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GroEL/ES Chaperone and Low Culture Temperature Synergistically Enhanced the Soluble Expression of CGTase in *E. coli*

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Abstract The effect of culture temperature on the production of soluble form of *B. macerans* cyclodextrin glucanotransferase (CGTase) in recombinant *E. coli* was investigated. *E. coli* cell was cotransformed with two plasmids (pTCGT1 and pGro11) in which the *cgt* and *groEL/ES* genes are under the control of T7 promoter and *pzt-1* promoter, respectively. When tetracycline (10 ng/ml) and IPTG (1 mM) were added as inducers at the early-exponential phase (2 h) and mid-exponential phase (3 h), respectively, the solubilization of the inclusion body CGTase was greatly dependent on the temperature of the culture. At low culture temperature of 25°C, 2- or 3-fold higher activity and specific activity were obtained over 37°C. SDS-PAGE analysis revealed that about 62% of CGTase in the total CGTase protein was found in the soluble fraction by applying overexpression of GroEL/ES chaperone and by cultivation of *E. coli* at 25°C, whereas 33% of CGTase was detected in the soluble fraction at 37°C. Therefore, the expression of GroEL/ES and cultivation at 25°C greatly enhanced the soluble production of CGTase in *E. coli.*

Key words: Culture temperature, coexpression, chaperone GroEL/ES, cyclodextrin glucanotransferase, *Escherichia coli*

Although *E. coli* is the most commonly used host microorganism for genetic manipulation, recombinant proteins produced in *E. coli* often fail to fold into their native state and intracellularly accumulate in the form of insoluble inclusion bodies [8]. However, it has been already demonstrated that folding of many protein can be facilitated by proteins called molecular chaperone GroEL/ES and DnaK-DnaJ-GrpE [21, 23, 27]. The molecular chaperone complex DnaK-DnaJ-GrpE interacts with nascent polypeptide chains to prevent irreversible polypeptide aggregation and mediate partial folding [3, 20]. GroEL/ES then interacts with the partially folded proteins and completes the folding process [24, 25]. Coexpression of the above molecular chaperones can assist in protein folding and, at least in some cases, this leads to increased production of active proteins [4, 8, 11, 13, 14].

In this study, *Bacillus macerans* cyclodextrin glucanotransferase (E.C. 2.4.1.19, CGTase) was used as the target protein. Cyclodextrins are synthesized from starch by cyclodextrin glucanotransferase. They are important compounds in industry due to their ability to form complexes with a number of materials. They are widely used in foods, pharmaceuticals, agrochemicals, and cosmetics. Previously, it was reported that most of the CGTase expressed in recombinant *E. coli* was produced as aggregated insoluble particles known as inclusion bodies [5, 6, 16]. Therefore, the main steps to be considered in inclusion body formation may be peptide translation, protein folding, and aggregation of partially folded peptides. All of the steps depend on translational rate, peptide concentration, and diffusion kinetics, and these parameters are affected by environmental factors including temperature, pH, and salt concentration, as well as molecular chaperone. In our previouse study, it was found that the coexpression of molecular chaperone GroEL/ES greatly enhanced the soluble expression of CGTase in *E. coli* [9]. The other experiments suggested that the formation of inclusion body is closely related to the culture temperature [15, 17, 19, 21, 26].

In this report, the synergistic effect of coexpression and low culture temperature on the production of soluble form of *B. macerans* CGTase in *E. coli* was 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

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from *B. macerans* [10]. The transcription of the *cgt* gene in the plasmid pTCGT1 is controlled by the T7 promoter [16]. 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 [13].

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 of ampicillin (selection for pTCGT1) and 20 μg/ml of 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 presence of 20 μg/ml of ampicillin and 20 μg/ml of chloramphenicol. Bioreactor cultures were performed in a 2.5-l jar fermentor (KoBiotech Co., Korea) with 1 l of the working volume on LB plus 2% glucose medium. Operational temperature was maintained either at 25°C or 37°C with pH of 7.0 In order to maintain the pH level of the medium at a desired value, 50% NH_4OH or 1 N of HCl was used. To induce the expression of *groEL/ES* and *cgt* genes, tetracycline (10 ng/ml) and isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM) were added at the early-exponential phase (2 h) and mid-exponential phase (3 h), respectively. The optimal timing of induction and inducer concentration were described in our previous study [9].

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

A spectrophotometric assay for the measurement of CGTase enzyme activity was carried out by the methyl orange method [12]. Reactions were carried out at 50°C in a total volume of 1.5 ml containing 0.03 mM of methyl orange and 1% soluble starch in 50 mM of 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.

