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Overproduction of *Bacillus macerans* Cyclodextrin Glucanotransferase in *E. coli* by Coexpression of GroEL/ES Chaperone

KWON, MI-JUNG[1], SO-LIM PARK[1], SUNG-KOO KIM[2], AND SOO-WAN NAM[3]*

[1]*Department of Microbiology, Dong-Eui University, Busan 614-714, Korea*
[2]*Department of Biotechnology & Bioengineering, Pukyong National University, Busan 608-737, Korea*
[3]*Department of Biotechnology & Bioengineering, Dong-Eui University, Busan 614-714, Korea*

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Abstract The effects of GroEL/ES chaperone on the production of soluble form of *B. macerans* cyclodextrin glucanotransferase (CGTase) in recombinant *E. coli* were investigated. The *cgt* gene and *groEL/ES* genes are under the control of T7 promoter and *Pzt*-1 promoter, respectively. The optimal concentrations of inducers, IPTG and tetracycline, were found to be 1.0 mM and 10 ng/ml, respectively. When tetracycline and IPTG were added at the early exponential phase (2 h) and exponential phase (3 h) of growth, respectively, about 1.5-fold increase of soluble CGTase activity and 1.6-fold increase of soluble CGTase protein were obtained. An SDS-PAGE analysis revealed that about 37.2% of total CGTase protein was in the soluble fraction when GroEL/ES chaperone was overexpressed.

Key words: Coexpression, cyclodextrin glucanotransferase, GroEL/ES chaperone, *Escherichia coli*

Escherichia coli is the most commonly used host microorganism for the production of recombinant proteins. However, foreign proteins produced in *E. coli* often form inclusion bodies, which are insoluble and inactive aggregates of the overexpressed polypeptides. In some cases, overexpression of molecular chaperones, such as GroEL/ES and DnaK-DnaJ-GrpE, facilitate the protein folding and enhance the production of active proteins [5, 19, 20, 23]. The molecular chaperone complex DnaK-DnaJ-GrpE interacts with nascent polypeptide chains to prevent irreversible polypeptide aggregation and mediate partial folding [2, 17]. GroEL/ES then interacts with the partially folded proteins and completes the folding [21, 22]. Trigger factor is thought to bind to ribosomes, has both chaperone and peptidyl-prolyl cis/trans isomerase functions, and mediates the folding of nascent polypeptides [13, 18]. In the case of secretory proteins, which are disulfide-bonded, the reduced state of cysteine residues is maintained during transportation by cytoplasmic thioredoxin [3]. Coexpression of the above molecular chaperones can assist protein folding, and in some cases, this leads to increased production of active proteins [6, 8, 9, 11, 12, 13, 14]. The target protein used in this work was *Bacillus macerans* cyclodextrin glucanotransferase (E.C. 2.4.1.19, CGTase). Cyclodextrins are synthesized from starch by cyclodextrin glucanotransferase. They are important compounds in industry because of their ability to form complexes with a number of materials. They are widely used in foods, pharmaceuticals, agrochemicals, and cosmetics [1]. Previously, it was reported that most of the CGTase expressed in recombinant *E. coli* was produced as aggregated insoluble particles known as inclusion bodies [4, 7, 15]. In this report, the application of the GroEL/ES coexpression system for the production of soluble CGTase in *E. coli* is described.

E. coli BL21(DE3)[F^-, *ompT*, r_B^-, m_B^-,(DE3)] strain was used in all experiments. The plasmid pTCGT1 was composed of the ribosome-binding site (SD sequence), signal sequence and structural gene of the *cgt* gene from *B. macerans* [16]. The transcription of the *cgt* gene in the plasmid pTCGT1 is controlled by T7 promoter. The plasmid pGro11 is a pACYC184-based chloramphenicol-resistant plasmid. The transcription of *groEL/ES* genes in the plasmid pGro11 is controlled by *Pzt*-1 promoter [12].

Equal amounts (1 μg) of pTCGT1 and pGro11 were cotransformed into *E. coli* BL21(DE3). The transformed *E. coli* cells were selected on LB agar plates containing 20 μg/ml ampicillin (selection for pTCGT1) and 20 μg/ml chloramphenicol (selection for pGro11).

