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Characterization of Cyclodextrin Glycosyltransferase of the Same Gene Expressed from *Bacillus macerans*, *Bacillus subtilis*, and *Escherichia coli*

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The plasmid pHG contains a cyclodextrin glycosyltransferase (CGTase) gene (*cgt*) derived from *Bacillus macerans*. Two transformants, *Bacillus subtilis* (pHG) and *Escherichia coli* (pHG), were found to produce CGTases with the same primary structure as the enzyme from *B. macerans*. However, the β-cyclodextrin coupling activity of the CGTase from *E. coli* (pHG) was 14-fold higher than that of the enzymes from the other strains. By contrast, no differences in α-cyclodextrin coupling activities were observed among these CGTases. CGTase from *E. coli* (pHG) was found to be less thermostable than the other CGTases. When the CGTase produced by *B. subtilis* was treated with increasing urea concentrations (10−1000 mM) to promote increasing degrees of protein unfolding, a bell-shaped β-cyclodextrin coupling activity profile was obtained. Subtle differences in the conformation of the CGTase produced by *E. coli* are therefore proposed to be responsible for the markedly increased β-cyclodextrin coupling activity of this enzyme.

KEYWORDS: Cyclodextrin glycosyltransferase; expression; *Bacillus macerans*; *Bacillus subtilis*; *Escherichia coli*; conformation

Cyclodextrin glycosyltransferase (EC 2.4.1.19, CGTase) is a starch-degrading enzyme belonging to the α-amylase family (family 13) of glycosyl hydrolases (*1*). CGTase converts starch to a mixture of cyclodextrins (CDs) consisting mainly of six, seven, or eight α-1,4-linked glucose residues termed α-, β-, or γ-CDs, respectively. CDs are cyclic molecules with hydrophilic exteriors and hydrophobic cavities that enable inclusion complexes to form with many hydrophobic molecules. As a consequence of complex formation, the properties of the included molecules are altered (*2*). CDs therefore have potential applications in the food, cosmetics, agricultural, and pharmaceutical industries (*3*−*5*), but industrial use is limited by the poor product specificity of CGTases. For example, all known CGTases produce a mixture of α-, β-, and γ-CDs (*6*−*8*). In addition to these cyclization reactions, the enzyme can perform coupling, hydrolysis, and disproportion reactions.

The structural and functional properties of specific CGTases have been examined in several laboratories (*9*−*12*). In previous studies in this laboratory, the *Bacillus macerans* cyclodextrin glycosyltransferase gene (*cgt*) was expressed in *Escherichia coli* (*13*) and in *Bacillus subtilis* (*14*), and properties of the yield enzymes were investigated. It was observed that certain biochemical properties of the mutant CGTase produced by *E. coli* were usually not reproduced when the identical mutant CGTase produced by *B. subtilis* was examined (unpublished finding). The present study was therefore undertaken to characterize purified CGTases from various hosts and to explore further the differences among these enzymes. Surprisingly, the CGTase expressed from *E. coli* was found to exhibit significantly higher β-coupling activities than identical enzymes produced from *B. macerans* and *B. subtilis*. New findings are provided that explain the observed diversities in CGTase activities and properties as a consequence of their expression in different hosts.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *B. macerans* IAM1243, the source of the *cgt* gene and CGTase, was obtained from the Institute of Agricultural Microbiology of Tokyo University. *E. coli* XL1-Blue (*sup* E44, *hsd* R17, *rec* A1, *end* A1, *gyr* A46, *thi*, *rel*, *lac*$^-$) and *B. subtilis* DB430 (*trpC2*, *npr*, *apr*, *epr*, *bpf*, *ispl*) were used as hosts for the expression of *cgt*. The recombinant plasmid, pHG, containing *cgt* was constructed as described previously (*14*) for expression in *E. coli* and *B. subtilis*.

