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Refolding of *Bacillus macerans* Cyclodextrin Glucanotransferase Expressed as Inclusion Bodies in Recombinant *Escherichia coli*

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Abstract This research was undertaken to restore the biological activity of cyclodextrin glucanotransferase (CGTase) of *Bacillus macerans* origin expressed as inclusion bodies in recombinant *Escherichia coli.* The optimum concentration of urea used as a denaturant was 8 M. The supplementation of 0.5 M urea into a dialysis buffer increased the refolding efficiency by preventing any protein aggregation. The influence of the protein concentration, temperature, and pH were also investigated. The protein concentration was found to be the most important factor in the refolding efficiency. The optimum temperature was 15–25°C and the optimum pH was 6.0. The maximum specific activity of the CGTase refolded under the optimum conditions was 92.2 U/mg, corresponding to 72% of the native CGTase. A comparison of the secondary structure between the native and the refolded CGTases showed that the relative ratio of the α-helix content in the native to the refolded CGTase was 1:0.82.

Key words: CGTase, recombinant *E. coli*, inclusion body, refolding

Escherichia coli is one of the most widely-used host cells for the production of recombinant proteins on an industrial scale as the single-celled microorganism has a number of advantages, including an accumulated knowledge-base regarding its molecular biology, genetics, physiology, and biochemistry, as well as a wide range of possible expression systems [19, 30]. Furthermore, fed-batch fermentation techniques that produce a high level of cloned proteins have been well established [30].

The overexpression of a cloned protein frequently involves insoluble inclusion body formation in recombinant bacterial systems [18], especially in recombinant *E. coli* [2]. An inclusion body contains a large number of cloned proteins and is seen as a dense granule under a microscope. There is no prevalent theory about the mechanism for inclusion body formation. However, experimental results indicate that the formation of an inclusion body for a given protein expressed in recombinant *E. coli* is dependent on the nature of the protein as well as the environmental conditions such as temperature, pH, and osmotic pressure [3]. The production of soluble proteins is enhanced by lowering culture temperature, controlling the induction of the promoter, and, in some cases, supplementing the growth medium with metal ions such as Zn^{2+} and Cu^{2+} [1]. The coexpression of a fusion partner [29] or chaperone [15], such as Skp, has also been demonstrated to be helpful in eliminating inclusion bodies. Mutation in a structural gene also helps to reduce or eliminate inclusion body formation [30, 31]. The formation of inclusion bodies can also be advantageous, in that the expressed protein is resistant to cytoplasmic proteases and can be easily purified from the cell lyzate [16]. This allows for the easy removal of any contaminating intracellular proteins before the solubilization of the desired product. Even with this advantage, the uncertainty of the *in vitro* refolding of an inclusion body can reduce some of the merits of *E. coli* as a high workhorse for the production of target proteins [28]. Consequently, the problem of protein refolding is a major issue in the efficient production of recombinant proteins for research and industrial applications [14].

Cyclodextrin glucanotransferase (EC 2.4.1.19) is a multi-functional enzyme, which catalyzes intra- and intermolecular transglycosylation and the ring opening of cyclodextrins (CDs) [17, 20, 21, 26, 27]. The major role of CGTase is to catalyze the formation of CDs from starch or starch-related substrates. Three types of CDs, α-, β- and γ-CD, consist of six, seven, and eight glucose units, respectively. In our

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previous work, *B. macerans* CGTase was produced in recombinant *E. coli* as an insoluble form, which required *in vitro* refolding operations to recover its biological activity [26]. This work was performed to investigate effects of environmental factors on the refolding efficiency of CGTase expressed as inclusion bodies in a recombinant *E. coli* expression system and to maximize the recovery of biologically active CGTase.

MATERIALS AND METHODS

Bacterial Strain and Plasmid

E. coli BL21 (DE3) pLysE [F, *ompT*, *rB*$^-$, *mB*$^-$, (DE3), pLysE, Cm^r] was used as the host. The pTCGT1 [26] plasmid used as the expression vector harbored, along with the SD sequence for a ribosome binding site, the signal sequence and *cgt* gene coding for the CGTase from *B. macerans*.