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 pTCGT1 and pGro11 were grown in the

**Corresponding author*
Phone: 82-51-890-2276; Fax: 82-51-891-7740;
E-mail: swnam@dongeui.ac.kr

presence of 20 μg/ml ampicillin and 20 μg/ml chloramphenicol. To induce the expression of *groEL/ES* and *cgt* genes, tetracycline and isopropyl-β-D-thiogalactopyranoside (IPTG) were used.

To examine the extent of aggregation of the CGTase protein produced, cells were disrupted by sonication for 1 min on ice, and then centrifuged at 4,000 ×g for 10 min to separate the soluble and insoluble fractions. Both fractions were analyzed by SDS-PAGE (8% gel). The CGTase and GroEL/ES proteins were detected by staining the gel with Coomassie brilliant blue, and by scanning by an Image Analyzer (Image Master VDS, Pharmacia Biotech., NJ, U.S.A.).

A spectrophotometric assay was carried out by the methyl orange method [10]. Reactions were carried out at 50°C in a total volume of 1.5 ml containing 0.03 mM methyl orange and 1% soluble starch in 50 mM phosphate buffer (pH 6.0). One unit of enzyme activity is defined as the amount of enzyme catalyzing the formation of 1 μmol of cyclodextrin per minute under the assay conditions.

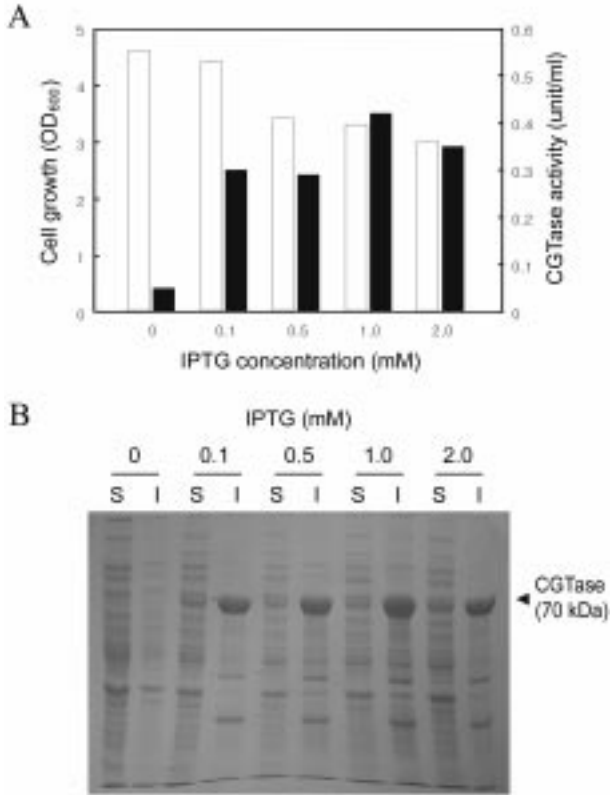

Fig. 1. Effect of IPTG concentration on the cell growth and soluble CGTase production in the recombinant *E. coli* BL21(DE3)/pTCGT1.

(A), cell growth (□) and soluble CGTase activity (■). (B), SDS-PAGE analysis of soluble (S) and insoluble (I) fractions. The cells were grown on 10 ml LB with IPTG (0–2 mM) which was added at the mid-exponential phase. After 4 h of induction, the cells were harvested, disrupted, and separated into soluble and insoluble fractions.

At first, the effect of IPTG concentration on the cell growth and CGTase expression was investigated by varying the IPTG concentration from 0 to 2 mM. Thus, the recombinant *E. coli* cell, BL21/pTCGT1, was cultivated on LB medium at 37°C and IPTG was added at the mid-exponential phase (optical density at 600 nm=0.8–1.0). As shown in Fig. 1A, the cell growth was slightly decreased with increasing IPTG concentration. The higest CGTase activity (0.42 unit/ml) was obtained at 1 mM IPTG. On SDS-PAGE analysis with soluble and insoluble fractions, only a small amount of CGTase was found in the soluble fraction, but most of the CGTase (70 kDa) expressed in *E. coli* was found in the insoluble fraction, indicating that the CGTase protein was accummlated within the cell as inclusion bodies (Fig. 1B). Similar result was also observed in the *cgt* expression in *E. coli* [3, 15].

