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Effects of Environmental Factors on *In Vivo* Folding of *Bacillus macerans* Cyclodextrin Glycosyltransferase in Recombinant *Escherichia coli*

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Abstract Effects of environmental factors on the expression of soluble forms of *Bacillus macerans* cyclodextrin glycosyltransferase in recombinant *Escherichia coli* BL21(DE3)pLysE:pTCGT1 were investigated. The amount of soluble CGTase produced in the cell was measured by determining its enzymatic activity. The soluble fraction of the enzyme was increased by lowering the culture temperature to 30°C and medium pH to 5.8 compared to the enzyme production in LB medium at 37°C and pH 7.0. Addition of 0.2 M NaCl enhanced enzyme expression levels at the expense of cell growth. Glycine betaine that was added after 3 h of induction protected not only the cell growth from high osmotic pressure but also helped *in vivo* folding of CGTase in recombinant *E. coli*. Addition of 1 mM $CaCl_2$ was also effective in the expression of soluble CGTase, resulting in 15 U/ml of the enzyme activity.

Key words: Cyclodextrin glycosyltransferase, recombinant *E. coli*, inclusion bodies, *in vivo* folding, environmental factors

Cyclodextrins (CDs) are synthesized from starch by cyclodextrin glycosyltransferase (E.C. 2.4.1.19, CGTase) [4, 14]. 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. Most of the CGTases have been isolated from the *Bacillus* genus, and the DNA and amino acid sequences of at least 12 different species have been determined. The *B. macerans* CGTase gene was cloned and expressed in recombinant *E. coli* by using the T7 promoter [10]. Several fermentation works were carried out to overproduce *B. macerans* CGTase in recombinant *E. coli* [15, 16]. Most of the CGTase expressed in recombinant *E. coli* aggregated into insoluble particles known as inclusion bodies [8, 9]. Inclusion body formation often offers advantages in purification; however, the *in vitro* refolding procedure of inclusion bodies is usually time-consuming, and inefficient at times. Furthermore, with the advent of an efficient purification method for CGTase using affinity chromatography [2], expression of CGTase as a soluble form became preferable.

It is generally accepted that the tendency of a foreign protein to aggregate is related to the kinetics of protein folding. Hasse-Pettingell and King [3] showed that the aggregation of recombinant P22 tail spike protein involved folding intermediates and that a competition existed between protein folding and aggregation. Klein and Dhurjati [7] found that ongoing translation facilitated the conversion of the soluble *Salmonella typhimurium* CheY protein to an insoluble form, implicating the translation rate in affecting formation of inclusion bodies. 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, salt concentration, and so on. In fact, earlier experiments suggested the correlation between temperature and inclusion body formation [17, 20].

The present work was conducted to systematically examine the effects of culture temperature, pH, and addition of various additives in the growth medium on *in vivo* folding of cyclodextrin glycosyltransferase in recombinant *E. coli* containing the gene coding for *B. macerans* CGTase.

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Materials and Methods

Plasmid and Bacterial Strain

E. coli BL21(DE3)[F^-, *ompT*, r_B^-, m_B^-, (DE3), pLysE, Cm^r] was used as a host for expression of the *cgt* gene coding for CGTase. The host cell contained the immunity region of the phage 21 and carried a DNA fragment with the *lacI* gene, the *lac*UV5 promoter, the beginning of the *lacZ* gene, and the gene for T7 RNA polymerase. The only promoter known to direct transcription of the T7 RNA polymerase gene was the *lac*UV5 promoter, which is inducible by isopropyl-β-D-thiogalacto-pyranoside (IPTG). Expressed T7 RNA polymerase, in turn, transcribed the *cgt* gene in the plasmid pTCGT1 which was controlled by the T7 promoter. The plasmid pTCGT1 was composed of the ribosome-binding site (SD sequence), signal sequence, and structural gene of the *cgt* gene from *B. macerans* cloned into the pET-21(+) system, an *E. coli* expression vector harboring the T7 promoter [10]. The plasmid pLysE provided T7 lysozyme, which bound specifically to T7 RNA polymerase [13]. The presence of pLysE increased the tolerance of *E. coli* BL21(DE3) against toxic target plasmids.

