the DnaK/DnaJ/GrpE chaperone was found to be 0.05 mg/mL. An analysis of the protein bands on SDS-PAGE gel indicated that at least 37% of total alginate lyase was produced in the soluble fraction when the DnaK/DnaJ/GrpE chaperone was coexpressed.

Keywords: alginate lyase, molecular chaperone, coexpression, *E. coli*

INTRODUCTION

Alginate lyase of *Pseudoalteromonas elyakovii* catalyzes the depolymerization of alginates by a β-elimination mechanism to form 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 β1,4-linked polymer of β-D-mannosyluronic acid (M) and its C-5 epimer α-L-gulosyluronic acid (G) [1,2]. Alginate is widely used in the food and pharmaceutical industries due to its chelating properties (metal ions) and tendency to form highly viscous solutions [3]. Recently, various polymers and oligosaccharides with novel physiochemical and physiological functions have been sought by the biopolymer-based industries. Their interests are in expanding the applications of polysaccharides [4,5]. However, it has proven difficult to obtain novel compounds with better effectiveness and safety profiles than that which already exist, simply by screening microorganisms. Since the enzyme alginate lyase is known to liquefy the viscous alginate excreted by mucous strains of *Pseudomonas aeruginosa*, alginate lyase is a potential good candidate as a potential therapeutic agent for treating the cystic fibrosis patient [6]. When the alginate lyase gene (*aly*) was expressed in *E. coli*, it yielded inactive aggregate known as inclusion bodies. The molecular chaperones, DnaK/DnaJ/GrpE and GroEL/ES, were used so that coexprssion with *aly* could be studied as a possible means of enhancing the bacterial production level of active alginate lyase enzyme.

E. coli is one of the most popular host organisms for the high-level production of recombinant proteins [7]. The high-level expression of recombinant gene products in *E. 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 [8-11]. The high-level expression of the cloned gene product as inclusion bodies limited significantly the total downstream processing yield of aggregated protein in the refolding step [12,13]. Ultimately, it is widely recognized that coexpression of molecular chaperones or foldases can assist protein folding which leads to an increased yield of active protein [14-17]. The formation of disulfide bonds in the periplasmic space of *E. coli* is catalyzed by two soluble, periplasmic cysteine oxioreductases (DsbA and DsbC), two membrane-bound enzymes (DsbB and DsbD), and various cytoplasmic proteins [18]. Coexpression of these Dsb proteins was found to dramatically increase by several-fold the total yield

***Corresponding author**
Tel: +82-51-890-2276 Fax: +82-52-890-2632
e-mail: swnam@deu.ac.kr

production of horseradish peroxidase (HRP) [19].

The Hsp70 chaperone machinery is one of the most conserved chaperone families across the archaeal, bacterial and eukaryotic domains. The coexpression of small heat shock proteins such as *E. coli* small heat shock proteins, IbpA and IbpB, facilitated the production of recombinant proteins in *E. coli* and played important roles in protecting recombinant proteins from degradation by cytoplasm proteases [20]. In bacteria, the chaperone function of DnaK, a bacterial homologue of Hsp70 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 [21]. 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 division, DNA replication, and regulation of the heat shock response [22]. The coexpression system of DnaK recognized the glucose-6-phosphate dehydrogenase aggregates as authentic substrates and specifically solubilized and refolded the protein into its native enzyme state [23]. The hsp60s chaperone systems exist as ~800 kDa homotetradecamers, which are arranged in two stacked heptameric rings with a central cavity. At elevated temperatures, overexpression of both the GroEL and GroES can protect newly synthesized proteins from aggregating in *E. coli* cells that lacking a normal heat shock response. The molecular chaperone complex DnaK/DnaJ/GrpE interacts with nascent polypeptide chains to prevent irreversible polypeptide aggregation and mediate partial folding process [24,25]. GroEL/ES then interacts with the partially folded proteins and completes the folding [9,10,23,25,26].