In order to decrease the formation of inclusion body, we investigated the coexpression of CGTase with the GroEL/ES chaperone. *E. coli* transformants harboring pTCGT1 alone and together with pGro11 were cultivated at 25°C and 37°C and the results of cell growth and CGTase activity are shown in Fig. 1. The cell growth and glucose consumption at 25°C were greatly decreased over those at 37°C. However, the CGTase activities in the coexpression system were increased from 1.24- 1.94 unit/ml at 37°C to 2.45- 2.91 unit/ml at 25°C. Meanwhile, the activities in the CGTase single expression system were in the range of 0.94- 1.30 unit/ml at 37°C and 1.6- 2.41 unit/ml at 25°C.

Table 1. Ratio of CGTase protein in soluble and insoluble forms. Each fraction was separated on 8% SDS-PAGE, followed by Coomassie staining, and scanned by the image analyzer. The total amount of CGTase produced was taken as 100%.

Condition		CGTase		
		Soluble (%)	Insoluble (%)	Specific activity* (unit/ml OD_{600})
pTCGT1	37°C	21.2	78.8	0.06
	25°C	40.4	59.6	0.14
pTCGT1+pGro11	37°C	33.1	66.9	0.07
	25°C	61.6	38.4	0.17

*Activity and cell concentration were at 7 h of cultivation.

The effect of decreasing the formation of CGTase inclusion body was obtained by simply lowering the culture temperature. Cultivation of the cells harboring only pTCGT1 at 37°C yielded 21% of soluble form of CGTase and the specific activity was 0.6 unit/ml at OD_{600} (Table 1).

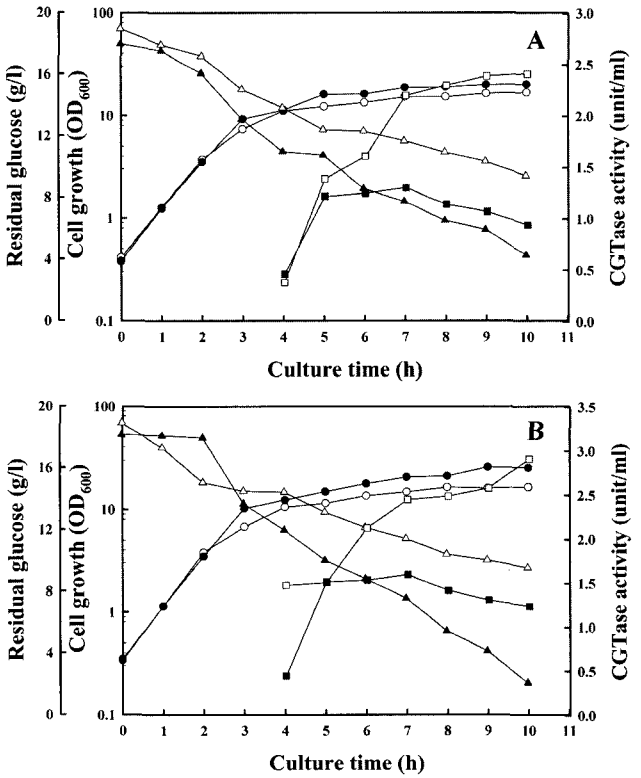

Fig. 1. Time profiles of cell growth, glucose consumption, and CGTase activity in the batch cultures of *E. coli* BL21/pTCGT1 (A) and *E. coli* BL21/pTCGT1+pGro11 (B) at 37°C and 25°C. The cells were grown on LB medium containing 2% glucose.
Closed symbols, 37°C, Open symbols, 25°C. (○, ●), Cell growth; (□, ■), CGTase activity; (△, ▲), residual glucose.

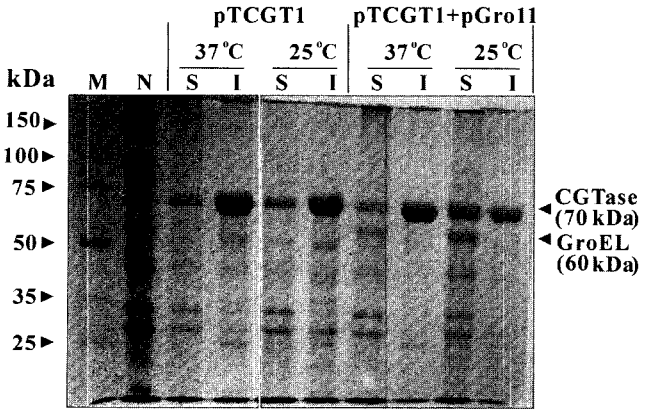

Fig. 2. SDS-PAGE analyses of expression and solubility of recombinant CGTase at 37°C and 25°C.
Expression of CGTase and GroEL/ES were induced by addition of 1 mM IPTG at 2 h and 10 ng/ml tetracycline at 3 h. At 10 h of cultivation, the cells were harvested, disrupted, and separated into soluble (S) and insoluble (I) fractions. M, molecular makers; N, whole-cell lysate of transformants before IPTG induction.

