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The Roles of Tryptophan and Histidine Residues in the Catalytic Activities of β-Cyclodextrin Glucanotransferase from *Bacillus firmus* var. *alkalophilus*

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Abstract In order to investigate the critical amino acid residues involved in the catalytic activities of β-cyclodextrin glucanotransferase (β-CGTase) excreted by *Bacillus firmus* var. *alkalophilus*, the amino acid residues in β-CGTase were modified by various site-specific amino acid modifying reagents. The cyclizing and amylolytic activities of β-CGTase were all seriously reduced after treatment with Woodward's reagent K (WRK) modifying aspartic/glutamic acid, *N*-bromosuccinimde (NBS) modifying tryptophan, and diethylpyrocarbonate (DEPC) modifying histidine residues. The roles of tryptophan and histidine residues in β-CGTase were further investigated by measuring the protection effect of various substrates during chemical modification, comparing protein mobility in native and affinity polyacrylamide gel electrophoresis containing soluble starch, and comparing the K_m and V_{max} values of native and modified enzymes. Tryptophan residues were identified as affecting substrate-binding ability rather than influencing catalytic activities. On the other hand, histidine residues influenced catalytic ability rather than substrate-binding ability, plus histidine modification had an effect on shifting the optimum pH and pH stability.

Key words: β-Cyclodextrin glucanotransferase, *Bacillus firmus* var. *alkalophilus*, active site, catalytic activities, substrate binding, chemical modification

Cyclodextrin glucanotransferase (CGTase; EC 2,4,1,19) is a multifunctional enzyme which catalyzes intramolecular transglycosylation (cyclization), intermolecular transglycosylation (coupling, disproportionation), and hydrolytic action on starch and cyclodextrins (CDs). CDs are cyclic oligosaccharides, consisting of six (α-CD), seven (β-CD), or eight (γ-CD) glucose units, respectively. CD molecules are composed of a hydrophilic outside and a hydrophobic central cavity, and they can form an inclusion complex with guest molecules located inside the central cavity. Accordingly, CDs have been widely utilized in food, pharmaceutical, chemical, cosmetic, and agricultural industries [34].

The relative proportion of α-, β-, or γ-CD depend on the type of CGTase and the enzyme reaction conditions [19]. CGTase is mainly produced by *Bacilli* [19] yet it is also created by *Klebsiella pneumoniae* M5al, *Klebsiella oxytoca*, and *Thermoanaerobactor* sp. [33].

CGTase amino acid sequences have been researched intensively, at least fourteen sequences have been investigated at the DNA level and a 60~90% identity was found to belong to *Bacilli* [16, 18, 33, 35]. However, the similarity between CGTase sequences and α-amylases is a somewhat low homology of 15~25%, as only four strongly conserved regions, identified as catalytic and Ca^{2+} binding sites for α-amylase, have been discovered [12, 23, 26].

Through X-ray crystallographic studies, CGTase chain fold is known to resemble a four domain α-amylase chain fold with one additional domain, and these domains have been designated as the A-, B-, C-, D- and E-regions, respectively [10, 20]. The N-terminal region in CGTase contributes to the starch-degrading and transglycosylation activities in combining the reducing and non-reducing end of oligosaccharide [7]. The C-terminal region may be involved in catalyzing activities and pH stability, however, its roles are not yet well defined [17]. The functions of the specific amino acid residues involved in the catalytic activities of CGTase have already been subjected to intensive study, however, their behaviors have not as yet been accurately defined.

Nakamura *et al.* [27] compared the refined structure of β-CGTase with α-amylase, and suggested that the amino acid residues of Asp^{229}, Glu^{257}, and Asp^{328} play an important role in catalysis. CGTase histidine residues have often been presented as being involved in glycosyl transfer reactions and the binding of substrates [2, 24, 25, 28]. Aromatic amino acid residues, such as tyrosine, phenylalanine, and tryptophan residues, have also been

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proposed as being involved in catalysis through substrate binding or the determination of the CD ratio of CGTase [14, 29, 30, 31]. A further understanding of amino acid residues at the CGTase active site is still required for a more accurate comprehension of the various mechanisms of catalytic reactions.

In our previous works, the alkalophilic strain *Bacillus firmus* var. *alkalophilus*, which mainly produces β-CD from starch without the accumulation of any significant amount of α-CD, was isolated [5]. A catabolite-derepressed mutant and constitutive mutant overproducing CGTase were then selected [6], and their regulation mechanisms for CGTase production were investigated [15]. A novel β-CGTase from the above strain was successfully purified, and its enzymatic properties were investigated [32].

