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Functional relationships between cyclodextrin glucanotransferase from an alkalophilic *Bacillus* and α-amylases

Site-directed mutagenesis of the conserved two Asp and one Glu residues

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Comparison of the amino acid sequences of cyclodextrin glucanotransferases (CGTases) with those of α-amylases revealed that two Asp and one Glu residues, which are considered to be the catalytic residues in α-amylases, were also conserved in CGTases. To analyze the function of the three conserved amino acid residues in CGTases, site-directed mutagenesis was carried out. The three mutant CGTases, in which Asp^{229}, Glu^{257} and Asp^{328} were individually replaced by Asn or Gln, completely lost both their starch-degrading and β-cyclodextrin-forming activities, whereas another mutant CGTase, in which Glu^{264} was replaced by Gln, retained these activities. The three inactive enzymes retained the ability to be bound to starch. These results suggest that Asp^{229}, Glu^{257} and Asp^{328} play an important role in the enzymatic reaction catalyzed by CGTase and that a similar catalytic mechanism is present in both CGTases and α-amylases.

Active site; Amylase; Catalytic center; Cyclodextrin glucanotransferase; Site-directed mutagenesis

1. INTRODUCTION

Although both cyclodextrin glucanotransferases (CGTases) and α-amylases degrade starch and amylose, the degradation products are quite different. α-Amylases degrade starch and amylose to linear maltooligosaccharides and glucose by hydrolysis whereas CGTases degrade them to cyclodextrins through an intramolecular transglycosylation reaction. Many CGTase genes have been cloned and sequenced [1–4]. Most CGTases consist of approximately 686 amino acids, and most α-amylases of approximately 500 amino acids. Although the sequence similarity between them is usually less than 30%, 3 highly conserved regions, which are located at approximately the 100, 200 and 300 amino acid positions from their NH_2-termini, have been identified, and these are designated as A-, B- and C-regions, respectively [1,2]. On the basis of crystallographic studies of α-amylases [5–7], approximately 400 amino acid residues of the NH_2-terminal region of the α-amylases are folded to given an $(\alpha/\beta)_8$-barrel structure, in which an active center is present. The catalytic residues of the α-amylases have been restricted to 3 acidic amino acid residues; Asp in the B-region; Glu, which is located approximately 30 amino acid residues on the COOH-terminal side of the B-region (designated as B′-region); and Asp in the C-region. Crystallographic studies on CGTases from *B. circulans* [8] and *B. stearothermophilus* [9] also showed that the NH_2-terminal half of CGTases was folded to a barrel structure and that 3 conserved amino acid residues were present in this domain. In order to analyze the function of the 3 conserved residues in the CGTases, 4 acidic amino acid residues, which are candidates for the catalytic residues of a CGTase from an alkalophilic *Bacillus* sp. #1011, were replaced by Asn or Gln residues, and the effects on the enzyme activity were analyzed. In this paper, we demonstrated that Asp^{229}, Glu^{257} and Asp^{328} play important roles in the enzymatic reaction as observed in the 3 conserved residues in α-amylases, but that Glu^{264} does not.

Abbreviations: BSA, bovine serum albumin; CGTase, cyclodextrin glucanotransferase; kb, kilobase pairs; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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2. MATERIALS AND METHODS

2.1. *Bacterial strains and plasmids*

Escherichia coli JM109 [*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 Δ(lac proAB)/F′ traD36 proAB lacI*^{q} *ZΔM15*] was used as a host for recombinant DNA manipulations. A protease-deficient mutant, *E. coli* ME8417 [*lon::Tn10(tet*^{r}*) thr leu lacY*] was provided by Dr. H.

Takahashi. The strain was used as the host for production of the wild-type and mutant CGTases. *E. coli* BW313 {*Hfr*KL16PO/45[*lysA*(61-62)] *dut1 ung1 thi-1 relA1*} was used for the site-directed mutagenesis experiments. The plasmid pTUE254 was constructed by the insertion of the 4.5 kb *Hin*dIII fragment of pTUE217 [1], in which the CGTase gene region of an alkalophilic *Bacillus* sp. #1011 is inserted into the *Hin*dIII site of pUC13.