In this work, we have investigated and report on the effects of DnaK/DnaJ/GrpE and GroEL/ES chaperone teams on the production of soluble and active forms of the enzyme alginate lyase as a soluble and active form in *E. coli*.

MATERIALS AND METHODS

Bacterial Strain and Plasmids

E. coli BL21 (DE3 F^-, ompT, r_B^-, m_B^-) strain was used in all experiments. Recombinant plasmids, pG-KJE6 and pALP4, were used in this work. The alginate lyase gene (*aly*, 1.19 kb) from *P. elyakovii* [27] was subcloned into the pET_{25}-vector, resulting in the plasmid pALP4 (6.7 kb). The plasmid pG-KJE6 is a pACYC184-based chloramphenicol-resistant plasmid. The transcription of *groEL/ES* and *dnaK/dnaK/grpE* in the plasmid pG-KJE6 is controlled by *Pzt*-1 promoter and *araB* promoter, respectively [28]. The transformed *E. coli* cells were grown and selected on LB agar plates containing 50 μg/mL ampicillin (selection for pALP4) and 50 μg/mL chloramphenicol (selection for pG-KJE6).

Culture Medium and Culture Conditions

E. coli cells were grown in LB medium (1% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.5% NaCl). *E. coli* transformants harboring pALP4 were grown in LB supplemented with 50 μg/mL ampicillin, and *E. coli* cell harboring pALP4 and pG-KJE6 were grown in LB supplemented with 50 μg/mL ampicillin and 50 μg/mL chloramphenicol. To induce the expression of *groEL/ES* and *dnaK/dnaK/grpE*, tetracycline and L-arabinose were used, respectively.

Measurement of Cell Growth and Protein Concentration

Cell growth was estimated by optical density at 600 nm (OD_{600}) with a spectrophotometer (Shimadzu, Japan). Cells were disrupted by sonication (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 the separation into the soluble and insoluble fractions. The protein concentrations of total cell lysates and soluble and insoluble fractions were determined by the modified micro method of Lowry.

SDS-PAGE Analysis

Each fraction obtained from 10 μg-cell lysate protein/mL was analyzed by SDS-PAGE (10% gel). The GroEL/ES, DnaK/DnaJ/GrpE and alginate lyase proteins were detected by staining gels with Coomassie brilliant blue RT250. The protein bands on the gels were scanned by an Image Analyzer (FluorChem 5500, Alpha Innotech., USA). The total intensity of protein bands in each fraction were calculated and taken to be 100%. The intensity of the alginate lyase protein band was then described as percentage of the total.

Assay of Alginate Lyase Activity

The assay for alginate lyase activity was conducted as follows. In each assay, reaction products were confirmed to increase proportionally with time and enzyme concentration in the reaction mixture. Alginate lyase assayed in a mixture containing 0.2% sodium alginate (Sigma, USA) in 1 M Tris-HCl (pH 7.5) and 0.3 M NaCl. The reaction was monitored at 37°C for 5 min, depending on the increase in absorbance at 235 nm in comparison with that without enzyme. One unit of alginate lyase was defined as the amount of enzyme required to increase the absorbance by 0.1 at 235 nm per min [1,29].

RESULTS AND DISCUSSION

Expression of Alginate Lyase in *E. coli*

The transformed *E. coli* cells with pALP4 were selected on LB agar plates containing 50 μg/mL ampicillin. IPTG was used to induce the expression of *aly*. The optimal