Production and Purification of CGTases. Both *B. macerans* IAM 1243 and *B. subtilis* DB430/pHG secreted CGTases into the culture medium, which was collected. The CGTases were subsequently purified by β-CD coupling affinity chromatography and DEAE-Sepharose chromatography as described previously (*14*, *15*). A different approach was used with *E. coli* XL1-blue/pHG, which did not secrete the enzyme. The cells were collected after being cultured in a 180 rpm shaking incubator at 37 °C for 8 h, then resuspended in lysis buffer [50 mM (pH 8.0) phosphate buffer, 300 mM sodium chloride, 0.2 mM PMSF], and subjected to sonication. Enzyme in the crude extract was purified using β-CD coupling affinity chromatography as described previously

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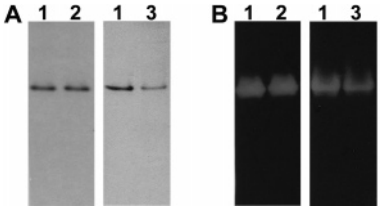

Figure 1. Purified CGTases were separated by native-PAGE and stained by CBR staining (**A**) or iodine staining (**B**): lane 1, CGTase (BM); lane 2, CGTase (BS); lane 3, CGTase (EC).

(*13*). The collected fractions with enzyme activity were dialyzed against 10 mM phosphate buffer (pH 7.0) before use in enzymatic assays. Each protein concentration of all purified CGTases was determined according to the method of Lowry et al. (*16*). The *B. macerans* IAM 1243 *cgt* gene products obtained from *B. macerans* IAM 1243, *B. subtilis* DB430/pHG, and *E. coli* XL1-blue/pHG were designated CGTase (BM), CGTase (BS), and CGTase (EC), respectively.

N-Terminal Sequencing of the CGTases. The purified CGTase preparations were subjected to SDS-PAGE (10%), and proteins were then transferred to poly(vinylidene fluoride) (PVDF) membranes (Millipore, Bedford, MA). The desired species were excised and eluted from the membranes and sequenced using the Applied Biosystems 476A protein sequencer.

Assay of CGTase Activity. The coupling and starch digestion activities of the CGTases were measured as described previously (*17*, *18*). In the coupling reaction, enzyme was added into the substrate mixture [50 mM sucrose, 1.0 mg/ mL glucoamylase, 25 mM Tris-HCl (pH 7.5), and 12.5 mM indicated CD] and allowed to stand at 40 °C for 10 min. Then the released glucose was determined by DNS reagent; 1 unit of activity was defined as 1 mmol of glucose produced per minute at 40 °C. In the starch-digesting reaction, enzyme was added into the substrate mixture (10 mM Tris-HCl and 0.5% soluble starch) and allowed to stand at 40 °C for 10 min. Then the reaction was terminated by 1 N HCl, and residual starch was determined by iodine reagent (10 mM I_2 and 250 mM KI). One unit of activity was defined as 50% absorbance at 660 nm reduced per 10 min at 40 °C. Cyclodextrin formation was measured using HPLC according to the method of Jeang and Sung (*19*).

To determine the effects of temperature on enzyme stability, the coupling activity remaining after incubation of the enzymes in assay buffer for 30 min at different temperatures (20−80 °C) was measured using α-CD as the substrate.

To determine the kinetics of enzyme inactivation (k_d values), the coupling activity remaining after incubation of the enzymes in assay buffer at 60 °C for various times (5−40 min) was measured with α-CD as the substrate.

Urea-Induced Unfolding of the CGTases. Equal amounts of CGTase (BS) were incubated with urea (10−1000 mM) at 4 °C for 2 h, followed by immediate measurement of residual α- and β-CD coupling activities.

