Characterization of Two γ -Cyclodextrin-specific Enzymes from *Bacillus clarkii* 7364

(Received December 1, 2009; Accepted January 5, 2010)

Yoshinori Nakagawa,^{1,*} Wataru Saburi,¹ Takeshi Yamamoto,¹ Masayasu Takada,¹ Koichi Ogawa,¹ Mikio Yamamoto,¹ Yuji Hatada,² Nobuyuki Nakamura² and Koki Horikoshi²

¹*Nihon Shokuhin Kako Co., Ltd. (30, Tajima, Fuji 417–8530, Japan)*

²*Extremobiosphere Research Center, Japan Agency for Marine–Earth Science and Technology (JAMSTEC) (2–15, Natsushima, Yokosuka 237–0061, Japan)*

Abstract: The alkaliphilic soil bacterium *Bacillus clarkii* 7364 was found to produce cyclodextrin glucanotransferase (CGTase), an enzyme which converts starch into γ -cyclodextrin (γ -CD) with high specificity. The bacterium also intracellularly produced cyclodextrinase (CDase). The gene encoding the CDase (Cda) was located about 200 bases downstream of the gene encoding the CGTase (Cgt). Comparison of the amino acid sequence of Cgt with those of other CGTases revealed that several amino acids which contribute to substrate binding are absent or different at subsites +3, +2, –3 and –7 in Cgt. The replacement of Ala223 at subsite +2 by three basic amino acids, histidine, lysine and arginine, strongly enhanced γ -CD-forming activity in the neutral pH range, although the optimum pH for the activity (pH 10.0) and CD production specificity remained the same. The interaction between the protonated amino group in the side chain of these basic amino acids and the linear substrate is thought to play an important role in the enhancement of the activity. Cda had extremely high hydrolytic specificity for γ -CD. Cda also displayed a transglycosylation activity, where a maltotriose moiety could be transferred, unlike other CD-degrading enzymes. In Cda, the N-domain normally found in other CD-degrading enzymes which functions in the dimerization, thus contributing to the preference for CDs, was deleted and instead, a long extra sequence was present in the C-terminus. Despite the lack of the N-domain, Cda showed a dodecameric structure. These unique features indicate that Cda is a novel CD-degrading enzyme.

Key words: γ -cyclodextrin, cyclodextrin glucanotransferase, cyclodextrinase

Cyclodextrin (CD) has a closed ring structure consisting of D-glucose residues linked through α -(1,4)-linkages. The most common CDs consist of six, seven, or eight glucose residues, and are named α -, β - and γ -CD, respectively. CDs have a hydrophilic outer surface and a relatively hydrophobic internal cavity. CDs are able to form complexes with various molecules. The formation of complexes leads to changes in the chemical and physical properties of the molecule including stability and solubility; thus there have been extensive applications of CDs in the food, cosmetic, agricultural and pharmaceutical industries.¹⁾

Cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19) forms CDs from starch and related α -(1,4)-linked glucose polymers through intramolecular transglycosylation.²⁾ This enzyme belongs to glycoside hydrolase family 13 (α -amylase family), based on its amino acid sequence.^{3–5)} Members of this family show various reaction specificities but have the following common features: a (β/α)₈ barrel structure and four highly conserved regions, regions I–IV, in the primary sequence. The conserved regions include invariant catalytic residues, two Asp residues and one Glu residue, and other amino acids involved in transition state stabilization.³⁾ A number of bacteria and archaea (e.g., *Bacillus*, *Thermoanaerobacter* and *Thermococcus* species)

have been isolated as CGTase producers.⁶⁾ Many studies on X-ray crystallographic structures of CGTases complexed with substrates, products, and inhibitors have revealed that CGTase possesses a substrate binding groove that accommodates at least 7 glucose residues at the donor subsites and 3 at the acceptor subsites (subsite –7 to +3), and contains an aromatic amino acid in a dominant position at the center of the substrate binding groove.^{2,7–9)}

As all known CGTases produce a mixture of CDs, they are subdivided into α -, β - and γ -CGTases based on the major CD product. The majority of CGTases reported are β -CGTases while α - and γ -CGTases are less frequent. γ -CD is the most expensive CD due to the low yield of this substance from the current CGTases. Several attempts have been made to enhance the γ -CD yield, but no satisfactory results have been obtained to date.^{10,11)} In addition to the ability of CD synthesis (cyclization reaction), CGTase catalyzes three other reactions: coupling, disproportionation and starch hydrolysis. In the coupling reaction, CD is cleaved and transferred to an acceptor substrate. In the disproportionation reaction and starch hydrolysis reaction, a linear maltooligosaccharide is cleaved and then glycosyl residue transfers to another linear substrate and water, respectively. Thus CGTase is also utilized for the synthesis of glycosides.^{12,13)}