Growth Conditions

LB (10 /l NaCl, 10 g/l Tryptone, 5 g/l yeast extract) containing 2 g/l of glucose and 50 μg/ml of ampicillin was used for cultures of the recombinant *E. coli* BL21(DE3)pLysE:pTCGT1. Flask cultures with 200 ml of culture volume in 500-ml flasks were incubated at 37°C and shaken at 200 rpm. Each flask was inoculated with 2 ml of the preculture in the exponential growth phase. Bioreactor cultures were performed in a 500-ml jar fermentor (Biostat Q, B.Brown Biotech International, Melsungen, Germany) with 300 ml of the working volume. Operational pH and temperature were maintained at 7.0 and 37°C. In order to maintain the pH of the medium at a desired value, 1 N HCl or 1 N NaOH was used. Induction was made by adding 0.5 mM of IPTG at the late exponential phase since these conditions were found to be the optimal induction timing and inducer concentration in our previous study [15].

Determination of CGTase Activity and Glucose Concentration

To prepare soluble CGTase samples, the culture broth was centrifuged at 5,000 rpm and 4°C for 5 min. The cell pellet was resuspended in an equal volume of 50 mM phosphate buffer (pH 6.0). After sonication of the cell suspension at 40% output for 4 min with an ultrasonic processor (50-watt Model, Cole-Parmer, IL, U.S.A.), the crude CGTase solution was obtained by centrifugation of the cell-disruptured suspension at 12,000 rpm and 4°C for 30 min. To assay the α-CD forming activity of CGTase, a spectrophotometric method with methyl orange was employed according to the method of Imanaka [11]. This method involved the formation of an inclusion complex between α-CD and methyl orange. The reaction was carried out at 50°C in a total volume of 3 ml containing 0.03 mM methyl orange and 1% soluble starch in 50 mM phosphate buffer (pH 6.0). The reaction was started by adding 100 μl of the enzyme solution that was appropriately diluted with phosphate buffer. After 5 min, the reaction was stopped by adding 150 μl of 6 N HCl. The test tubes were then cooled to 16°C for 15 min, and the absorbance at 505 nm was determined against the blank. One unit of the enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmole of α-CD per minute under the assay condition.

Glucose concentrations were measured by using either a glucose analyzer (YSI 1500 Sidekick, Ohio, U.S.A.) or a glucose kit (Yongdong Pharm. Co., Seoul, Korea).

Results and Discussion

Effects of Culture Conditions on *In Vivo* Folding of CGTase in *E. coli*

Recombinant *E. coli* BL21(DE3)pLysE:pTCGT1 was grown at various pHs (5.8, 6.4, and 8.0) in the LB medium to explore the effects of pH on *in vivo* folding of CGTase, using a 500-ml jar fermenter at 37°C. The profiles for cell growth, glucose consumption, and soluble CGTase activity at various pHs were plotted in Fig. 1. Few variations among cell growth rates and glucose consumption rates

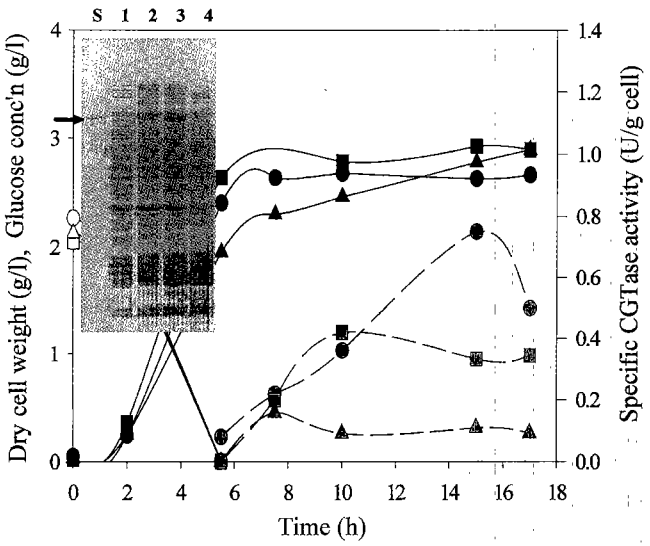

Fig. 1. Fermentation profiles and SDS-PAGE analysis of CGTase expressed in recombinant *E. coli* BL21(DE3)pLysE:pTCGT1 with various medium pHs.

Closed: dry cell weight; open: glucose concentration; gray: specific CGTase activity; pH 5.8 (●), pH 6.4 (■), pH 8.0 (▲). Inner panel shows SDS-PAGE analysis of soluble fraction of cell lyzates. Lane S: purified CGTase; Lane 1: pH 7.0; Lane 2: pH 6.4; Lane 3: pH 5.8; Lane 4: pH 8.0.