In this paper, the functions of the amino acid residues at the active site of the β-CGTase produced from *Bacillus firmus* var. *alkalophilus* have been studied using site-specific chemical modification. The amino acid residues involved in catalytic activities were identified using site-specific chemical modification and the protection effect by various substrates against inactivation from chemical modification was investigated. The roles of these amino acid residues, in both substrate-binding and catalytic activities, were further studied through the comparison of the kinetic constants of modified and native β-CGTases, and the comparison of protein mobility in native-polyacrylamide gel electrophoresis (PAGE) and affinity PAGE containing soluble starch. These results are essential both for understanding the detailed reaction mechanisms of CGTase, and for developing a suitable industrial CGTase through site-directed mutagenesis.

MATERIALS AND METHODS

Enzyme and Strain

β-Cyclodextrin glucanotransferase (β-CGTase) was separated from a culture filtrate of alkalophilic *Bacillus firmus* var. *alkalophilus* screened in our laboratory [5], that was cultivated at 37°C for 2 days in Horikoshi's alkaline basal medium [11].

Purification of the β-CGTase

The culture filtrate was concentrated by ultrafiltration. The concentrated β-CGTase was purified by starch adsorption/desorption, DEAE-cellulose ion-exchange chromatography, and gel filtration with a Sephacryl HR-100, as described in our previous paper [32]. It was then further purified with affinity chromatography using β-CD-liganded acrylic bead [1]. The homogeneity of the enzyme was confirmed by SDS-PAGE [21] and the specific activity of the β-CGTase was measured as 653 units/mg.

Assay of CGTase Activities

Cyclizing activity was determined by the phenolphthalein method proposed by Kaneko *et al.* [13], and 1 unit of CGTase was defined as the amount of enzyme that produced 1 mg of β-CD per min. Amylolytic activity was assayed using soluble starch as the substrate by measuring the transmittance at 660 nm according to Fuwa's method [9], and 1 unit of the amylolytic activity was defined as the amount of enzyme which increased the transmittance by 1.0% during one minute.

Substrates and Chemicals

The substrates used included glucose, maltose, linear maltooligosaccharides (DP3-6), and cyclodextrins (from Sigma Co., St. Louis, U.S.A.). The chemicals used in the site-specific chemical modification of CGTase included diethylpyrocarbonate (DEPC), Woodward's reagent K (*N*-ethyl-5-phenylisoxazolium-3-sulfonate), 2,3-butanedoine (BD), *N*-acetylimidazole (NAI), 2,4,6-trinitrobenzensulfonate (TNBS), phenylglyoxal (PG), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), and *N*-ethylmaleimide (NEM) (from Sigma Co., St. Louis, U.S.A.), chloramine T, and hydrogen peroxide (from Junsei Chemicals Co., Tokyo, Japan), and *N*-bromosuccinimide (NBS) (from Jansseen Chimica Co., Geel, Belgium).

Chemical Modification of β-CGTase

The site-specific chemical modifications of the amino acid residues were carried out according to the methods described by Lundblad [22]. For chemical modification, the β-CGTase was incubated with specific chemicals of a suitable molar ratio at room temperature for 10 min. The reactions were then stopped by the addition of an excessive amount of a corresponding amino acid and the remaining activity was measured. The number of modified residues was calculated from the molar extinction coefficients and the changes in absorbance measured at wavelengths corresponding to each reaction mixture. For the substrate protection experiments, the β-CGTase was pre-incubated with CDs, soluble starch, or maltooligosaccharides (G_1~G_6) for 10 min at 4°C before chemical modification.

Polyacrylamide Gel Electrophoresis

The native-PAGE and affinity PAGE were performed using the Davis' system at pH 8.9 with a 7.5% gel [4]. A separating gel containing 0.2% (w/v) soluble starch was used for the affinity PAGE. The protein was stained by Coomassie Brilliant blue and the amylolytic zymogram was detected by the I_2-staining method [27].

Evaluation of K_m and V_{max}

The kinetic constants of the native and modified β-CGTases were evaluated, and the K_m and V_{max}

values were then determined using Lineweaver-Burk plotting.

Analytical Methods

The protein concentration was measured according to the Bradford method [3]. The CDs and maltooligosaccharides were analyzed by HPLC (Gilson Medical Electronics Inc., Villiers-le-Bel, France); a Cosmosil packed column $5NH_2$ column (Nacalai Tesque Co., Kyoto, Japan), acetonitrile/water (65:35), 1.0 ml/min, and an RI detector.