2.2. *Construction of mutant CGTase genes*

Four 21-mer oligonucleotide, 5'-ATTCGCGTGAACGCGGTCAAG-3' (replacement of Asp229 by Asn: D229N), 5'-ACCTTCGGCCAATGGTTCCTA-3' (replacement of Glu^{257} by Gln: E257Q), 5'-GGCGTCAATCAGATCAGTCCG-3' (replacement of Glu^{264} by Gln: E264Q), 5'-GACAATCATAACATGGAGCGT-3' (replacement of Asp^{328} by Asn: D328N) were synthesized with an Applied Biosystems model 380A DNA synthesizer. The nucleotides were purified using oligonucleotide purification cartridges (Applied Biosystems). For the introduction of the mutations, the *Sph*I–*Sma*I fragment (1.8 kb), containing His 126–124 base pairs downstream from the termination codon of the CGTase gene, was inserted into M13mp18. Site-directed mutagenesis was carried out by the method of Kunkel et al. [10], and the mutations were verified by DNA sequencing [11]. The 4 mutagenized *Sph*I–*Afl*II fragments (1.8 kb) were then introduced into the *Sph*I–*Afl*II fragment (5.3 kb) of pTUE254.

2.3. *Purification of wild-type and mutant enzymes*

The *E. coli* ME8417 strains carrying the constructed plasmids were grown in 1 l of L-broth (1% Bacto tryptone (Difco), 0.5% Yeast extract (Difco), 0.5% NaCl) containing 250 μg/ml ampicillin and 20 μg/ml tetracycline at 37°C for 12 h. CGTases in the periplasm of the cells were extracted by the osmotic shock method [12], and subsequently ammonium sulfate was added to 25% saturation. All the steps were performed at 4°C. The fractions were centrifuged and the supernatants were loaded onto a column of Toyopearl HW-55F (Tosoh; 15 × 150 mm) pre-equilibrated with 10 mM sodium phosphate buffer (pH 6.0) containing 25% saturated ammonium sulfate. The enzymes were eluted with 10 mM sodium phosphate buffer (pH 6.0). The protein peak fractions were dialyzed against the same buffer. Protein concentrations were determined by BCA Protein Assay Reagent (Pierce), using bovine serum albumin (BSA) as the standard.

2.4. *Assay of CGTase*

Starch-degrading activity was measured at 37°C by the method of Fuwa [13] with slight modifications using soluble starch (Merck; Art. 1252) as the substrate. One unit of starch-degrading activity was defined as the amount that gave a 1% decrease per min of absorbance at 660 nm.

Since the main initial product from starch by *Bacillus* sp. #1011 CGTase is β-cyclodextrin, the β-cyclodextrin-forming activity was measured. The assay was performed according to the phenolphthalein method of Vikmon [14] with modifications. One unit of activity was defined as the amount that formed 1 μmol of β-cyclodextrin per min.

3. RESULTS

From the crystallographic studies, catalytic residues in *Aspergillus oryzae* α-amylase (Taka-amylase A) [5], porcine pancreatic α-amylase [6] and *A. niger* α-amylase [7] have been proposed to Glu^{230} and Asp^{297}, Asp^{197} and Asp^{300}, and Asp^{206} and Glu^{230}, respectively in the B-, B'- and C-regions. Among these residues, Asp residues in the B- and C-regions of the α-amylases corresponded to Asp^{229} and Asp^{328} of *Bacillus* sp. #1011 CGTase. How-