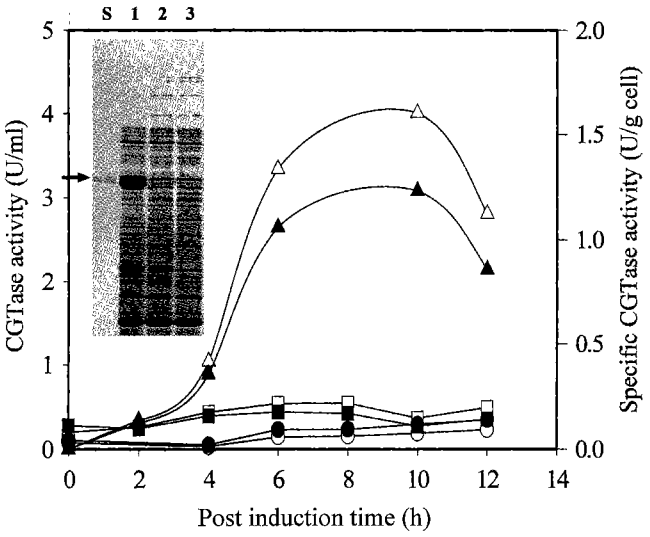

Fig. 2. Effect of temperature on CGTase expression in recombinant *E. coli* BL21(DE3)pLysE:pTCGT1.
Open: CGTase activity (activity per culture volume); closed: specific CGTase activity, 42°C (●), 37°C (■), 30°C (▲). Inner panel shows SDS-PAGE analysis of the soluble fraction of the cell lyzates. Lane S: purified CGTase; Lane 1: 30°C; Lane 2: 37°C; Lane 3: 42°C.

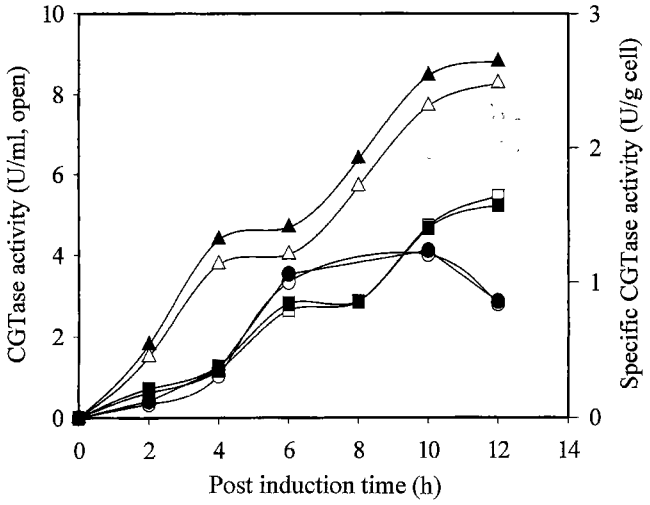

Fig. 3. Synergistic effect of temperature and pH on CGTase expression in recombinant *E. coli* BL21(DE3)pLysE:pTCGT1.
Open: CGTase activity (activity per culture volume); closed: specific CGTase activity, pH 7.0 at 30°C (●), pH 6.4 at 30°C (■), pH 5.8 at 30°C (▲).

were observed for all cultures. After 15 h of inoculation, the maximum CGTase activity per cell mass (0.8 units/g cells) was obtained in the culture at pH 5.8, which was about two-fold higher than that of the control at pH 7.0 (0.4 units/g cells). This result suggested that lower pH levels in the medium provided a much favorable condition for solubilization of CGTase in *E. coli* compared to the neutral or alkaline conditions.

Temperature is one of the most important environmental factors in microbial fermentations affecting metabolic reaction rates of the cell. Therefore, the effect of temperature on the folding of CGTase in *E. coli* was examined by growing the cells at various temperatures (Fig. 2). Both the specific growth rate and glucose consumption rate became slower with lowering culture temperatures, resulting in a delay of induction time at the late exponential phase. However, the final cell concentrations cultured at 30°C and 37°C were identical even with different specific growth rates. The highest CGTase activity per culture volume (4.0 units/ml) and the highest CGTase activity per cell mass (1.2 units/g cells) were obtained at 30°C, which was three- and four-fold higher than the corresponding activities of the control case at 37°C (1.1 units/ml of CGTase activity and 0.4 units/g cells of specific activity).

To observe the combined effects of pH and temperature, the recombinant *E. coli* BL21(DE3)pLysE:pTCGT1 was grown at 30°C at pHs 7.0, 6.4, and 5.8 (Fig. 3). As expected, the maximum CGTase activity was obtained under the optimum condition, at pH 5.8 and 30°C, yielding 8.3 units/ml and 2.6 units/g cells. The specific CGTase acitivity (activity per cell mass) was 6-fold higher than that obtained from the control.