RESULTS AND DISCUSSION

Identification of Amino Acid Residues Involved in the Catalytic Activity of β-CGTase

The critical amino acid residues involved in the catalytic activities of β-CGTase were identified after chemical modification with various amino acid modifiers. The effects of the various modifications on the cyclizing and amylolytic activities of the modified β-CGTase are summarized in Table 1. Most of the modifying reagents had no effect on the cyclizing and amylolytic activities. However, when the β-CGTase was treated with NBS modifying tryptophan, DEPC modifying histidine, and WRK modifying glutamic/aspartic acid residues, these activities were seriously reduced by 9~20% compared to the native enzyme.

In particular, NBS and DEPC produced a significant effect on the inactivation of β-CGTase, and the inactivation occurred at relatively low NBS and DEPC concentrations ranging from 0~0.1 mM and 0~1.0 mM, respectively, compared to WRK concentration ranging from 0~10 mM. Accordingly, the most important amino acid residues involved in β-CGTase catalytic activities seem to be tryptophan, histidine, and glutamic/aspartic acid residues; consequently, their catalytic functions were further investigated.

Table 1. Effect of various chemical modifications on cyclizing and amylolytic activities of β-CGTase.

	Target residue (s)	Concentration of reagent (mM)	pH	Residual activity (%)		Number of modified residues
				Cyc.	Amy.	
WRK	Asp/Glu	10.0	8.0	19.6	17.6	≒5.2
BD	Arg	10.0	8.0	95.8	96.1	ND
PG	Arg	10.0	8.0	97.8	97.2	ND
NEM	Cys	10.0	6.0	97.9	96.8	ND
DTNB	Cys	10.0	6.0	98.6	97.8	≒0.2
DEPC	His	1.0	6.0	9.3	9.7	≒3.8
TNBS	Lys	10.0	8.0	95.2	97.5	≒9.2
CT	Met	10.0	6.0	93.4	90.3	ND
NBS	Trp	0.1	5.0	8.7	9.6	≒2.3
NAI	Tyr	10.0	8.0	96.2	96.8	≒17.8

β-CGTase was incubated with specific reagents of suitable concentration at room temperature for 10 min, an excessive amount of corresponding amino acid was added to stop the reaction, and then remaining cyclizing and amylolytic activities were measured. Cyc., cyclizing activity; Amy., amylolytic activity; ND, not determined; WRK, Woodward's reagent K (*N*-ethyl-5-phenylisoxazolium-3'-sulfonate); BD, 2,3-butanedion; PG, phenylglyoxal; NEM, *N*-ethylmaleimide; DTNB, 5,5'-dithiobis-(2-nitrobenzoate); DEPC, diethylpyrocarbonate; TNBS, 2,4,6-trinitrobenzensulfonate; NBS, *N*-bromosuccinimide; NAI, *N*-acetylimidazole.

Role of Glutamic/Aspartic Acid Residues in β-CGTase Modified with WRK

To examine the role of glutamic/aspartic acid residues in the catalytic activities of β-CGTase, β-CGTase was treated with various molar ratios of WRK (0-10,000:1, [WRK]/[β-CGTase]) at pH 8.0 for 10 min. Figure 1 shows the effect of a molar ratio of WRK on the cyclizing and amylolytic activities of β-CGTase. The cyclizing and amylolytic activities were proportionally decreased by down to 18~20% of the initial level as the molar ratio of WRK increased, plus the number of modified glutamic/aspartic acid residues increased proportionally to around five per β-CGTase molecule. The above results show that glutamic/aspartic acid residues play an important role in maintaining either the catalytic function or the structural stability of β-CGTase.

The relative residual activity of β-CGTase modified with WRK in the presence of various substrates was also

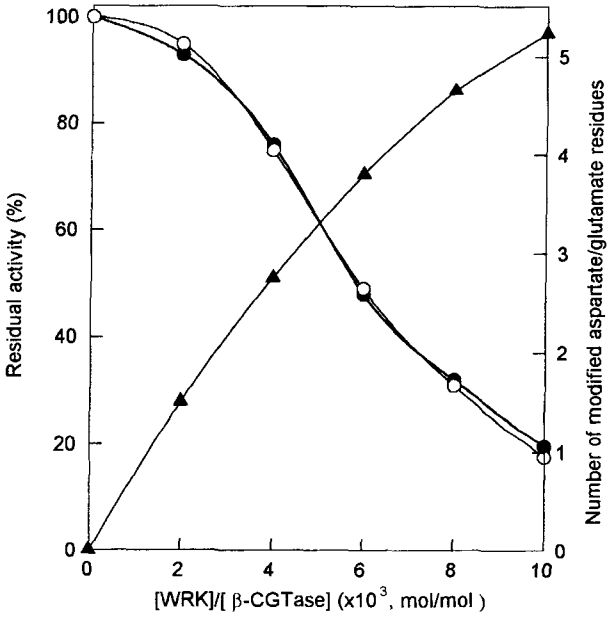

Fig. 1. Effect of molar ratio of WRK on cyclizing and amylolytic activities of βCGTase.