Next, the effects of coexpression of GroEL/ES chaperone on the synthesis and solubilization of CGTase was examined by using strain BL21(DE3) harboring a pair of plasmids, pTCGT1 and pGro11. When different concentrations of tetracycline ranging from 0 to 40 ng/ml were added at 2 h followed by the addition of 1 mM IPTG at 3 h, the cell growth was decreased with increasing tetracycline concentration. The highest soluble CGTase activity (0.58 unit/ml) was obtained at 10 ng/ml tetracycline concentration (Fig. 2A). This activity level is 38% higher than that of *cgt* single expression. An SDS-PAGE analysis showed that the GroEL protein (60 kDa) was overexpressed and in the soluble fraction, irrespective of tetracycline concentration. When scanned on the gel, the percentage of GroEL protein band in the soluble fraction was found to have increased from 31% to 48%, due to increase of tetracycline concentration from 10 ng/ml to 40 ng/ml. The higher the concentration of tetracycline, the greater the expression level of GroEL protein (Fig. 2B). Since the molecular weight of GroES protein is relatively small (10 kDa), GroES protein was not clearly revealed on the gel. The decreases of CGTase activity and cell growth in the absence tetracycline in Fig. 2A were most likely due to the metabolic stress caused by replication and maintenances of the two plasmids.

In spite of the lower production of GroEL/ES chaperones at low concentrations of tetracycline, the largest amount of soluble CGTase protein was detected at 10 ng/ml tetracycline. By scanning the gel, it was found that the percentage of soluble CGTase protein band was decreased from 38% to 24% by increasing the tetracycline concentration from 10 ng/ml to 40 ng/ml, thus indicating that an optimal concentration of GroEL/ES chaperone exists for the overproduction of soluble and active CGTase.

To monitor the expression level of soluble CGTase with the culture period, *E. coli* BL21(DE3) cells harboring pTCGT1 and pTCGT1+pGro11 were cultivated in the flasks containing 80 ml of LB. The *groEL/ES* and *cgt*

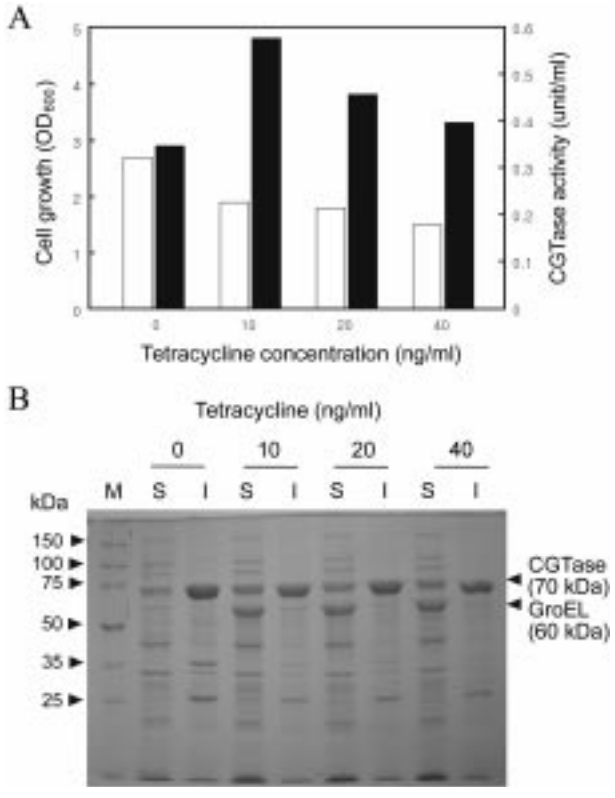

Fig. 2. Effect of tetracycline concentration on the cell growth and soluble CGTase production in the recombinant *E. coli* BL21(DE3)/pTCGT1+pGro11.
(A), cell growth (□) and soluble CGTase activity (■). (B), SDS-PAGE analysis of soluble (S) and insoluble (I) fractions. The cells were grown on 10 ml LB, in which tetracycline (0–40 ng/ml) was added at the early-exponential phase (2 h), and 1 mM IPTG at the mid-exponential phase (3 h). After 4 h of IPTG induction, the cells were harvested, disrupted, and separated into soluble and insoluble fractions.

