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Analysis of Mutations in Cyclodextrin Glucanotransferase from *Bacillus stearothermophilus* Which Affect Cyclization Characteristics and Thermostability

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Cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19) produces cyclodextrin from starch. The CGTase molecule is composed of four globular domains, A, B, C, and D. In order to gain better understanding of the amylolytic and cyclization mechanisms of CGTase, mutant CGTases were constructed from a CGTase gene (*cgtl*) of *Bacillus stearothermophilus* NO2. Cgtl-F191Y (Phe at position 191 was replaced by Tyr), Cgtl-F191Y-F255Y, Cgtl-W254V-F255I, Cgtl-W254V, and Cgtl-F255I were constructed for the analysis of the NH_2-terminal region. It was revealed that amino acids surrounding a spiral amylose are important for cyclization characteristics and that hydrophobic amino acids just after the Glu catalytic site play an important role in the hydrolysis characteristics of the enzyme. Mutant CGTases Cgtl-T591F and Cgtl-W629F were also constructed to study the role of a second substrate-binding site in domain D, and it was suggested that substrate binding at both domains A and D stabilized the enzyme and optimized cyclodextrin production.

Cyclodextrin (CD) is a closed-ring structure in which six or more glucose units are joined by means of α-1,4-glucosidic bonds (15). They are able to form inclusion complexes with many organic and inorganic molecules, thereby changing the physical and chemical properties of the included compounds. According to the number of their glucose units (G6, G7, and G8), they are named α-, β-, and γ-CDs, respectively. Cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19), which catalyzes the degradation of starch into CD, is used as an important enzyme by the food and pharmaceutical industries.

Recently, tertiary structures of CGTases from *Bacillus circulans* no. 8 and *Bacillus stearothermophilus* TC-91 were determined (5, 7, 8), and those CGTases were found to be composed of five and four domains, respectively. On the basis of their analyses, NH_2-terminal domains were similar to the structure of Taka-amylase and COOH-terminal domains were unique to the CGTase. Hence, it was supposed that the COOH region of CGTase was related to the cyclization of linear maltooligosaccharide produced by the hydrolysis of starch with the enzyme (5–7, 19). However, we recently found that the NH_2-terminal region of CGTase was important for cyclization characteristics (3). In this paper, we describe the roles of the NH_2-terminal and COOH-terminal regions in cyclization, determined by constructing mutant CGTases from *B. stearothermophilus* NO2 as listed in Table 1.

Enzyme characteristics of Cgtl-F191Y and Cgtl-F191Y-F255Y. The nucleotide sequence of the CGTase gene (*cgtl*) from *B. stearothermophilus* NO2 was determined previously (3). The amino acid sequence deduced from *cgtl* exhibited 99% homology to that of the CGTase of *B. stearothermophilus* TC-91 (3). Therefore, the molecular structure of Cgtl is thought to be similar to that of the CGTase of *B. stearothermophilus* TC-91, which has a size of 80 by 70 by 50 Å (8 by 7 by 5 nm) and has four globular domains, A, B, C, and D. Kubota et al. reported that substrate (spiral amylose) was considered to be fixed in domains A and D of CGTase (8). Hence, amino acid residues surrounding a spiral amylose in domain A are supposed to affect the cyclization. Phe-191 and Phe-255 of Cgtl were considered to be located around a spiral amylose. In comparison with those of α-specific CGTase from *Bacillus macerans* IFO3490 (3), Tyr residues instead of Phe residues were located at these positions. The difference between Phe and Tyr is the OH group in the side chain, and this difference may affect cyclization characteristics. We constructed mutant CGTases Cgtl-F191Y (Phe at position 191 was replaced by Tyr) and Cgtl-F191Y-F255Y (Phe-191 and Phe-255 were replaced by Tyr residues) to test the effect of these residues on the cyclization reaction. Cgtl-F191Y and Cgtl-F191Y-F255Y were expressed in the recombinant plasmid carrier of *Bacillus subtilis* NA-1 cells (1) and secreted into the culture medium. Each enzyme was partially purified and used to examine enzyme characteristics according to the procedure reported previously (3). CGTase from *B. macerans* IFO3490 was also purified and used as the control enzyme for an α-specific CGTase. A total of 100 U of each enzyme was used for the reaction, and the products were analyzed by high-performance liquid chromatography (HPLC). As shown in Fig. 1, CGTase from *B. macerans* IFO3490 produced mainly α-CD from starch. On the other hand, Cgtl produced α-CD at the initial step and the ratio of β-CD gradually increased and became almost equal to the level of α-CD at the stationary step of the reaction. Cgtl-F191Y produced more α-CD than did Cgtl. Cgtl-F191Y-F255Y produced more α-CD than did Cgtl-F191Y, especially at the initial step. These results indicate that Phe-191 and Phe-255 are important for cyclization characteristics.