The β-CGTase was incubated with different molar ratios of WRK for 10 min at room temperature, and then the residual activities in reaction mixtures were measured. The number of modified aspartate/glutamate residues was calculated from the change of absorbance at 340 nm and molar extinction coefficient of 7,000 M ^{1}cm ^{1}. ●, cyclizing activity; ○, amylolytic activity; ▲, number of modified aspartate/glutamate residues per molecule of β-CGTase.

Table 2. Relative residual activity of β-CGTase modified with WRK, NBS, and DEPC under the presence of various substrates.

Substrates	Relative activity (%)		
	WRK	NBS	DEPC
Native β-CGTase	100	100	100
Modified β-CGTase	19	8	9
Glucose (G_1, 10 mM)	23	9	10
Maltose (G_2, 10 mM)	28	11	13
Maltotriose (G_3, 10 mM)	45	35	32
Maltotetraose (G_4, 10 mM)	56	44	46
Maltopentaose (G_5, 10 mM)	65	60	62
Maltohexaose (G_6, 10 mM)	86	78	82
α-CD (10 mM)	86	85	88
β-CD (10 mM)	92	94	92
γ-CD (10 mM)	90	91	90
Soluble starch (1.0%, w/v)	91	95	88

β-CGTase was pre-incubated with CDs, soluble starch, or maltooligosaccharides (G_1~G_6) for 10 min at 4°C before chemical modification, and then chemical modifications were carried out at WRK, NBS, and DEPC molar ratios of 10,000:1 ([WRK]/[β-CGTase]), 100:1 ([NBS]/[β-CGTase]), and 1,000:1 ([DEPC]/[β-CGTase]) for 10 min at room temperature.

investigated as shown in Table 2. Soluble starch and α-, β-, γ-CD all indicated the most effective protection effect on β-CGTase from the inactivation of WRK, higher than 86%, and high DP maltooligosaccharides (DP≥3) also partially protected 45~86% of the native enzyme, while, glucose and maltose demonstrated a lower protection effect.

The above substrate affinity differences on β-CGTase might be caused by competing the substrates, used for CD synthesis, with WRK at the active site, indicating that glutamic/aspartic acid residues modified by WRK seem to be located at or near the β-CGTase active site. Nakamura *et al.* [27] similarly reported that Asp^{229}, Glu^{257}, and Asp^{328} play important roles in the catalytic activities of CGTase excreted by *Bacillus* sp. Additionally, glutamic/aspartic acid residues have been known to be essential in various carbohydrate degrading enzymes having acid-base catalysis mechanism including amylases [23, 26, 27], thus, the reaction of CGTases seems to be operated by a similar mechanism to that of these enzymes. The accurate position and functions of these residues should be further studied through a site-directed mutagenesis of the β-CGTase gene and an investigation of the enzymatic properties of mutant enzymes.

Role of Tryptophan Residues in β-CGTase Modified with NBS

The role of the tryptophan residue of β-CGTase was investigated after chemical modification with various molar ratios of NBS (0-100:1, [NBS]/[β-CGTase]) at pH 5.0 for 10 min. As shown in Fig. 2, the cyclizing and amylolytic activities of the β-CGTase were both also seriously reduced (about 8~9% of the initial level proportionally), at an even lower molar ratio of NBS.

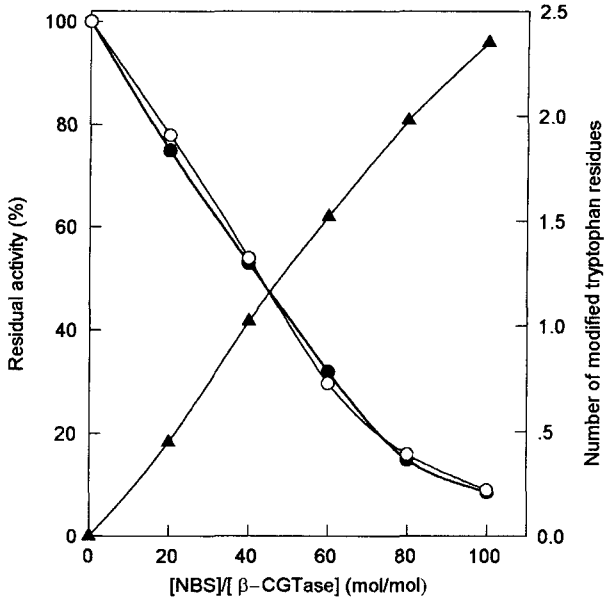

Fig. 2. Effect of molar ratio of NBS cyclizing and amylolytic activities of β-CGTase.