Sugimoto *et al.* [18] suggested that formation of inclusion bodies was caused by inhibition of protein folding and aggregation of peptide intermediates at high pH medium. Our experimental results were consistent with the results derived from the above experimental observations. It was suggested that the translation rate at low temperatures in *E. coli* decreased to maintain unfolded polypeptides inside the cell at low concentrations, and the diffusion rate of polypeptides also decreased so as not to aggregate but rather result in the enhancement of the productivity of soluble protein.

Effects of Additives in the Culture Medium

More than 70% of the CGTase expressed in the recombinant *E. coli* was found to accumulate in the periplasmic space (data now shown), and it was suggested that the addition of various chemical compounds in the medium would affect the folding and aggregation kinetics of the CGTase. Therefore, several experiments were carried out to examine the effects of NaCl, glycine betaine, or $CaCl_2$ in the medium on *in vivo* folding of CGTase in recombinant *E. coli* BL21(DE3)pLysE:pTCGT1.

The recombinant *E. coli* cells were grown in a 500-ml jar fermenter at 30°C in LB medium containing 2 g/l of glucose at various concentrations of NaCl (200, 300, and 400 mM). Cell mass, glucose concentration in the medium, and CGTase activity in the cells were measured at time intervals during the fermentation process. Increasing NaCl concentrations exerted an inhibitory effect on cell growth, decreasing both the cell growth rate and glucose consumption rate. Figure 4 shows the maximum CGTase

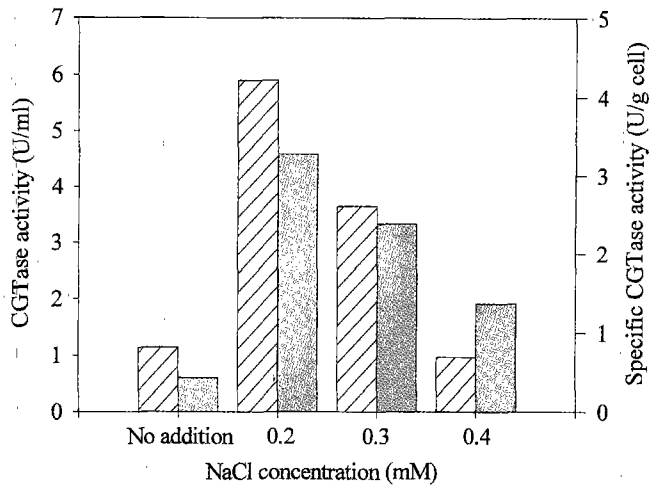

Fig. 4. Effect of NaCl addition on CGTase expression in recombinant *E. coli* BL21(DE3)pLysE:pTCGT1.
Slashed bar: CGTase activity (activity per culture volume); dark bar: specific CGTase activity (activity per cell mass).

activity and its corresponding specific activity as a function of NaCl concentrations in the medium. In the culture with 200 mM NaCl, the specific CGTase activity was 3.3 units/g cells, which was about 8 times higher than that without NaCl addition.

Glycine betaine is an osmotic effector to protect cells from high osmotic pressure [12, 19]. It penetrates and accumulates inside the cell and reduces the osmotic stress. Even in a high salt concentration of 0.65 M NaCl, addition of 1 mM glycine betaine in the medium can reduce osmotic stress, allowing fast cell growth. In the above experiment, the cell growth rate was reduced when NaCl was added to the medium. In order to recover the growth rate and to maintain high yield of soluble CGTase in 0.2 M NaCl, glycine betaine was added at 1 mM and then CGTase activity was measured. The results are summarized in Table 1 which shows the maximum cell mass and maximum CGTase activity. When both glycine betaine and NaCl were added in the medium, the recombinant cells grew to 2.2 g/l and CGTase expression reached 6.1 units/ml with 2.8 units/g cells. When glycine betaine was added, the inhibition of cell growth at high salt concentration was reduced, thus allowing the cells to grow to the level of the control without additives. Enzyme activity did not change, resulting in a reduction of specific CGTase activity. However, when glycine betaine was added 3 h after induction, the CGTase activity increased by approximately two-fold while maintaining the same cell density. This result indicated that the addition of glycine betaine conferred stability to CGTase during protein folding in the cell, as well as an osmotolerance to the recombinant *E. coli* cell.