When the cotransformants with the GroEL/ES chaperone and CGTase gene were cultivated at 25°C, over 60% of the expressed CGTase was localized in the soluble fraction and the specific CGTase activity was increased to 0.17 unit/ml·OD_{600} (Table 1). Therefore, with the coexpression system, the amount of soluble CGTase at 25°C and specific activity were increased by 2- or 3-fold, compared with that at 37°C. It was also found that about 73-76% of CGTase activity in the total activity was detected in the periplasmic space at both temperatures, and this indicates that the localization of soluble CGTase was independent on the culture temperature.

This higher production of soluble CGTase at 25°C may be explained with the decreased expression rate of *cgt* and the concentration of CGTase protein produced within the cell. Generally, a rapid accumulation of the overproduced protein may increase a tendency to form inclusion bodies. When cultured at 25°C, the production rate of CGTase enzyme could be slower than that at 37°C by a lower cell growth rate, and the resulting lower protein concentration might help prevent the formation of the insoluble aggregate. This observation, *i.e.*, in which a soluble production of foreign proteins caused a decreased expression rate at a lower culture temperature, has also been reported in the production of β-glucosidase, human C1 inhibitor, and D-carbamoylase in *E. coli* [2, 18, 22].

Consequently, the coexpression of GroEL/ES and lowering the culture temperature to 25°C cooperatively enhanced the conversion of inactive CGTase form into an active form, resulting in a greater production of soluble enzyme. This result may be useful for the commercial production of recombinant proteins in *E. coli* as active and soluble form.

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References

1. Chrunyk, B. A., J. Evans, J. Lillqust, P. Young, and R. Wetzel. 1993. Inclusion body formation and protein stability in sequence variants of interleukin-1. *J. Biol. Chem.* **268:** 18053-18061.
2. Dipti, S., S. Rakesh, and M. W. Rakesh. 2001. Chaperone-assisted overexpression of an active D-carbamoylase from *Agrobacterium tumefaciens* AM10. *Proteion Expression Purif.* **23:** 374-379
3. Gragerov, A., E. Nudler, N. Komissarova, G. A. Gaitanaris, M. E. Gottesman, and V. Nikiforov. 1992. Cooperation of GroEL/GroES and DnaK/DnaJ heat shock proteins in preventing protein misfolding in *Escherichia coli. Proc. Natl. Acad. Sci. USA* **89:** 10341-10344.
4. Han, N. S. and B. Y. Tao. 1999. Enhancement of solubility of *Bacillus macerans* cyclodextrin glucanotransferase by thioredoxin fusion. *Food Sci. Biotechnol.* **8:** 276-279.
5. Jin, H. H., N. S. Han, D. K. Kweon, Y. C. Park, and J. H. Seo. 2001. Effects of environmental factors on *in vivo* folding of *Bacillus macerans* cyclodextrin glycosyltransferase in recombinant *Escherichia coli. J. Microbiol. Biotechnol.* **11:** 92-96.
6. Kim, C. I., M. D. Kim, Y. C. Park, N. S. Han, and J. H. Seo. 2000. Refolding of *Bacillus macerans* cyclodextrin glucanotransferase expressed as inclusion bodies in recombinant *Escherichia coli. J. Microbiol. Biotechnol.* **10:** 632-637.
7. Klein, J. and P. Dhurjati. 1995. Protein aggregation kinetics in an *Escherichia coli* strain overexpressing a *Salmonella typhimurium* CheY mutant gene. *Appl. Environ. Microbiol.* **61:** 1220-1225.
8. Kondo, A., J. Kohda, Y. Endo, T. Shiromizu, Y. Kurokawa, K. Nishihara, H. Yanagi, T. Yura, and H. Fukuda. 2000. Improvement of productivity of active horseradish peroxidase in *Escherichia coli* by coexpression of Dsb proteins. *J. Biosci. Bioeng.* **90:** 600-606.
9. Kwon, M. J., S. L. Rark, S. K. Kim, and S. W. Nam. 2002. Overproduction of *Bacillus macerans* cyclodextrin glucanotransferase in *E. coli* by coexpression of GroEL/ES chaperone. *J. Microbiol. Biotechnol.* **12:** 1002-1005.
10. Lee, P. K. C. and B. Y. Tao. 1994. High-level expression of cyclodextrin glucanotransferase in *E. coli* using a T7 promoter expression system. *Starch* **46:** 67-74.
11. Lee, S. C. and P. O. Olins. 1992. Effect of overproduction of heat shock chaperones GroESL and DnaK on human procollagenase production in *Escherichia coli. J. Biol. Chem.* **267:** 2849-2852.