The β-CGTase was incubated with various molar ratios of NBS. The number of modified tryptophan residues were calculated from the decrease of absorbance at 280 nm and molar extinction coefficient of 4,000 $M^{-1}cm^{-1}$. ●, cyclizing activity; ○, amylolytic activity; ▲, number of modified tryptophan residues per molecule of β-CGTase.

The number of the modified tryptophan residue was increased up to approximately 2.3 in relation to the molar ratio of NBS. The above results indicate that tryptophan residues are also essential in maintaining either the catalytic activities or the structural stability of β-CGTase.

CDs and soluble starch also effectively protected up to 95% of the β-CGTase from the inactivation of NBS, however, glucose, maltose and maltotriose could only protect slightly as shown in Table 2. Ohnishi *et al.* [30] studied the chemical modification of tryptophan residues of CGTase from *B. stearothermophilus*, and also obtained similar results finding that Trp^{97} from CGTase plays an important role in catalytic activities and is located at the CGTase substrate-binding site.

Substrate Binding Characteristics of β-CGTase Modified with NBS

The substrate binding characteristics of β-CGTase were investigated comparing the protein mobility of native and modified enzymes in native-PAGE and affinity PAGE containing soluble starch. As can be observed in the zymogram in Fig. 3A, the amylolytic activities of the modified β-CGTases gradually decreased as inactivation occurred more severely.

The mobilities of native and modified β-CGTase with NBS in native (Fig. 3B) and affinity PAGE (Fig. 3C) were also compared. In native PAGE, the mobility differences between native and modified β-CGTase were

not observed (lanes 2, 3, 4, and 5 of Fig. 3B). However, in affinity PAGE, the mobility of the native enzyme (lane 2 in Fig. 3C) was shifted up compared to the native enzyme in lane 2 in Fig. 3B. Also, the modified β-CGTase was separated into two portions, retarded like that of native enzyme and not retarded, as shown in lanes 3 and 4 of Fig. 3C. The fully inactivated β-CGTase was not retarded at all as shown in lane 5 of Fig. 3C, indicating that β-CGTase modified with NBS started to lose its

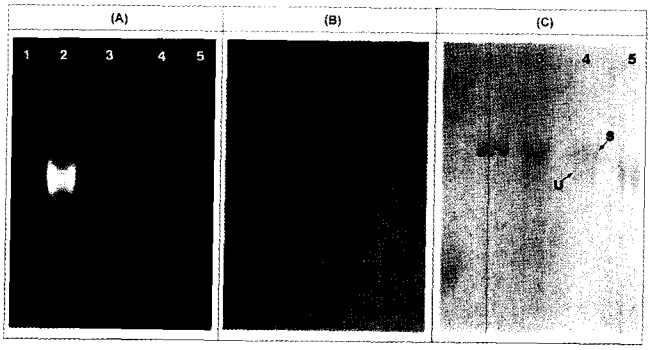

Fig. 3. Comparison of mobilities of native and NBS-modified β-CGTases in native and affinity PAGE.

Native and affinity PAGE were carried out at 4°C according to the method of Davis [4]. (A) is amylolytic zymogram of native and NBS-modified β-CGTases, (B) is native-PAGE, and (C) is affinity PAGE containing 0.2% soluble starch. Each sample (10 μg) was loaded onto polyacrylamide gels, electrophoresed, and then stained by Coomassie Brilliant blue. To develop the amylolytic zymogram of (A), the electrophoresed gel was immersed into 5.0% (w/v) soluble starch solution for 30 min at 50°C, and then stained with 0.2% KI and 0.02% I_2 solution. Lane 1, lysozyme (Sigma Co.); Lane 2, native β-CGTase; Lane 3, modified β-CGTase (20:1, [NBS]/[β-CGTase]); Lane 4, modified β-CGTase (50:1, [NBS]/[β-CGTase]); Lane 5, modified β-CGTase (100:1, [NBS]/[β-CGTase]). S, starch bound β-CGTase; U, unbound β-CGTase.