Culture Conditions

A colony from an LB-agar plate (0.5% yeast extract, 1% NaCl, 1% tryptone, 2% agar) was picked up and cultivated in an LB medium. The seed culture was carried out in a rotary shaking incubator for 10 h at 37°C, and transferred into a flask with 200 ml of the LB medium and incubated again for 10 h. Isopropyl β-D-thiogalactoside (IPTG) was used to induce the CGTase expression in recombinant *E. coli*.

Refolding and Purification of CGTase

The culture broth was centrifuged for 5 min at 8,000 ×g and 4°C to harvest the cells. The cell pellet was resuspended in an equal volume of 50 mM phosphate buffer (pH 6.0). After the disruption of the cell suspension with an ultrasonic processor (Cole-Parmer, Vernon Hills, U.S.A.), the crude CGTase solution was prepared by centrifugation of the cell lysate for 30 min at 10,000 ×g and 4°C. The supernatant and cell debris were defined as a soluble and inclusion body fraction of CGTase, respectively. The inclusion body fraction was washed twice with 1% Triton X-100 (Sigma Chemical Co., St. Louis, U.S.A.) and any residual Triton was removed by washing the pellet with 50 mM phosphate buffer (pH 6.0). Urea was added to unfold the insoluble CGTase and the mixture was then incubated by shaking for 1 h at room temperature. The unfolded CGTase was transferred into a dialysis tube (MW cut-off 12,000, Millipore, Bedford, U.S.A.) and dialyzed for 16 h at various temperatures against 50 mM Tris-Malate buffer supplemented with 5 mM $CaCl_2$. Any insoluble material after dialysis was removed by centrifugation. The soluble fraction of CGTase after dialysis was estimated by subtracting the insoluble aggregate removed by centrifugation from the total protein concentration.

The refolded CGTase was purified by affinity chromatography to determine its concentration and enzyme activity. Alpha-CD immobilized Sepharose 6B (Sigma Chemical Co., St. Louis, U.S.A.) was packed in a column and equilibrated with 10 mM sodium phosphate buffer (pH 6.0). The refolded CGTase in 10 mM sodium phosphate buffer (pH 6.0) was then loaded onto the column and eluted with 1% β-CD solution. The CGTase was concentrated using an ultrafiltration kit (Millipore, Bedford, U.S.A.). The protein concentrations at each purification step were determined by a protein assay kit (Bio-Rad, Hercules, U.S.A.).

Circular Dichroism Spectroscopy

The secondary structure of the native and refolded CGTases was analyzed by a spectropolarimeter (Jasco, Tokyo, Japan) within a wavelength range of 190-250 nm. The cell path length and resolution were 10 mm and 0.1 mm, respectively. The secondary structure was estimated by the computer program installed in the instrument.

CGTase Activity

The reaction mixture was composed of 5% soluble starch and 50 mM phosphate buffer (pH 6.0) to make a total volume of 0.8 ml and pre-warmed at 50°C for 10 min. The enzymatic reaction was started by adding 0.2 ml diluted solution of the purified CGTase to the reaction mixture and then the reaction was continued for 5 min. The reaction was stopped by adding 1 ml of acetonitrile followed by filtering the mixture with a 0.2 µm filter (Millipore, Bedford, U.S.A.). The reaction products were analyzed by HPLC (Knauer, Berlin, Germany) with the Licrosorb NH_2 column (Merck, Berlin, Germany). The column temperature was maintained at 80°C. The flow rate of the solvent (CH_3CN: H_2O=[65:35, v/v]) was 1.0 ml/min. One unit of CGTase activity was defined as the amount of enzyme that produced one mmole of α-CD under the reaction conditions.

RESULTS AND DISCUSSION

Urea Concentration

Urea was used to unfold the aggregated CGTase in the inclusion body. The inclusion body was dissolved in 6 and 8 M urea and refolded for 16 h at 4°C. When 223 mg/l of crude CGTase was applied, 6,500 CGTase units were recovered in the 8 M urea, while only 3,940 CGTase units were recovered in the 6 M urea. When 408 mg/l of crude CGTase was refolded using the above conditions, the similar trend was observed, with more CGTase recovered in the 8 M urea than at 6 M. In the above experiment, the difference in the refolding efficiency may have been caused by partial denaturation of insoluble CGTase in the 6 M urea. Eight molar urea was then used to unfold the inclusion body in the subsequent experiments.