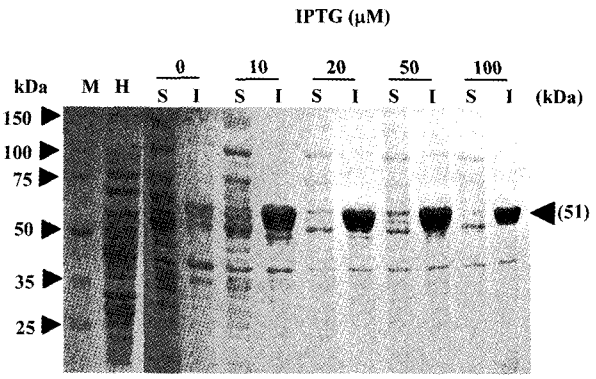

Fig. 1. Effect of IPTG concentration on the alginate lyase production in the recombinant *E. coli* BL21/pALP4. The cell was grown in 10 mL LB with IPTG (0 ~100 μM), which was added at the mid-exponential phase. After 5 h induction, the cells were harvested, disrupted with sonication, and separated as soluble (S) and insoluble (I) fractions. Analyses of SDS-PAGE (10% gel) of each fractions revealed that the expressed alginate lyase protein migrated as a 51 kDa polypeptide.

IPTG concentration for the maximal expression of alginate lyase was decided to be 20 μM as compeared with other IPTG concentration. The protein band (51 kDa) corresponding to alginate lyase was clearly shown in the insoluble fraction of cell lysates (Fig. 1). Because most alginate lyase produced was in the inactive inclusion body form and in the insoluble fraction, the enzyme activity in the soluble fraction was very low. This finding is probably due to a high expression rate leading the protein to accumulate with an abnormal conformation [30].

Effect of DnaK/DnaJ/GrpE and GroEL/ES Chaperone Teams

The effects of coexpressing the DnaK/DnaJ/GrpE chaperone system on the synthesis and solubilization of alginate lyase was tested. L-Arabinose was used to induce the expression of *dnaK/dnaJ/grpE*. The *E. coli* BL21 (DE3) cells harboring two plasmids of pALP4 and pG-KJE6 were cultivated on LB medium at 37°C. L-Arabinose was added at OD_{600} = 0.4~0.5 and then 20 μM IPTG was added at OD_{600} = 0.8. After 5 h of induction, the cells were harvested, determined the protein concentration, and separated as soluble and insoluble fractions. When the effect of various L-arabinose concentrations (0 to 1 mg/mL) were examined, the chaperone proteins of DnaK (70 kDa), DnaJ (40 kDa), and GrpE (26 kDa) were drastically overexpressed at the over 0.1 mg/mL L-arabinose concentration and were identified in the soluble fraction (Fig. 2A). However, the greatest amount of soluble alginate lyase protein was detected at 0.05 mg/mL L-arabinose. In parallel with the SDS-PAGE results, the enzyme activity of alginate lyase in the soluble fraction was also increased from 1.4 unit/g-soluble protein to 20.0 unit/g-soluble proteins by elevating the L-arabinose concentration from 0 to 0.05 mg/mL (Fig. 2B). At higher than 0.1 mg/mL L-arabinose, DnaK/DnaJ/