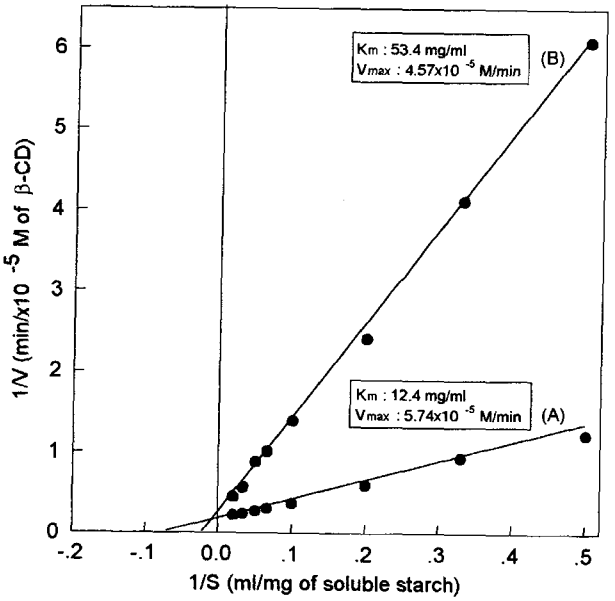

Fig. 4. Lineweaver-Burk plot for the cyclizing activities of the native (A) and the NBS-modified (B) β-CGTase.

The NBS-modified β-CGTase was prepared with an NBS molar ratio of 50:1 ([NBS]/[β-CGTase]) for 10 min at room temperature.

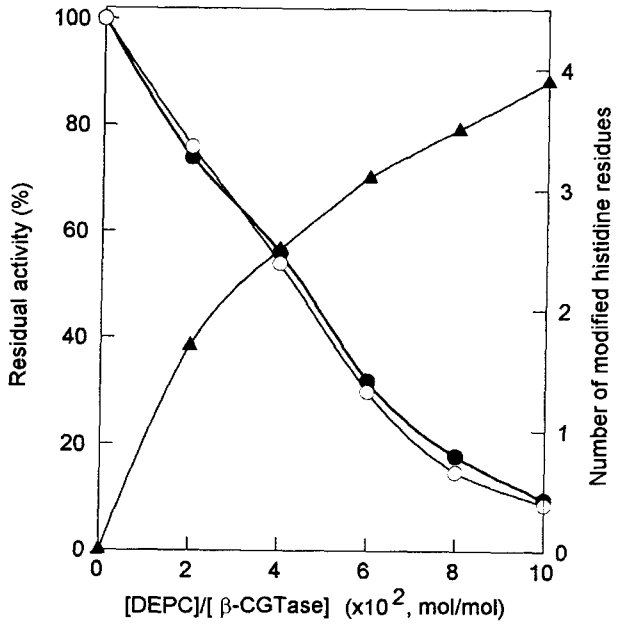

Fig. 5. Effect of molar ratio of DEPC on cyclizing and amylolytic activities of β-CGTase.

The β-CGTase was incubated with different molar ratios of DEPC. The number of modified histidine residue was calculated from the increase of absorbance at 240 nm and molar extinction coefficient of 3,200 $M^{-1}cm^{-1}$. ●, cyclizing activity; ○, amylolytic activity; ▲, number of modified histidine residues per molecule of β-CGTase.

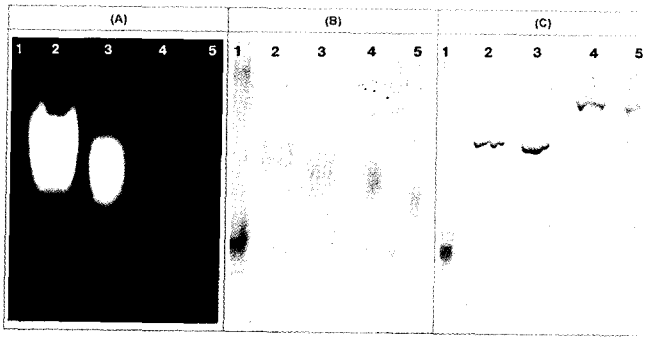

Fig. 6. Comparison of mobilities of native and DEPC-modified β-CGTases in native and affinity PAGE.

Native and affinity PAGE were carried out at 4°C according to the method of Davis [4]. (A) is amylolytic zymogram of native and DEPC-modified β-CGTases, (B) is native-PAGE, and (C) is affinity PAGE containing 0.2% soluble starch. Each sample (10 μg) was loaded onto polyacrylamide gels, electrophoresed, and then stained by Coomassie Brilliant blue. To develop the amylolytic zymogram of (A), the electrophoresed gel was immersed into 5.0% (w/v) soluble starch solution for 30 min at 50°C, and then stained with 0.2% KI and 0.02% I_2 solution. Lane 1, lysozyme (Sigma Co.); Lane 2, native β-CGTase; Lane 3, modified β-CGTase (200:1, [DEPC]/[β-CGTase]); Lane 4, modified β-CGTase (500:1, [DEPC]/[β-CGTase]); Lane 5, modified β-CGTase (1,000:1, [DEPC]/[β-CGTase]).

substrate-binding ability. The facts show a similar mobility in native PAGE, yet a different mobility according to the degree of inactivation in affinity PAGE. This indicates that tryptophan residues participate in substrate binding rather than catalytic activities.