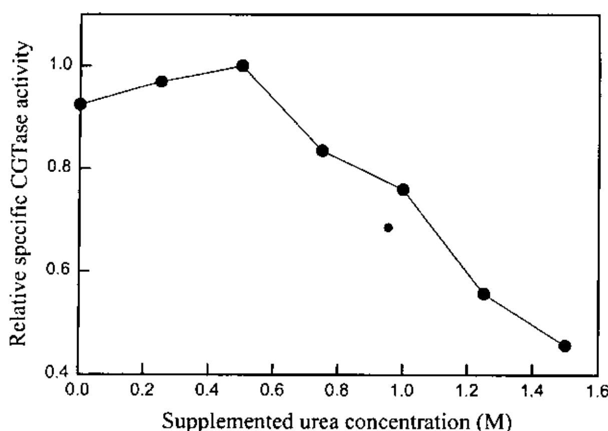

Fig. 1. Effects of urea supplementation in dialysis buffer on CGTase refolding performed at pH 6.0 and 4°C.

Various concentrations of urea were also supplemented in the dialysis buffer to investigate its influence on the refolding efficiency. The addition of 0.5 M urea in the dialysis buffer increased the refolding efficiency by 10%, while the addition of more than 0.5 M urea reduced the yield (Fig. 1). It would seem that low concentrations of urea assisted the refolding of CGTase by preventing any irreversible protein aggregation during the dialysis, which is consistent with the results of Daisuke *et al.* [4] who studied the refolding of denatured human interleukin-6. They revealed that a low concentration of denaturants could suppress the aggregation of the refolding intermediates even at high-protein concentrations, thereby increasing the yield of the desired protein.

Protein Concentration

Various concentrations of insoluble CGTase (85-210 mg/l) were dissolved in 8 M urea and dialyzed for 16 h at 4°C. As shown in Fig. 2, the recovery yield of CGTase decreased as the protein concentration increased. Concomitantly, the

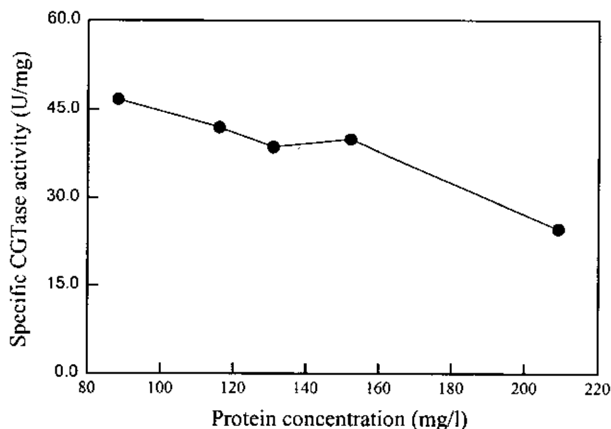

Fig. 2. Effects of protein concentrations on CGTase refolding performed at pH 6.0 and 4°C.

amount of insoluble protein that reaggregated during the dialysis increased with an increasing protein concentration (data not shown). The above result suggests that high protein concentrations caused a decrease in the specific CGTase activity due to either protein reaggregation or misfolding.

Refolding experiments are generally performed with low protein concentrations to avoid aggregation [24]. It has been commonly observed that the final yield of a refolding process decreases drastically with increasing concentrations of the unfolded protein due to hydrophobic interactions of the denatured peptide chains [8]. The influence of the protein concentration has been highlighted in relation to the competition between intramolecular interactions (which promote folding) and intermolecular interactions (which promote aggregation) [13]. In some cases, aggregation competes efficiently with intramolecular folding, particularly at higher protein concentrations, and often leads to a low yield of the refolded protein. However, the refolding of active proteins in very dilute solutions is not practical, especially when large quantities of a product protein are needed [25]. Recently, a novel preparation method aimed at improving the efficiency of refolding with high protein concentrations has been developed [14]. The isolation of protein molecules in a reversed micellar matrix significantly reduces the intermolecular interactions, thereby improving the refolding process [14].

pH and Temperature

The pH during refolding can affect the yield. Exposure to an alkaline pH (>9.0) without urea or guanidine hydrochloride leads to unfolded proteins [23]. As a result, dilution of urea in buffers at an alkaline pH has been applied to produce active prochymosin [8]. The pH is also critical for the development of a correct disulfide bond formation, since a thiol-disulfide interchange proceeds more rapidly with an alkaline pH [8, 23].