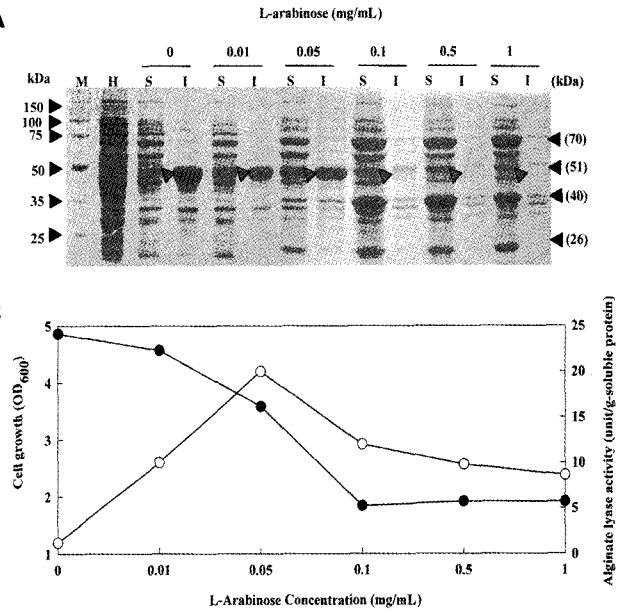

Fig. 2. Effect of DnaK/DnaJ/GrpE chaperones on the alginate lyase production in the recombinant *E. coli* BL21/pALP4 + pG-KJE6. The cell was grown in 10 mL LB with L-arabinose (0~1 mg), which was added at the early-exponential phase. After 5 h induction, the cells were harvested, disrupted with sonication, and separated as soluble (S) and insoluble (I) fractions. Each fraction was analyzed by SDS-PAGE (10% gel) (A). Dnak, DnaJ, GrpE, and alginate lyase proteins are clearly visible on the gel as bands with the expected molecular weights of 70, 40, 26, and 51 kDa, respectively. The soluble fractions were used to assay the alginate lyase activity (B). Symbols: (○), alginate lyase; (●), cell growth.

GrpE chaperone proteins were dramatically overproduced in the soluble fraction, whereas the cell growth and expression level of alginate lyase in the soluble fraction was decreased. These results seem due to metabolic burdens or metabolite depletions caused by the overexpression of DnaK/DnaJ/GrpE chaperones [31]. Therefore, it can be suggested that an optimal expression level of DnaK/DnaJ/GrpE chaperones exists that is required for the maximum expression of alginate lyase.

In order to investigate the effects of the GroEL/ES chaperone, tetracycline at 0~100 ng/mL as an inducer of *groEL/ES* was added at OD_{600} = 0.4~0.5, followed by the addition of IPTG (20 μM) at OD_{600} = 0.8. The results, indicated that the GroEL/ES chaperone did not affect the production of soluble alginate lyase (data not shown). Previously, it was observed that the DnaK/DnaJ/GrpE chaperone team was more effective in producing soluble and active human granulocyte-colony stimulating factor (hG-CSF) than was the GroEL/ES chaperone team [32]. The DnaK/DnaJ/GrpE molecules were shown to bind to hydrophobic segments of the unfolding polypeptide thereby maintaining solubility and preventing aggregation [33]. In contrast, the GroEL/ES chaperone system binds to misfolded polypeptides and allows them to refold when released [34]. Therefore, it seems that the

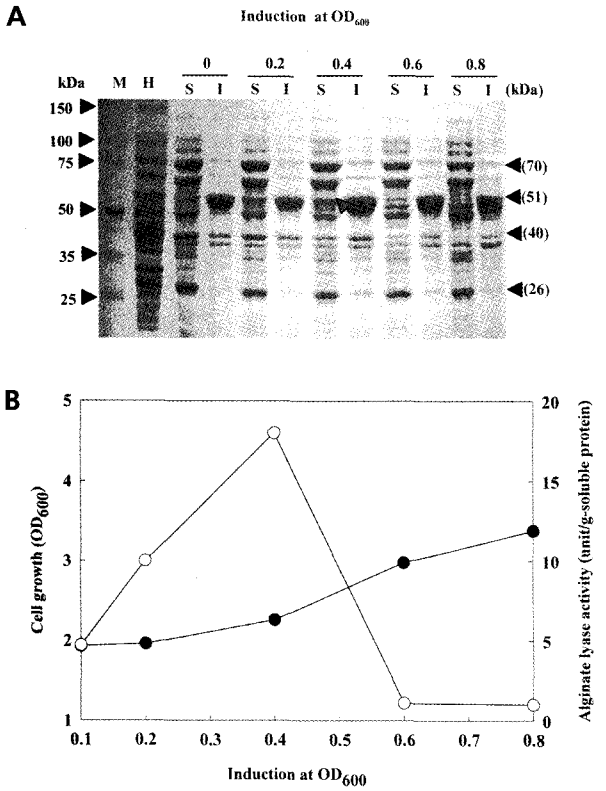

Fig. 3. Effect of induction time of DnaK/DnaJ/GrpE chaperone on the production of alginate lyase in *E. coli* BL21/pALP4 + pG-KJE6. The cell was grown in 10 mL LB with L-arabinose (0.05 mg), which was added at the OD_{600}=0.1, 0.2, 0.4, 0.6, and 0.8. After 5 h induction, the cells were harvested, disrupted with sonication, and separated as soluble (S) and insoluble (I) fractions. (A) The positions of Dnak, DnaJ, GrpE, and alginate lyase are indicated by arrows. The soluble fractions were used to assay the alginate lyase activity (B). Symbols: (○), alginate lyase; (●), cell growth.