Cyclodextrinase (CDase; EC 3.2.1.54) hydrolyzes CDs much faster than starch and pullulan.¹⁴⁾ Many CDases have been isolated from various bacteria, including *Alicy-*

* Corresponding author (Tel. +81-545-53-5995, Fax. +81-545-53-2986, E-mail: yoshinori.nakagawa@nisshoku.co.jp).

clobacillus acidocaldarius,¹⁵⁾ *Anoxybacillus flavithermus*,¹⁶⁾ *Bacillus coagulans*,¹⁷⁾ *Bacillus sphaericus*,¹⁸⁾ *Bacillus* sp. A2-5a,¹⁹⁾ *Bacillus* sp. I-5,²⁰⁾ *Clostridium thermohydrosulfuricum* 39E,²¹⁾ *Flavobacterium* sp. 92,²²⁾ *Klebsiella oxytoca* M5a1,²³⁾ *Paenibacillus* sp. A11²⁴⁾ and *Thermococcus* sp. B1001.²⁵⁾ The genes encoding CDases from *K. oxytoca* M5a1 and *Thermococcus* sp. B1001 have been found in the gene cluster for starch degradation pathway via CD. The gene cluster is composed of genes encoding CGTase and proteins involved in the transport of CD as well as a CDase. Maltogenic amylase and neopullulanase are also known to be enzymes capable of efficiently degrading CDs.¹⁴⁾ These CD-degrading enzymes all belong to glycoside hydrolase family 13. They are structurally related and possess an extended sequence at the N-terminus not found in a typical α -amylase (e.g., TAKA amylase A of *Aspergillus oryzae*²⁶⁾). Determination of the three dimensional structures of CDase from alkaliphilic *Bacillus* sp. I-5, maltogenic amylase from a *Thermus* species, and neopullulanase from *Geobacillus stearothermophilus* revealed a relationship between structure and substrate specificity.^{27–29)} In these enzymes, the N-domain is separated from the catalytic domain and is involved in dimerization. The N-domain of one subunit covers a part of the top of the (α/β)₈ barrel of the other subunit. As a result, the dimer formation shapes a deep and narrow active site cleft contributing to the preference of CDs.

Recently, we found a novel alkaliphilic bacterium, *Bacillus clarkii* 7364, which produces γ -CGTase.³⁰⁾ The CGTase from *B. clarkii* 7364 (Cgt) can convert pre-

gelatinized potato starch into γ -CD with high specificity. In addition, it was revealed that this bacterium had the gene (*cda*) encoding CDase (Cda) by sequence analysis of the adjacent region of the *cgt* gene encoding Cgt.³¹⁾ Cda shows high hydrolytic specificity for γ -CD. In this study, we describe the recent knowledge about two γ -cyclodextrin-specific enzymes, Cgt and Cda.

Characterization of Cgt.

Cgt was purified from the culture supernatant of *B. clarkii* 7364 by affinity chromatography on a γ -CD immobilized column and the purified Cgt was characterized.³⁰⁾ The molecular mass of Cgt was determined to be 68 and 73 kDa by SDS-PAGE and gel filtration chromatography, respectively. This indicates that Cgt is a monomer. The optimum pH and temperature for γ -CD-forming activity of Cgt were 10.0 and 60°C, respectively. The enzyme was completely stable over the pH range of 6.0–11.0 at 4°C for 24 h and below 40°C for 15 min of heat treatment at pH 10.0. These results showed that Cgt was an alkalophilic enzyme. Cgt (0.15 mg/g dry starch) produced 3.5% β -CD and 27.5% γ -CD from 1% soluble starch at pH 10.0 and 50°C after 48 h of incubation. At this time, α -CD was not detected in the reaction mixture.

Comparison of the amino acid sequence of Cgt with those of other typical CGTases.

The amino acid sequence of Cgt was moderately homologous (~70%) compared with those of CGTases from *Bacillus macerans* (α -CGTase), *Bacillus circulans* 251

Table 1. Comparison of the amino acid residues around the active center in the four types of CGTase.