Accordingly, we investigated the effects of pH on CGTase refolding. The maximum specific CGTase activity of 47 U/mg was obtained at pH 6.0, corresponding to a 9.8-fold increase compared with that at pH 8.0. As shown in Fig. 3, the soluble fraction increased with an increasing pH, yet the specific activity dropped abruptly above pH 6.0. The refolding at pH 7.0 yielded only 22 U/mg of specific CGTase activity. The amount of soluble fraction and specific activity were very low between pH 4 and 5, which could be explained by the pI value of *B. macerans* CGTase being, 4.65. The solubility of CGTase around an isoelectric point is generally very low. However, it was of interest to note that there was a discrepancy between the solubility and the specific activity of CGTase at a high pH; an increase in protein solubility was not necessarily accompanied by an enhanced specific activity. Therefore, it would appear, in view of the tertiary structure of the protein of interest, that attenuated specific activity of

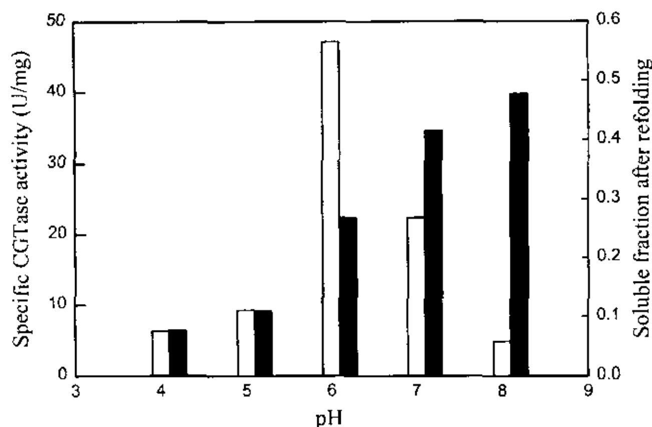

Fig. 3. Dependencies of specific CGTase activity (□) and soluble fraction (■) after refolding in dialysis buffer pH.
The refolding was carried out for 16 h at 4°C

CGTase at a higher pH may result from a structural change or the misfolding of the CGTase.

The effect of temperature was also examined. The protein concentration was adjusted to 150 mg/l before the dialysis. The refolding carried out at 25°C yielded the maximum specific activity of 28 U/mg (Fig. 4). However, there was no remarkable difference in the specific activity of CGTase after refolding between 15°C and 25°C. Decreases in both both the specific activity and the soluble fraction were observed at 37°C, which would seem to be due to protein aggregation caused by an elevated level of hydrophobic interactions between the unfolded proteins. The specific activity of CGTase at 37°C was 56% and the soluble fraction was 74% compared with the corresponding values at 25°C.

It is generally accepted that the initial folding intermediates aggregate largely through hydrophobic interactions, which are highly dependent upon temperature [22, 32]. The above

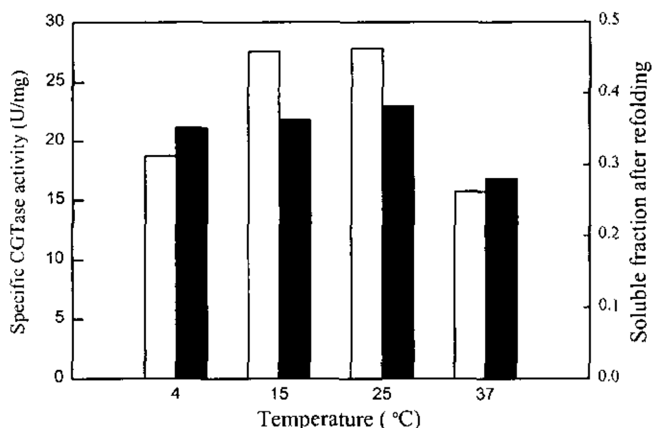

Fig. 4. Refolding efficiencies of CGTase at various temperatures.
The pH was fixed at 6.0 and the protein concentration was 150 mg/l before the dialysis. □: specific CGTase activity; ■: soluble fraction after refolding.