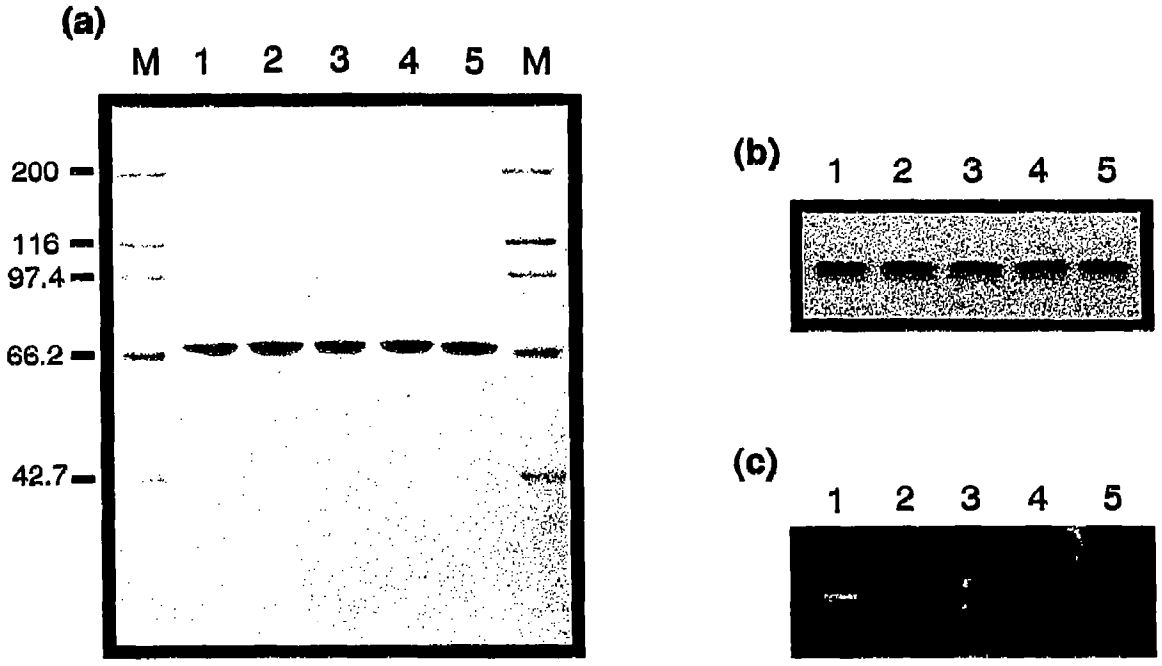

Fig. 1. SDS-PAGE of the purified wild-type and mutant enzymes. SDS-PAGE was carried out on 8.5% gels by the method of Laemmli [24]. The CGTases were purified from the periplasm of the *E. coli* strains carrying pTUE254 (lane 1), pTUE254-D229N (lane 2), pTUE254-E257Q (lane 3), pTUE254-E264Q (lane 4) or pTUE254-D328N (lane 5). (a) The purified protein samples (2 μg) were loaded onto a SDS-polyacrylamide gel, electrophoresed and stained with Coomassie brilliant blue. (Lane M) Bio-Rad high molecular weight protein standards. The sizes of the molecular weight markers are given in kDa. (b) Western immunoblot analysis [25] of the purified samples (0.5 μg). After electrophoresis, the samples were transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon; Millipore Corporation). CGTase bands were detected using a rabbit antiserum against *Bacillus* sp. #1011 CGTase and ^{125}I-labeled anti-rabbit Ig antibody F(ab')$_2$ fragment (Amersham International) followed by autoradiography. (c) Regenerated CGTase bands of the purified samples (0.2 μg). Detection of starch degrading activity of CGTase bands after SDS-PAGE was carried out as described by Itoh et al. [26] with slight modifications.

ever, we could not identify which Glu residue was involved in catalysis, because 2 residues (Glu^{257} and Glu^{264}) were considered to be the candidates at about 30 amino acids on the COOH-terminal side of the B-region. Therefore, 4 mutant CGTase genes D229N, E257Q, E264Q and D328N were constructed.