Effects of mutations of hydrophobic residues in the vicinity of the catalytic site on CD production. A comparison of the

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TABLE 1. Recombinant plasmids used

Plasmid	CGTase type or mutation position(s)
pKB1	Wild type (Cgtl)
pKBM1	Wild type (CgtM)
pKB1-F191Y	Phe-191 → Tyr
pKB1-F191Y-F255Y	Phe-191 → Tyr, Phe-255 → Tyr
pKB1-W254V	Trp-254 → Val
pKB1-F255I	Phe-255 → Ile
pKB1-W254V-F255I	Trp-254 → Val, Phe-255 → Ile
pKB1-T591F	Thr-591 → Phe
pKB1-W629F	Trp-629 → Phe
pKB1-T591F-W629F	Thr-591 → Phe, Trp-629 → Phe

amino acid sequence of CGTase with that of α-amylase indicates that the NH_2-terminal region at domain A (approximately 400 amino acids) of CGTase contains the four conserved consensus amino acid sequences, which were found in all reported α-amylases and other starch-degrading enzymes (2, 9, 11, 14). Therefore, it is supposed that the NH_2-terminal region in the CGTase has the starch-degrading activity to cleave the α-1,4-glucosidic bonds. On the basis of the structural analysis of amylase (7, 8, 12, 13), Glu-253, Asp-225, and Asp-324 in *B. stearothermophilus* NO2 CGTase (Cgtl) were considered to be the catalytic residues for the hydrolysis, and these residues are conserved in all CGTases. In CGTases, Trp, Phe, and Tyr are located just after the catalytic site Glu-253 and are all hydrophobic residues having aromatic side chains. However, in most α-amylases or other starch-degrading enzymes, there are aliphatic hydrophobic residues such as Ile or Val at this position.

Such a microenvironmental difference may be related to the cyclization reaction. Therefore, we constructed a mutant CGTase, Cgtl-W254V-F255I, which had Val and Ile at residues 254 and 255 instead of Trp and Phe, respectively. Val and Ile are typically observed in amylases. The enzyme solution (4 mg/ml) was obtained from the extracellular fraction of *B. subtilis* NA-1 cells harboring the mutant plasmid pKB1-W254V-F255I. CD-producing activity and amylase activity were measured as α-CD specific activity (10) and both saccharifying (1) and liquefying (4) activities, respectively (Table 2). The mutant CGTase Cgtl-W254V-F255I lost α-CD-forming activity, and the liquefying activity decreased drastically. However, the saccharifying activity remained. In order to clarify the product profile with Cgtl-W254V-F255I, we analyzed individual CDs and other sugars. The