RESULTS AND DISCUSSION

Expression of *cgt* in Various Hosts. *B. macerans* and *B. subtilis* (pHG) secrete newly formed CGTase directly into the medium, but the *E. coli* (pHG) enzyme is localized to the periplasm and cytosol (*14*). It was therefore considered essential to verify that the amino acid sequences of the enzymes from these different hosts were identical. The purified CGTases, from each of the three hosts, migrated as a single band in native-PAGE by Coomassie Brilliant Blue R-250 (CBR) staining and iodine staining (**Figure 1**). The N-terminal sequences of these enzymes were determined and found to be identical (**Table 1**). This indicated that the signal peptides of CGTases were processed at the same cleavage site in *E. coli*, *B. subtilis*, and *B. macerans*, regardless of expression of the homologous *cgt* gene in these different hosts. The CGTases from these different hosts also displayed the same molecular size when analyzed by SDS-PAGE (*14*). Collectively, these results indicate that the primary amino acid sequences of the CGTases produced by the *Bacillus* strains and by *E. coli* are identical.

Table 1. N-Terminal Sequences of CGTases from Various Sources

source of CGTase	N-terminal sequence
B. macerans	S P D T S V D N K V N F S T D V I Y
B. subtilis/pHG	S P D T S V D N K V N F .....
E. coli/pHG	S P D T S V ... N K V .. F .....

Table 2. Activities of Coupling and Starch Digesting of Different CGTases

source of CGTase	coupling reaction (units[a]/mg)		starch-digesting reaction (units[b]/mg)
	α-CD	β-CD	
B. macerans	56.5 ± 4.9	1.3 ± 0.1	159.3 ± 3.5
B. subtilis/pHG	62.4 ± 5.0	1.1 ± 0.1	164.5 ± 3.5
E. coli/pHG	50.6 ± 6.0	18.2 ± 1.6	151.5 ± 1.6

[a] One unit of activity is defined as 1 mmol of glucose produced per minute at 40 °C. [b] One unit of activity is defined as 50% absorbance at 660 nm reduced per 10 min at 40 °C.

Table 3. k_d Values of CGTases from Various Sources at 60 °C

source of CGTase	k_d (min^{-1})
B. macerans	1×10^{-2}
B. subtilis/pHG	2×10^{-2}
E. coli/pHG	6×10^{-2}

Catalytic Properties of the CGTases. The purified CGTases were examined for their abilities to perform cyclization, starch digestion, and coupling reactions. All CGTases had similar CD production profiles (data not shown) and starch digestion capacities (**Table 2**). When the coupling reaction was measured utilizing β-CD as substrate, however, CGTase (EC) displayed a 14-fold greater catalytic activity as compared to CGTase (BM) or CGTase (BS). By contrast, the coupling activity of CGTase (EC) was not significantly different from that of CGTase (BM) or CGTase (BS) when α-CD was used as the substrate (**Table 2**). Nakamura et al. (*20*) hypothesized that the coupling reaction is modulated by a random ternary complex-based mechanism. It was also proposed that, in the process of forming the ternary complex, the acceptor binds to acceptor subsites +2 and +3 (*21*). Because the binding of larger CDs (β- or γ-CDs) interferes with binding of these acceptors at subsite +2, the coupling reaction is hindered (*22*). A slightly more flexible local conformation in CGTase (EC) as compared to CGTase (BM) or CGTase (BS) was therefore proposed to increase the binding of acceptor at subsite +2 during the β-CD coupling reaction.

Thermostability of the CGTases. In certain investigations (*23*−*25*), proteins with good conformational packing of the polypeptide chain were found to exhibit increased thermostability. When the thermostabilities of the CGTases were examined during the coupling reaction, CGTase (EC) was observed to be significantly less stable than the other CGTases only at 60 °C (**Figure 2**). Additionally, the k_d value for CGTase (EC) ($6 \times 10^{-2} min^{-1}$) was found to be higher than those for CGTase (BM) and CGTase (BS) (1×10^{-2} and 2×10^{-2} min^{-1}, respectively) at 60 °C (**Table 3**). These findings are consistent with lesser degrees of folding of CGTase (EC) as compared to the other two enzymes and with the presence of a relatively more flexible active center in CGTase (EC) during the coupling reaction.