GroEL/ES chaperone was not able to binds to the target protein (alginate lyase) and that only the interaction of DnaK/DnaJ/GrpE chaperone might be effective in yielding soluble alginate lyase protein.

Effect of Induction Time

To monitor the effect of induction time of the DnaK/DnaJ/GrpE chaperone, 0.05 mg/mL L-arabinose was added at different growth phases (OD_{600} = 0.1, 0.2, 0.4, 0.6, and 0.8). When the DnaK/DnaJ/GrpE chaperone was induced at OD_{600} = 0.1, the cell growth and expression of alginate lyase were significantly decreased (Fig. 3). These decreases were most likely due to metabolic stresses caused by intracellular metabolite shift into a hypersynthesis mode for the DnaK/DnaJ/GrpE chaperone proteins [32]. When L-arabinose was added at the mid-logarithmic phase of growth (OD_{600} = 0.4), an elevated level of alginate lyase protein was detected for both in the soluble and insoluble fractions (Fig. 3A). The induction of the chaperone at the late-logarithmic phase of growth (OD_{600} = 0.6 or 0.8) produced no activity of alginate lyase (Fig. 3B). This growth phase-dependency in the soluble production of a foreign protein in *E. coli* has also been observed in the expression of cyclodextrin glycosyltransferase and hG-CSF [14,32].

Table 1. Ratio of alginate lyase protein in the soluble and insoluble fractions of cell lysate of *E. coli* BL21 harboring pALP4 or pALP4 + pGKJE6.

Condition	Alginate lyase		
	Soluble (%)	Insoluble (%)	Activity(unit/g-soluble protein)
pALP4	3	97	1.4
pALP4+pGKJE6 (Dnak/DnaJ/GrpE)	37	50	20.0

*The total amount of alginate lyase was taken as 100%, and only the intensity of alginate lyase protein band was represented as percentage.

Image Analysis of Alginate Lyase

The alginate lyase expression in *E. coli* cells, (SDS-PAGE gel staining) was scanned enabling a quantitative analysis statement. The total intensity of protein bands in each fraction was calculated, summed and taken as 100%, and only the intensity of alginate lyase protein band was described as a percentage of the total. As shown in Table 1, when the protein bands scanned on the gel (Fig. 2) were form cells harboring only pALP4, a yield of only 3% soluble form of alginate lyase was measured. When the DnaK/DnaJ/GrpE chaperone and alginate lyase were coexpressed, however, the alginate lyase detected in the soluble fraction was increased from 3 to 37% with the L-arabinose concentration held at 0.05 mg/mL. By considering the amount of loaded protein in SDS-PAGE for each sample, the amount of alginate lyase protein in the soluble fractions was estimated to increase from about 15 mg/g-total cellular protein to 185 mg/g-total cellular protein by coexpressing the DnaK/DnaJ/GrpE chaperones. Such a dramatic improvement of soluble protein production has also been observed for hG-CSF, in which the signal intensity of hG-CSF protein band in the soluble fraction increased from 3.5% at only hG-CSF expression to 13.9% at DnaK/DnaJ/GrpE coexpression (L-arabinose concentration of 1 mg/mL) [32].

In conclusion, the establishment of an optimal production of DnaK/DnaJ/GrpE rather than GroEL/ES chaperone could effectively improve the formation of active and soluble alginate lyase protein. The coexpression concept followed in the present report may be useful in the commercial development of functional or medical recombinant proteins.

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