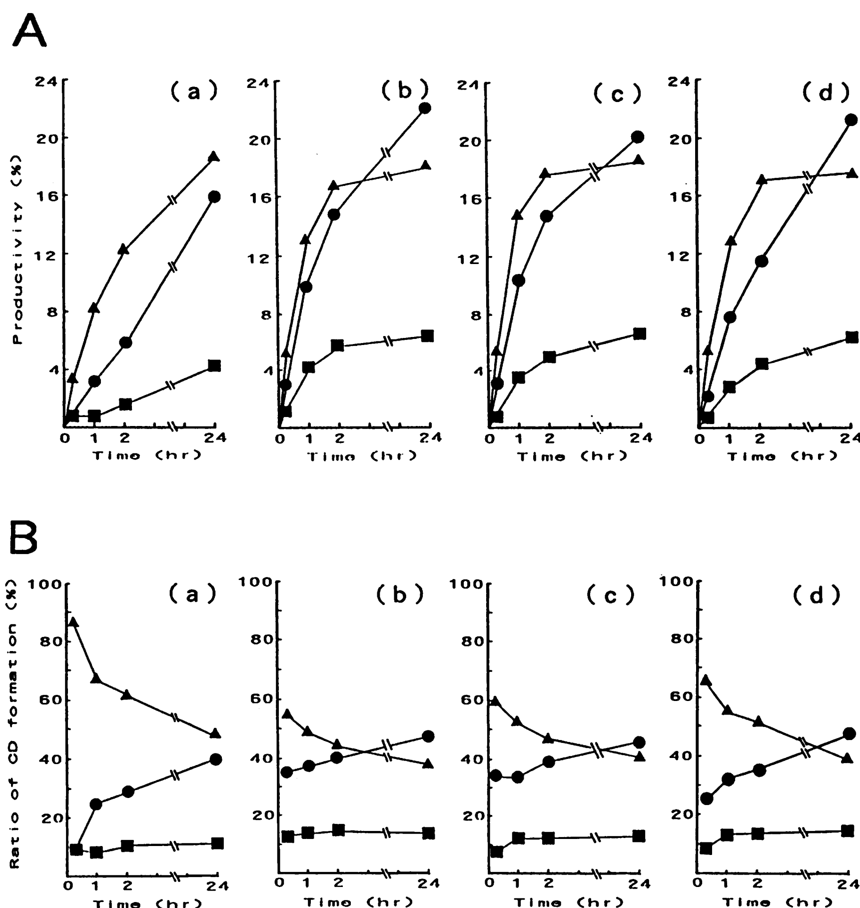

FIG. 1. Time course of CD production by Cgtl, CgtM, Cgtl-F191Y, and Cgtl-F191Y-F255Y. (A) Production of CDs from starch. (B) Ratio of CD produced from starch. The reaction mixture (pH 6.0) containing 5% soluble starch and 3 mM $CaCl_2$ was incubated with 100 U of the enzyme as liquefying activity in a total volume of 4 ml. The reaction was carried out at 65°C. Samples were withdrawn and kept in a boiling water bath for 5 min to stop the reaction. The CDs formed were analyzed by HPLC. a, b, c, and d, profiles of the reactions with CgtM, Cgtl, Cgtl-F191Y, and Cgt-F191Y-F255Y, respectively. ▲, α-CD; ●, β-CD; ■, γ-CD.

TABLE 2. CGTase and amylolytic activities of mutant CGTases

CGTase	Activity[a]		
	CGTase (μmol/ml/min)	Saccharifying (μmol/ml/min)	Liquefying (U/ml/min)
Cgt1	7.61 (100)	1.88 (100)	260.0 (100)
Cgt1-W254V-F255I	<0.01 (0)	1.79 (94)	35.8 (14)
Cgt1-W254V	1.70 (23)	1.86 (99)	56.5 (22)
Cgt1-F255I	<0.01 (0)	4.28 (227)	61.3 (24)

[a] Values in parentheses are relative activities.