The *E. coli* transformants harboring the wild-type and the mutant CGTase genes were grown in shaking flasks and the enzyme from each strain was purified. The purity of the enzymes obtained exceeded 90%, based on SDS-PAGE. The molecular weights were estimated to be 70 kDa (Fig. 1a). Western immunoblot analysis showed that all the mutant enzymes reacted against anti-CGTase antiserum to the same degree as the wild-type enzyme, and that the protein bands corresponded to the CGTases (Fig. 1b). After SDS-PAGE, the regenerated bands for the starch degrading activity were visualized (Fig. 1c). The mutant CGTase from pTUE254-E264Q, CGTase-E264Q, showed a similar level of activity from band staining as the wild-type enzyme (Fig. 1c, lanes 1 and 4), whereas no activity bands were detected in the 3 mutant enzymes, CGTase-D229N, E257Q and D328N (Fig. 1c, lanes 2, 3 and 5). Starch-degrading and β-cyclodextrin-forming specific activities of each enzyme were assayed and are summarized in Table I. For CGTase-E264Q, the starch-degrading activity was 82% and the β-cyclodextrin-forming activity was 107% of the wild-type enzyme, whereas neither starch-degrading nor β-cyclodextrin-forming activities were detected with the D229N-, E257Q- and D328N-enzymes. The two activities could not be detected even when the assay was performed using 1000-times greater amounts of the mutant enzymes than that of the wild-type enzyme. These results indicate that Asp^{229}, Glu^{257} and Asp^{328} are essential for the catalysis, unlike Glu^{264}. No clear difference was observed in the CD spectra among the wild-type and all mutant enzymes (data not shown), suggesting that these mutations were not associated with significant conformational changes.

To determine whether the inactive enzymes retained the ability to be bound to the substrate, native-PAGE was carried out in the presence or absence of soluble starch in the separation gels. As shown in Fig. 2a, the migration of the 3 inactive enzymes was similar to that of the wild-type enzyme in the absence of starch (lanes 2, 4, 5 and 6), but they were retarded in the presence of 0.2% soluble starch (Fig. 2b, lanes 4, 5 and 6). The migration of the wild-type and E264Q enzymes was insignificantly affected in this starch concentration (Fig. 2a,b, lanes 2 and 3), since these enzymes degrade starch during migration. In the presence of 1% starch, the migration of the wild-type and E264Q enzymes were retarded (data not shown). The migration of BSA, which does not bind to starch, was unaffected in the presence of 0.2% starch (Fig. 2a,b, lane 1). These results indicate that the 3 inactive enzymes retained the ability to be bound to starch, the substrate. The migration of CGTase-E264Q was slower than that of the wild-type enzyme.