The above observations were confirmed by comparing the K_m and V_{max} values of native and modified β-CGTase as illustrated in the Lineweaver-Burk plot in Fig. 4. The K_m value of modified β-CGTase increased approximately 4.3 times compared to native β-CGTase, whereas, the V_{max} value remained at a similar level, indicating that the inactivation of the tryptophan residues by NBS was mainly caused by the loss of substrate binding ability rather than catalytic ability.

Role of Histidine Residues of β-CGTase Modified with DEPC

The role of histidine residues was investigated after chemical modification with DEPC (0-1,000:1, [DEPC]/[β-CGTase]). As shown in Fig. 5, both cyclizing and amylolytic activities decreased drastically by about 9~10% of the initial level and the number of modified histidine residue gradually increased by approximately four, as the molar ratio of DEPC increased. This result also indicates the important role of histidine residues in either the catalytic activities or the structural stability of β-CGTase. As shown in Table 2, CDs, soluble starch, and long maltooligosaccharides (DP≥6) also successfully protected up to 92% of the native enzyme from inactivation, which indicates that histidine residues are also located at or near the active center.

Mattsson *et al.* studied the functions of histidine residues of CGTase from *B. circulans* through chemical modification [24] and site-directed mutagenesis [25]. They reported that cyclizing activity was remarkably reduced, while amylolytic activity did not decrease. Bender [2] also reported that the modification of histidine residues with DEPC caused a delayed formation of CDs and a marked increase in the production of reducing saccharides, indicating that histidine residues play an important role for substrate binding and/or in the glycosyl transfer reaction rather than starch hydrolysis.

The above reports differ from our results which observed serious reductions in both cyclizing and amylolytic activities and a negligible increment of reducing saccharides during the CD formation reaction from soluble starch. The coincidental decrement of cyclizing and amylolytic activities of β-CGTase modifying histidine residues has not yet been reported. The role of histidine residues of β-CGTase excreted by alkalophilic *B. firmus* var. *alkalophilus* should be clarified either by investigating a site-directed mutagenesis of the β-CGTase gene or the three dimensional structure of the active site of the above enzyme.

Substrate Binding Characteristics of the β-CGTase Modified with DEPC

The substrate binding characteristics of histidine modified β-CGTases is illustrated in Fig. 6. In the zymogram of Fig. 6A, the halo size indicating starch degradation was proportionally reduced as the inactivation by DEPC increased. The mobility of the modified β-CGTases was increased slightly compared to the native enzyme in native-PAGE as shown in lanes 2, 3, 4, and 5 of Fig. 6B. Meanwhile, all native and modified enzymes were retarded in affinity PAGE as shown in lanes 2, 3, 4 and 5 of Fig. 6C, and the mobility of fully inactivated β-CGTase (lanes 4 and 5) showed slightly lower values compared to native and partially inactivated β-CGTases. The above results indicate that the modification of β-CGTase with DEPC had a slight influence on its substrate binding ability. The mobility differences between the inactivated β-CGTase and the native enzyme in affinity PAGE may be caused by a conformational change because of additional modification at another site besides the active site of the enzyme.

The K_m and V_{max} values were also compared as shown in Fig. 7. The K_m value was not changed, however, the V_{max} value was substantially decreased by about 25%, indicating that the inactivation of β-CGTase with DEPC was mainly caused by the loss of catalytic ability rather than substrate binding ability. Accordingly, histidine residues are not involved in substrate binding but rather in catalytic activities including cyclization and starch hydrolysis.