Table 1. Comparison of enzyme properties between native and refolded CGTase*.

	Specific activity (U/mg)	Product distribution (mole %)			Relative α-helix content
		α-CD	β-CD	γ-CD	
Native	128	75	17	8	1
Refolded	92	64	29	7	0.82

*The enzymatic reactions were carried out using soluble starch as the substrate at pH 6.0 and 50°C.

experimental results are compatible with the fact that the major driving force in protein aggregation is hydrophobic interaction, which is enhanced by an increase in temperature. By taking advantage of a low temperature to attenuate the hydrophobic effect, a rational approach of temperature modulation has been used to control aggregation and achieve high refolding yields [32].

Comparison of Refolded and Soluble CGTase

The refolded native CGTases were purified by affinity column chromatography. The native CGTase was defined as the enzyme directly purified from the supernatant of the recombinant *E. coli* cell lysate. The native CGTase showed specific activity of 128 U/mg (Table 1). In contrast, the CGTase that refolded under the optimum conditions showed 92 U/mg specific CGTase activity, corresponding to 72% of the native CGTase. Consequently, it was concluded that refolding does not always lead to the correct folding of the product protein. In other words, the refolding process can lead to a misfolded form of CGTase even under the optimum refolding conditions. The reasons for the differences in specific activity observed between the two different protein preparations need to be clarified.

A circular dichroism analysis of the purified CGTases was performed to explain the differences in the specific activity in terms of the secondary structure of the protein. The far UV-CD spectra of the native and refolded CGTases are illustrated in Fig. 5. The native and refolded CGTase showed a major circular dichroism signal at approximately 210 nm, which is a region characteristic of α-helical structure [7]. The refolded CGTase illustrated a decrease in the α-helix content, thereby indicating a conformational change in the protein. Nevertheless, the refolded CGTase exhibited a substantial recovery of the secondary structure, closely resembling the native CGTase, except for a slightly larger intensity and narrower bandwidth on the far-UV spectra. Accordingly, it is speculated that the misfolded CGTase with altered α-helix content occurred during the refolding process and coexisted with the correctly folded CGTase. The misfolded protein fraction probably was trapped in the altered conformations while remaining as soluble and still possessing the CGTase activity. In order to characterize the enzymatic reaction

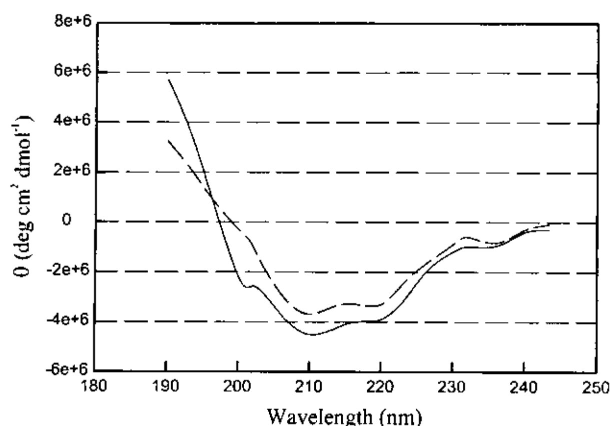

Fig. 5. CD spectra of native (line) and refolded (dashed line) CGTases.

The protein concentration was fixed at 179 mg/l.

patterns, the two different CGTases were reacted with a starch solution at 50°C and pH 6.0. An analysis of the reaction products showed a difference in the CD selectivity (Table 1). The relative concentration of the α-CD produced by the refolded CGTase decreased by about 15% compared with the native CGTase. The refolded CGTase produced more oligosaccharides, ranging from G1 to G5, from the soluble starch. The above results on the enzymatic characteristics of CGTase necessitate further investigation on the relationship between the structure and the biological function of the protein.

In conclusion, it would appear that a refolding strategy for a given system should be designed based on its folding kinetics, where the environmental conditions are optimized experimentally.

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