mutant enzyme produced neither CDs nor specific maltooligosaccharides (data not shown). These results mean that the mutant enzyme can hydrolyze α-1,4-glucosidic bonds but cannot cyclize the digested products and that aromatic hydrophobic residues such as Trp, Phe, and Tyr in the conserved region play an important role for cyclization. To examine which residue is critical for cyclization, Cgt1-W254V and Cgt1-F255I were also constructed. As shown in Table 2, Cgt1-W254V had CGTase activity and amylase activity, although their levels were decreased. Cgt1-F255I lost CGTase activity, and the liquefying activity decreased. However, the saccharifying activity of Cgt1-F255I was drastically increased. On the basis of these results, it was supposed that hydrophobic amino acid residues at position 255 were important not only for cyclization but also for the hydrolysis reaction of the enzyme. It seems necessary to provide a hydrophobic environment around the acid catalysis residue to raise the pK_a value. Mutant enzymes might have different affinities to the substrate starch.

Functional analysis of the COOH-terminal substrate-binding site of CGTase. The COOH-terminal region of CGTase also has homology with the raw-starch-binding sites of glucoamylase and G4-amylase (16, 18, 19). Amino acid residues in domain D are highly conserved among different CGTases. By substrate-binding analysis of the crystal, maltose has been found to bind at two sites of the CGTase molecule (8). One is in the cleft of domain A, which is the active site for hydrolysis, and the other is in domain D at a distance of about 30 Å (3 nm) from that of domain A. Therefore, starch as a substrate is thought to be fixed between two domains like a bridge. CGTase may be stabilized by such a starch bridge. If the starch bridge forms, the

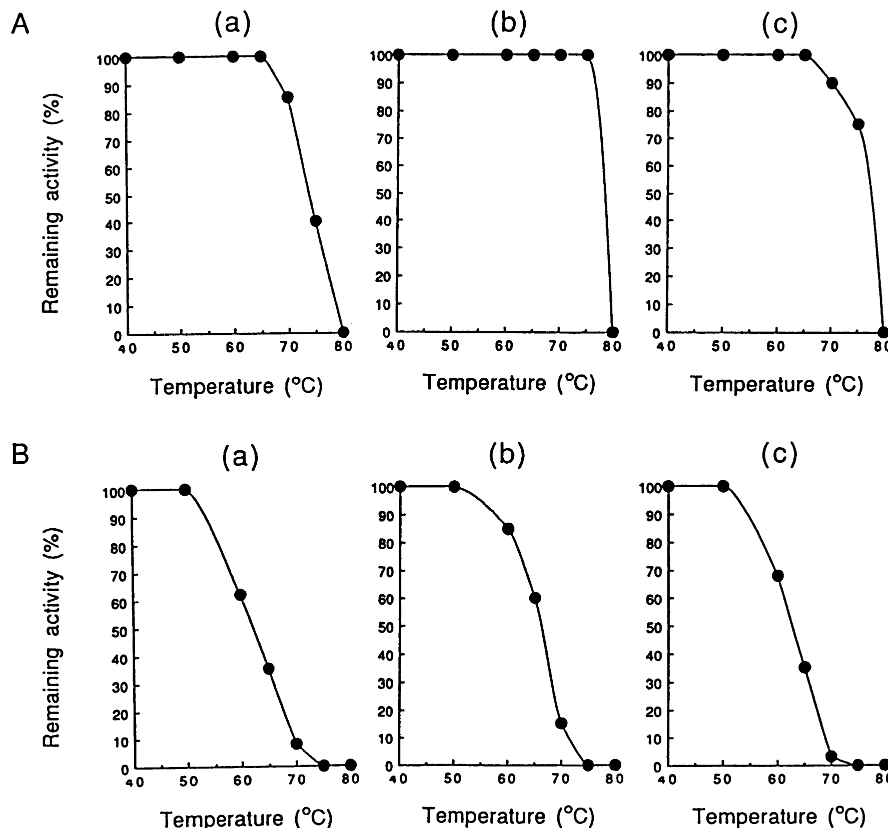

FIG. 2. Effect of substrate addition on the thermostability of Cgt1 (A) and Cgt1-T591F (B). CGTase (20 U) in 50 mM phosphate buffer (pH 6.0) was treated at constant temperatures (40, 50, 55, or 60°C) for 10 min and cooled quickly with ice water. The remaining CGTase activity was assayed. Starch and maltose were added to the enzyme solution at a concentration of 1% (wt/vol). (a) Control; (b) starch addition; (c) maltose addition.