Urea-Induced Unfolding of the CGTases. Urea disorders hydrogen-bonding interactions in proteins, promoting their

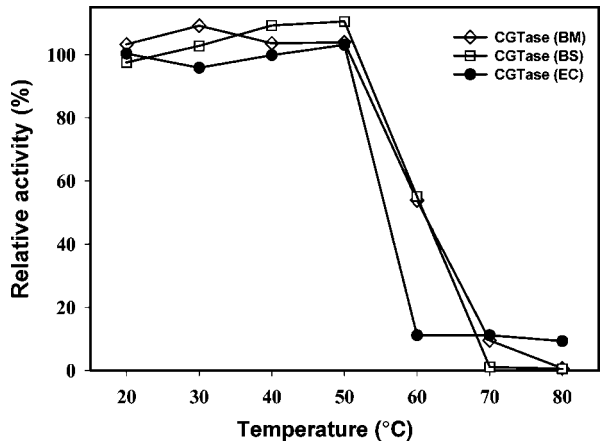

Figure 2. Thermostability of the studied CGTases. Coupling activities were determined for the α-CD substrate after incubation of the enzymes at various temperatures (20–80 °C) for 30 min.

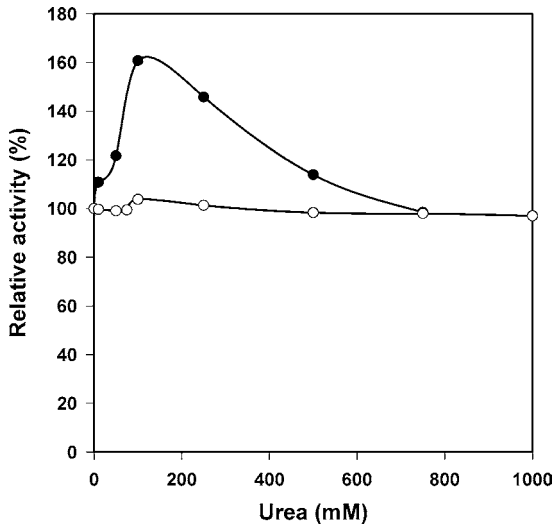

Figure 3. Urea induced-unfolding of the studied CGTases. The CGTase (BS) was unfolded using urea (10–1000 mM) at 4 °C for 2 h, and the residual α- and β-CD coupling activities were measured immediately: (○) α-CD; (●) β-CD.

unfolding. Ordinarily, the degree of protein unfolding by urea is directly proportional to its concentration. To ascertain whether the higher β-CD coupling activity observed with CGTase (EC) was also observed with partially unfolded forms of CGTase (BS), the latter was exposed to moderate urea concentrations in vitro followed by measurements of β-CD coupling activity (**Figure 3**). Significant increases in activity were observed when CGTase (BS) was treated with 150 mM urea, consistent with promotion of the coupling reaction through loss of structure at the acceptor subsite. Further increases in urea concentration reduced coupling activity, indicating that greater destabilization of hydrogen-bonding interactions within the ternary complex suppressed activity. By contrast, urea treatment failed to affect the α-CD coupling activity of CGTase (BS), supporting the hypothesis that enlargement of the active center is not required for optimal CGTase activity when a small CD serves as the donor substrate.

On the basis of findings from the studies of thermostability and urea-induced unfolding, the higher β-CD coupling activity of CGTase (EC) is concluded to result from the slightly more flexible structure of its active site. Conformational diversity of proteins may reflect the environment in which such proteins are folded. This coincides with Wulfing and Pluckthun's conclusion that proteins folded in the periplasm acquire distinctive conformations due to the unique physiological environment of this compartment (*26*). CGTase (EC), unlike the enzymes from *B. subtilis* or *B. macerans*, is secreted into the periplasm and is therefore anticipated to differ from the other enzymes in its degree of folding. Although CGTase (EC) exhibits unusally high β-CD coupling activity, *B. subtilis* is suggested to be the superior host in terms of elaboration of the underlying mechanisms of native CGTases and of the properties of foreign proteins.

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