4. DISCUSSION

CGTases have unique enzymatic characters. The en-

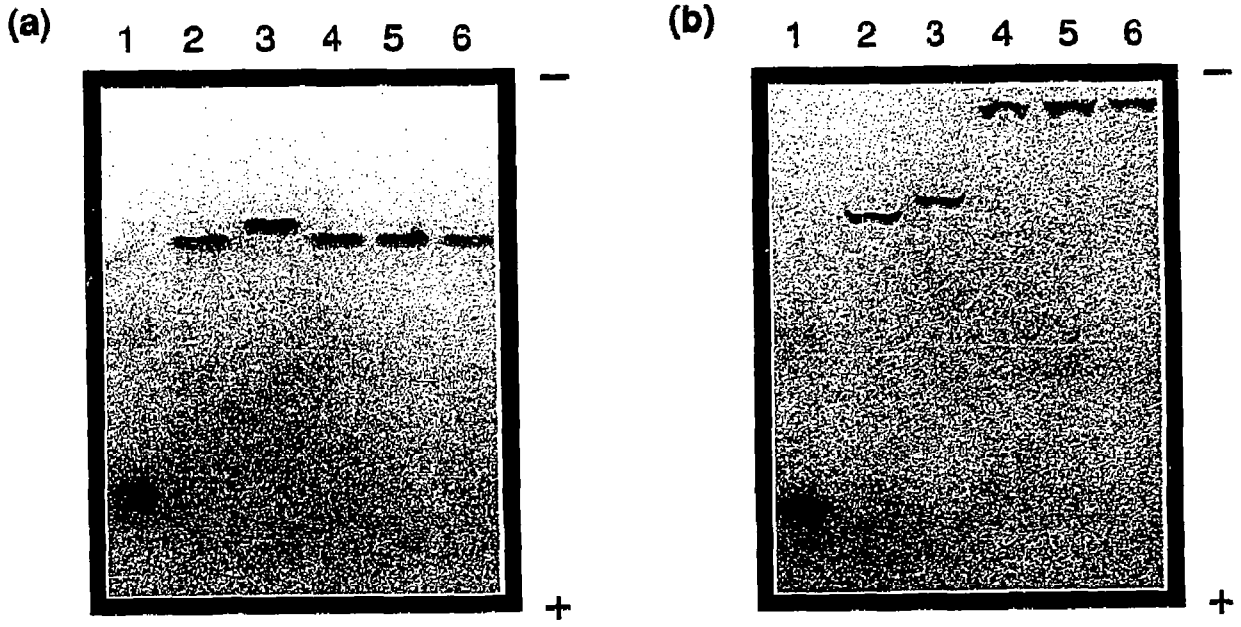

Fig. 2. Difference in migration between the wild-type and mutant enzymes in 7.5% native-PAGE. Native-PAGE was carried out according to the method of Davis [27] in the absence (a) or presence (b) of 0.2% soluble starch at 4°C. Each sample (3 μg) was loaded onto polyacrylamide gels, electrophoresed and stained by Coomassie brilliant blue. (Lane 1) BSA; (lane 2) wild-type enzyme; (lane 3) CGTase-E264Q; (lane 4) CGTase-D229N; (lane 5) CGTase-E257Q; (lane 6) CGTase-D328N.

Table 1

Specific activities of the wild-type and mutant enzymes on soluble starch

CGTases	Starch-degrading activity (U/mg protein)	β-cyclodextrin-forming activity (U/mg protein)
Wild-type	1920	39.4
D229N	<0.1	<0.001
E257Q	<0.1	<0.001
E264Q	1570	42.3
D328N	<0.1	<0.001

All the values given are an average of the results in 5 independent experiments.

zymes convert starch to cyclodextrins through an intramolecular transglycosylation reaction. They also catalyze the intermolecular transglycosylation reaction in the presence of acceptors. Furthermore, they display a weak hydrolyzing activity. Presently, we observed that the 3 mutants, D229N, E257Q and D328N of *Bacillus* sp. #1011 CGTase, showed neither starch-degrading nor transglycosylation activities, whereas they retained their ability to be bound to starch. Similar substitutions for the 3 conserved residues in *B. subtilis* and *B. stearothermophilus* α-amylases also showed that the 3 conserved residues are essential for their activity [15,16]. Therefore, it is suggested that the 3 conserved residues play an important role in the enzymatic reaction, not only in α-amylases, but also in CGTases.

α-Amylase is considered to catalyze the hydrolysis of starch based on the acid-base catalysis mechanism as proposed for lysozyme [5,17]. From the present results, it suggests that the reaction of CGTases is operated by a similar mechanism to that of the α-amylases. Even if it is true, it still remains to be determined which mechanism is used for the transglycosylation in CGTases. The 2 conserved Asp and 1 conserved Glu residues found in both α-amylases and CGTases are also recognized in pullulanase [18], neopullulanase [19], α-amylase-pullulanase [20], isoamylase [21] and α-glucosidases [22,23]. These findings suggest that all these enzymes may use a similar catalysis mechanism. The variations in the substrate specificity and products may be ascribed to the relationships between a similar catalytic center and different subsite structures. It is noteworthy that many residues proposed to constitute the subsites in Taka-amylase A are conserved in CGTases [3].

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