12. Lejeune, A., K. Sakaguchi, and T. Imanaka. 1989. A spectrophotometric assay for the cyclization activity of cyclomaltohexaose (α-cyclodextrin) glucanotransferase. *Anal. Biochem.* **181:** 6-11.
13. Nishihara, K., M. Kanemori, M. Kitagawa, H. Yanagi, and T. Yura. 1998. Chaperone coexpression plasmids: Differential and synergistic roles DnaK-DnaJ-GrpE and GroEL-GroES in assisting folding of an allergen of Japanese cedar pollen, Cryj2, in *Escherichia coli*. *Appl. Environ. Microbiol.* **64:** 1694-1699.
14. Nishihara, K., M. Kanemori, H. Yanagi, and T. Yura. 2000. Overexpression of trigger factor prevents aggregation of recombinant proteins in *Escherichia coli*. *Appl. Environ. Microbiol.* **66:** 884-889.
15. Oh, Y. P., S. T. Jeong, D.-W. Kim, E.-C. Kim, and K.-H. Yoon. 2002. Simple purification of Shiga toxin B chain from recombinant *Escherichia coli*. *J. Microbiol. Biotechnol.* **12:** 986-988.
16. Park, Y. C., C. S. Kim, N. S. Han, and J. H. Seo. 1995. Expression of cyclodextrin glucanotransferase from *Bacillus macerans* in recombinant *Escherichia coli*. *Foods Biotechnol.* **4:** 290-295.
17. Piatak, M., J. A. Lane, W. Laird, M. J. Bjorn, A. Wang, and M. Williams. 1988. Expression of soluble and fully functional ricin a chain in *Escherichia coli* is temperature sensitive. *J. Biol. Chem.* **263:** 4837-4843.
18. Sachiko, M., Y. Yu, S. P. Singh, J. D. Kim, K. Hayashi, and Y. Kawata. 1998. Overproduction of β-glucosidase in active form by an *Escherichia coli* system coexpressing the chaperonin GroEL/ES. *FEBS Microbiol Lett.* **159:** 41-46.
19. Standberg, L. and S. O. Enfors. 1991. Factors influencing inclusion body formation in the production of a fused protein in *Escherichia coli*. *Appl. Environ. Microbiol.* **57:** 1669-1674.
20. Szabo, A., T. Langer, H. Schroder, J. Flanagan, B. Bukau, and F. U. Hartl. 1994. The ATP hydrolysis-dependent reaction cycle of the *Escherichia coli* Hsp70 system-DnaK, DnaJ, and GrpE. *Proc. Natl. Acad. Sci. USA* **91:** 10345-10349.
21. Thomas, J. G., A. Ayling, and F. Baneyx. 1997. Molecular chaperones, folding catalysts, and the recovery of active recombinant proteins from *E. coli*. *Appl. Biochem. Biotechnol.* **66:** 197-238.
22. Troned, L., I. Monica, B. Camilla, M. Tarja, E. M. Tom, and W. N. Erik. 2001. Expression of active human C1 inhibitor serpin domain in *E. coli*. *Protein Expression Purif.* **22:** 349-358.
23. Wall, J. G. and A. Pluckthun. 1995. Effects of overexpressing folding modulators on the *in vivo* folding of heterologous proteins in *Escherichia coli*. *Curr. Opin. Biotechnol.* **6:** 507-516.
24. Weissman, J. S., C. M. Hohl, O. Kovalenko, Y. Kashi, S. Chen, K. Braig, H. R. Saibil, W. A. Fenton, and A. L. Horwich. 1995. Mechanism of GroEL action: Productive release of polypeptide from a sequestered position under GroES. *Cell* **83:** 577-587.
25. Weissman, J. S., H. S. Rye, W. A. Fenton, J. M. Beechem, and A. L. Horwich. 1996. Characterization of the active intermediate of a GroEL-GroES-mediated protein folding reaction. *Cell* **84:** 481-490.
26. Wetzel, R. and B. A. Chrunyk. 1994. Inclusion body formation by interleukin-1 depends on the thermal sensitivity of a folding intermediate. *FEBS Lett.* **350:** 245-248.
27. Ziemienowicz, A., D. Skowyra, J. Zeilstra-Ryalls, O. Fayet, C. Georgopoulos, and M. Zylicz. 1993. Both the *Escherichia coli* chaperone systems, GroEL/GroES and DnaK/DnaJ/GrpE, can reactivate heat-treated RNA polymerase. *J. Biol. Chem.* **268:** 25425-25431.