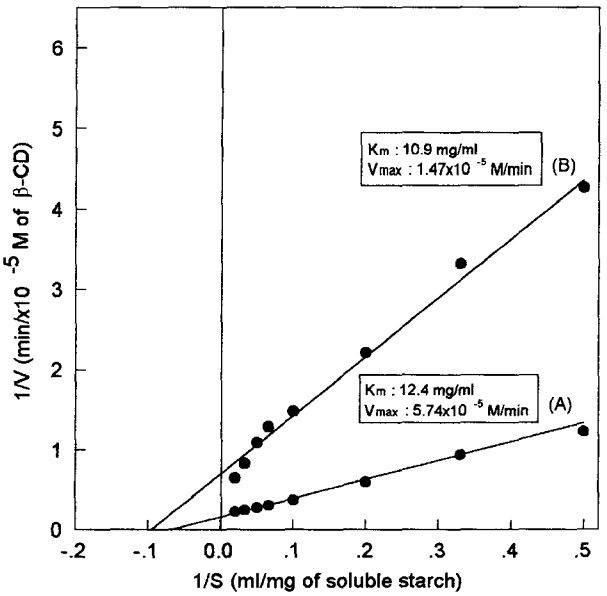

Fig. 7. Lineweaver-Burk plot for the cyclizing activities of the native (A) and the DEPC-modified (B) β-CGTase.

The DEPC-modified β-CGTase was prepared with DEPC molar ratio of 500:1 ([DEPC]/[β-CGTase]) for 10 min at room temperature.

The effects of the chemical modification of β-CGTase histidine residues by DEPC on the optimum pH and pH stability were also investigated as illustrated in Fig. 8. Histidine modification enlarged the optimum pH range from 5.5~7.0 to 5.5~9.0; however, the pH stability range was reduced from 6.0~10.0 to 7.0~9.0, indicating that histidine residues also play a critical role in the determination of pH ranges. Similar changes in the optimum pH and pH stability resulting from the site-directed mutagenesis of CGTase of alkalophilic *Bacillus* sp. have also been reported by Nakamura *et al.* [28].

In conclusion, tryptophan, histidine, and aspartic/glutamic acid residues seem to be located at or near the active site of β-CGTase, and play a critical role in the catalysis of β-CGTase from *B. firmus* var. *alkalophilus*. Tryptophan residues are involved in substrate binding rather than catalytic activities, whereas histidine residues contribute to catalytic activities rather than substrate binding and participate in the determination of the optimum pH and pH stability.

In addition to the above amino acid residues, phenylalanine residues at positions 191 and 255 of CGTase from *B. stearothermophilus* are also reported as playing an important role in determining the cyclization characteristics of CGTase by Fujiwara *et al.* [8]. Tyrosine residue at position 195 of CGTase from *B. circulans* participates in the cyclization reaction as reported by Penninga *et al.* [31]. The roles of the four aromatic amino acid residues of Phe^{183}, Tyr^{195}, Phe^{259}, and Phe^{283} of CGTase from *Bacillus* sp. in substrate binding and transition-state stabilization were reported by Nakamura *et al.* [29].

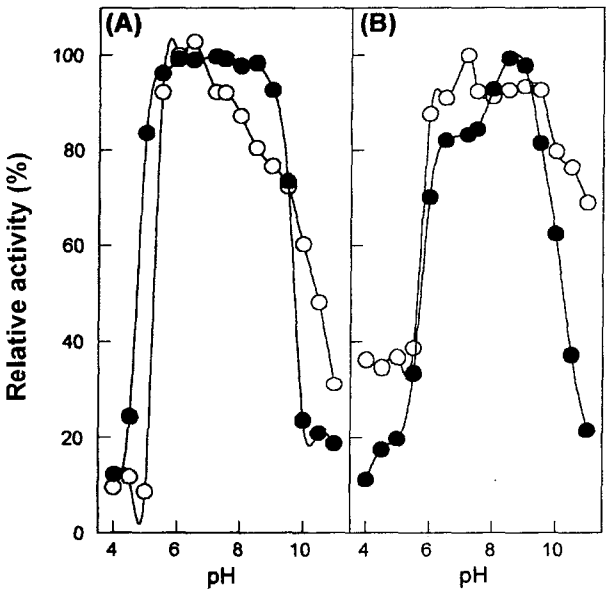

Fig. 8. Effects of pH on the activity (A) and stability (B) of the native β-CGTase (○) and the DEPC-modified β-CGTase (●).

The DEPC-modified CGTase was prepared from the treatment of β-CGTase with DEPC molar ratio of 500:1 ([DEPC]/[β-CGTase]) for 10 min at room temperature. To examine the pH stabilities of these CGTases, the enzymes in 100 mM various buffers containing 10 mM $CaCl_2$ having various pH values were kept for 1 h at 50°C.

However, the roles of the amino acid residues mentioned above were not recognized in our work. It is not clear whether these amino acid residues are unimportant in the catalytic activities of β-CGTase from alkalophilic *B. firmus* var. *alkalophilus*, or whether the chemical modification was insufficient to identify their roles. Detailed reaction mechanisms should be further clarified through a site-directed mutagenesis for the β-CGTase gene and an investigation of the enzymatic properties of mutant enzymes.

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