thermostability of CGTase may be increased by the addition of starch but not maltose because the maltose molecule is too small to make the bridge. We examined the thermostability of CGTase with and without starch or maltose. Partially purified Cgt1 was used for the analysis. As shown in Fig. 2, CGTase was stabilized by starch; however, it was not stabilized by the addition of maltose. Thr-591, Asn-596, Asn-620, Tyr-626, and Trp-629 of Cgt1 are considered important for maltose binding at domain D (8, 19). Especially Thr-591 and Trp-629 are conserved between CGTases and glucoamylase. This observation suggests that these two residues are the most functional for starch binding. We constructed three kinds of mutant enzymes which had mutations at positions 591 and 629 in domain D. Thr-591 and Trp-629 were replaced by Phe (Cgt1-T591F and Cgt1-W629F, respectively). Cgt1-T591F-W629F, which had a double mutation, was also constructed. These mutant enzymes were thought to decrease the efficiency of substrate binding at domain D. The recombinant plasmids harboring T591F, W629F, and T591F-W629F were designated pKB1-T591F, pKB1-W629F, and pKB1-T591F-W629F, respectively. Wild-type enzyme (Cgt1) was efficiently expressed in *B. subtilis* NA-1 cells and secreted into the culture medium. Cgt1-T591F and Cgt1-W629F were also expressed. However, Cgt1-T591F-W629F was not (data not shown). This deficiency might be due to the conformational change of the enzyme.

Enzyme samples of Cgt1-T591F and Cgt1-W629F were obtained, purified, and used for stability analysis. Cgt1 was used at 65°C, and both Cgt1-T591F and Cgt1-W629F were used at 55°C, the optimum temperature for these mutant enzymes. The thermostability of Cgt1-T591F was also examined, and it was decreased in comparison with that of the wild-type enzyme (Fig. 2). The effects of addition of starch or maltose on thermostability were also examined. Cgt1-T591F was stabilized by starch to some extent but not by maltose addition. The same effects were observed for Cgt1-W629F (data not shown). However, the ratio of individual CDs produced by Cgt1-T591F or Cgt1-W629F was not changed (data not shown). The CD productivity of mutant CGTases was slightly decreased (to around 90% of the wild-type level). These results mean that substrate binding at domain D is related to enzyme stability but not to cyclization characteristics. The substrate-binding site at domain D is close to that of domain A, at a distance of about 30 Å (3 nm). Hence, spiral amylose, which has a diameter of 13 Å (1.3 nm) and a pitch of 8 Å (0.8 nm) (17), is considered to be fixed like a bridge over two domains. This bridge might stabilize the CGTase molecule and optimize CD production because of the suitable positioning of amylose. Actually, Cgt1-T591F and Cgt1-W629F harboring mutations in the substrate-binding site caused deterioration of enzyme thermostability and CD productivity. More precise study is expected to clarify the role of substrate binding at domain D.