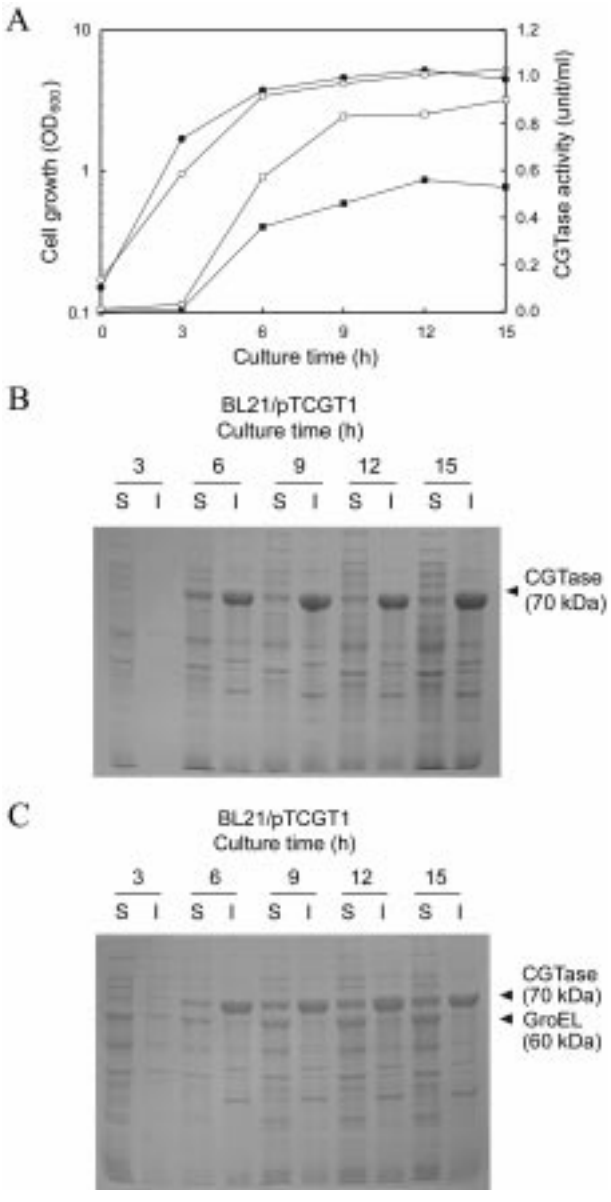

Fig. 3. Time courses of CGTase and GroEL/ES production in the recombinant *E. coli* BL21(DE3) cells.
(A), cell growth (○, ●) and soluble CGTase activity (□, ■). Closed symbols, CGTase single expression system (BL21/pTCGT1). Open symbols, coexpression system (BL21/pTCGT1+pGro11). (B), SDS-PAGE analysis of soluble (S) and insoluble (I) fractions in the CGTase single expression system. (C), SDS-PAGE analysis of soluble (S) and insoluble (I) fractions in the coexpression system. The cells were grown in 80 ml LB flask, in which 10 ng/ml tetracycline was added at 2 h and 1 mM IPTG at 3 h. After 9 h of IPTG induction, the cells were harvested, disrupted, and separated into soluble and insoluble fractions.

genes were then induced with 10 ng/ml tetracycline at the early-exponential phase (at 2 h) and 1 mM IPTG at the exponential phase (at 3 h). As shown in Fig. 3A, the cell growth of *E. coli* BL21/pTCGT1+pGro11 was retarded due to the overexpression of *groEL/ES* and *cgt* genes. The CGTase activity in the coexpression system at 12 h was about 0.84 unit/ml, whereas that of the CGTase in the single expression system was about 0.56 unit/ml. The CGTase activity was stably maintained at a maximum level of 0.83 unit/ml from 6 h after the IPTG addition. Based on the scanning of protein bands and the amount of protein loaded on the polyacrylamide gel, the percentage and concentration of soluble CGTase protein in the coexpression system at 12 h were estimated to be 37.9% and 28.9 μg/ml, respectively, while those in the single expression system were about 18.8% and 18.2 μg/ml (Figs. 3B, 3C). Therefore, the coexpression of GroEL/ES chaperone resulted in about 1.5-fold increase of soluble CGTase activity and about 1.6-fold increase of soluble CGTase protein. Furthermore, in the coexpression system, about 37.2% of total CGTase protein was found in the soluble fraction, whereas about 20.8% of CGTase protein in the single expression system was detected in the soluble fraction. The result, therefore, indicates that a large fraction of insoluble CGTase could be converted into soluble form with the assistance of GroEL/ES chaperones.

In conclusion, it is suggested that optimal production of GroEL/ES chaperone can effectively prevent the aggregation of CGTase and significantly enhance the production of active soluble CGTase in *E. coli*.

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