In many cases, metal ions play an important role in both enzyme activity and thermal stability by giving rigidity to the enzyme structure. Most enzymes belonging to the α-amylase family contain Ca^{2+} ion, and its presence is critical for activity and stability. It is known that Ca^{2+} ion is located in the catalytic sites around the TIM barrel structure of α-amylase, connecting α-helices and β-sheets by an ionic interaction [5]. CGTase possesses additional Ca^{2+} per molecule and the two Ca^{2+} confer heat stability to the enzyme [1, 5, 6]. Considering the location of the two Ca^{2+} atoms, incorporation of Ca^{2+} may occur in a moment of competition between a shuffling process of the secondary structures into the globular form and an aggregation process of those intermediates into inclusion bodies. Therefore, it was postulated that the addition of Ca^{2+} would protect the aggregation of the translated polypeptides into insoluble inclusion bodies.

The effect of Ca^{2+} on *in vivo* folding of CGTase in *E. coli* was examined by adding 1, 3, 5, and 10 mM $CaCl_2$ to the medium upon induction. When $CaCl_2$ was added at the late exponential phase of cell growth, no significant difference in final cell densities was observed, regardless of $CaCl_2$ concentrations (Fig. 5). The maximum values were obtained in the medium containing 1 mM $CaCl_2$ with the 15 units/ml CGTase activities and 7.1 units/g cells specific activity. This result was a 10-fold increase in enzyme activity and a 14-fold increase in specific CGTase activity as compared with those of the control without $CaCl_2$. However, a synergistic effect was not observed on the expression of soluble CGTase even with co-supplementation of other additives (data not shown).

In conclusion, *in vivo* folding of CGTase in recombinant *E. coli* was favored by lowering the culture temperature

Table 1. Effects of NaCl and glycine betaine on cell growth and CGTase activity in recombinant *E. coli* BL21(DE3) pLysE:pTCGT1.

	Maximum dry cell weight (g/l)	Maximum CGTase activity (units/ml)	Maximum specific CGTase activity (units/g cell)
Control experiment*	2.6	1.1	0.4
200 mM NaCl added	1.8	5.9	3.3
200 mM NaCl[a]	2.2	6.1	2.8
+1 mM glycine betaine[b]	2.3	11.6	5.1

*Control experiment was carried out in LB with 2 g/l glucose at 37°C and at pH 7.0.
[a]Glycine betaine was added in the medium initially.
[b]Glycine betaine was added in the medium after 3 h of induction.

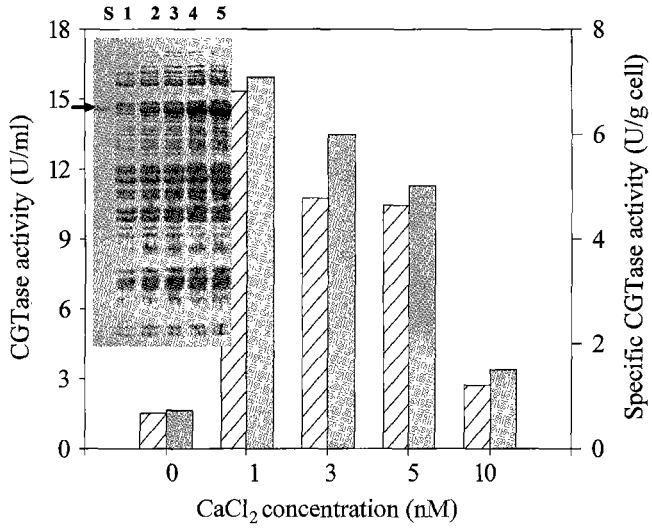

Fig. 5. Effect of $CaCl_2$ addition on CGTase expression in recombinant *E. coli* BL21(DE3)pLysE:pTCGT1.
Slashed bar: CGTase activity; dark bar: specific CGTase activity. Inner panel shows the SDS-PAGE analysis of the soluble fraction of the cell lyzates. Lane S: purified CGTase; Lane 1: no addition; Lane 2: 1 mM $CaCl_2$; Lane 3: 3 mM $CaCl_2$; Lane 4: 5 mM $CaCl_2$; Lane 5: 10 mM $CaCl_2$.

and by adding $CaCl_2$ as an osmotic effector. As commonly known, a low expression rate at low temperature resulted in a high final expression level of soluble CGTase. Supplementation of $CaCl_2$, a prosthetic group of CGTase, was the most effective additive among the various additives applied.

Acknowledgment

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