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REFERENCES

1. **Aiba, S., K. Kitai, and T. Imanaka.** 1983. Cloning and expression of thermostable α-amylase gene from *Bacillus stearothermophilus* in *Bacillus stearothermophilus* and *Bacillus subtilis*. Appl. Environ. Microbiol. **46:**1059–1065.
2. **Binder, S., O. Huber, and A. Boeck.** 1986. Cyclodextrin glycosyltransferase from Klebsiella pneumoniae M5a1: cloning, nucleotide sequence and expression. Gene **47:**269–277.
3. **Fujiwara, S., H. Kakihara, K. B. Woo, A. Lejeune, M. Kanemoto, K. Sakaguchi, and T. Imanaka.** Cyclization characteristics of cyclodextrin glucanotransferase are conferred by the NH_2-terminal region of the enzyme. Appl. Environ. Microbiol., in press.
4. **Fuwa, H.** 1954. A new method for microdetermination of amylase activity by the use of amylose as the substrate. J. Biochem. **41:**583–603.
5. **Hofman, B. E., H. Bender, and G. E. Schultz.** 1989. Three-dimensional structure of cyclodextrin glycosyltransferase from *Bacillus circulans* at 3.4Å resolution. J. Mol. Biol. **209:**793–800.
6. **Kimura, K., S. Kataoka, A. Nakamura, T. Takano, and S. Kobayashi.** 1989. Functions of the COOH-terminal region of cyclodextrin glucanotransferase of alkalophilic *Bacillus* sp. #1011: relation to catalyzing activity and pH stability. Biochem. Biophys. Res. Commun. **161:**1273–1297.
7. **Klein, C., and G. E. Schultz.** 1991. Structure of cyclodextrin glucanotransferase refined at 2.0 Å resolution. J. Mol. Biol. **217:**737–750.
8. **Kubota, M., Y. Matsuura, S. Sakai, and Y. Katsube.** 1991. Molecular structure of *B. stearothermophilus* cyclodextrin glucanotransferase and analysis of substrate binding site. Denpun Kagaku **38:**141–146.
9. **Kuriki, T., and T. Imanaka.** 1989. Nucleotide sequence of the neopullulanase gene from *Bacillus stearothermophilus*. J. Gen. Microbiol. **135:**1521–1528.
10. **Lejeune, A., K. Sakaguchi, and T. Imanaka.** 1989. A spectrophotometric assay for the cyclization activity of cyclomaltohexose (α-cyclodextrin) glucanotransferase. Anal. Biochem. **181:**6–11.
11. **MacGregor, E. A., and B. Svensson.** 1989. A super-secondary structure predicted to be common to several α-1,4-D-glucan-cleaving enzymes. Biochem. J. **259:**145–152.
12. **Matsuura, Y., M. Kusunoki, W. Harada, and M. Kakudo.** 1984. Structure and possible catalytic residues of Taka-amylase A. J. Biochem. **95:**697–702.
13. **Matsuura, Y., M. Kusunoki, and M. Kakudo.** 1991. Structure and catalytic implications of Taka-amylase A. Denpun Kagaku **38:**137–139.
14. **Nakajima, R., T. Imanaka, and S. Aiba.** 1986. Comparison of amino acid sequences of eleven different α-amylases. Appl. Microbiol. Biotechnol. **23:**355–360.
15. **Pulley, O. A., and D. French.** 1961. Studies on the Schardinger dextrins. XI. The isolation of new Schardinger dextrins. Biochem. Biophys. Res. Commun. **5:**11–15.
16. **Sakai, S., M. Kubota, K. Yamamoto, T. Nakada, K. Torigoe, O. Ando, and T. Sugimoto.** 1987. Cloning of cyclodextrin glucanotransferase genes from *B. stearothermophilus* and *B. macerans*. J. Jpn. Soc. Starch Sci. **34:**140–147.
17. **Seikagakujiten.** 1984. Dictionary of Biochemistry, p. 69. Tokyo Kagaku Dozin, Tokyo.
18. **Svensson, B., A. J. Clarke, and I. Svensson.** 1983. Amino acid sequence of tryptic fragments of glucoamylase G1 from *Aspergillus niger*. Carlsberg Res. Commun. **48:**517–527.
19. **Svensson, B., H. Jesperson, M. R. Sierks, and E. A. MacGreger.** 1989. Sequence homology between putative raw starch binding domains from different starch-degrading enzymes. Biochem. J. **264:**309–311.