Residue No.	Residue in α -CGTase	Residue in β -CGTase	Residue in γ -CGTase ^a	Residue in γ -CGTase ^b	Function in CGTase
Subsite +3					Acceptor
255	E	E	G	G	Disp. ^c
Subsite +2					Acceptor
174	F	F	F	F	Cycl. ^c
223	K	K	A	A	Disp. ^c
250	F	F	F	F	Cycl. ^c and disp. ^c
Subsite +1					Acceptor
185	L	L	L	L	Disp. ^c
221	A	A	A	A	—
224	H	H	H	H	General activity
Subsite -1					Catalytic
The catalytic residues (D220, E248 and D318) are completely conserved.					
Subsite -2					Donor
95	H	H	H	H	General activity
98	W	W	W	W	—
365	R	R	R	R	—
Subsite -3					Donor
88–92	NYSGVNNT	NYSGVNNT	PGG...FA	PEG...FS	Cycl. ^c and spec. ^c
361	D	D	D	D	Cycl. ^c
187	D	D	D	D	Cycl. ^c
47	K	R	T	T	Cycl. ^c
Subsites -4 and -5					
No side chain contacts subsite-6					Donor
158	Y	Y	Y	Y	—
170–171	GG	GG	GG	GG	—
184	N	N	N	N	Cycl. ^c
Subsite -7					Donor
142–143	SSTDPSFA	SSDQPSFA	D.....I	D.....I	Spec. ^c
Central					
186	Y	Y	Y	Y	Cycl. ^c

Reproduced from J.C.M. Uitdehaag, *et al.*⁸⁾ with some modifications. Dashes denote deletions. α -, β -, γ -^a and γ -CGTase^b indicate the CGTases from *B. macerans*,⁴⁴⁾ *B. circulans* 251,⁸⁾ *Bacillus* sp. G-825-6⁴⁵⁾ and *B. clarkii* 7364,³⁰⁾ respectively. ^c Disp., disproportionation; Cycl., cyclization; Spec., specificity.

(β -CGTase) and *Bacillus* sp. G-825-6 (γ -CGTase). Four highly conserved regions were found in Cgt and the catalytic residues, Asp220, Glu248 and Asp318, are completely conserved. Through comparison of the amino acids in the subsites and the center of the substrate binding groove among typical CGTases, several amino acids at subsites +3, +2, -3 and -7 were found to be different or absent (Table 1). By comparison to a glycine at subsite +3 and alanine at subsite +2 in Cgt and γ -CGTase from *Bacillus* sp. G-825-6, the corresponding amino acids in α -CGTase from *B. macerans* and β -CGTase from *B. circulans* 251 were glutamic acid and lysine, respectively. At subsites -3 and -7 in γ -CGTase, variations in several amino acids were shown. Both sequence and length of the

amino acids in position 88-92 and 142-143 of γ -CGTase were different compared to α - and β -CGTase. γ -CGTase lacked three and six amino acid residues at subsites -3 and -7, respectively. The deletion mutant [$\Delta(141-151)$ D] at subsite -7 of β -CGTase from *B. circulans* 8 yielded a greater amount of γ -CD than the native enzyme.¹¹ This result might support the idea that the deletion of amino acids at subsite -7 of γ -CGTase is necessary for a preference in γ -CD production.

Characterization of Cgt and its mutants by replacing Ala223 at subsite +2 with several amino acids.

The amino group in the side chain of the lysine at subsite +2 in β -CGTase from *B. circulans* 251 plays an im-

Fig. 1. Effects of pH on the γ -CD forming activity of Cgt and its mutants.

Cgt (circles), A223H (diamonds), A223K (squares) and A223R (triangles) were used as γ -CGTase. The buffers used were $1/4 \times$ McIlvaine buffer (pH 3-7), 25 mM HEPES-NaOH buffer (pH 7.3-8.2), 25 mM Gly-NaCl-NaOH buffer (pH 8.8-10.2) and 25 mM Na_2HPO_4 -NaOH buffer (pH 10.5-11.5). The value is shown as percentage of the specific activity of Cgt at pH 10.0, which was taken as 100%.

Fig. 2. Courses of CD production from soluble starch by Cgt and its mutants.

A reaction mixture containing 10% (w/v) soluble starch, a (mutant) CGTase (0.15 mg/g dry starch) at pH 7.5 (A) and 10.0 (B) was incubated at 50°C. The buffers used were 25 mM HEPES-NaOH buffer (pH 7.5) and 25 mM Gly-NaCl-NaOH buffer (pH 10.0). The proportion of reaction products was analyzed by HPLC. The enzymes used were Cgt (circles), A223H (diamonds), A223K (squares) and A223R (triangles). The production of β - and γ -CD is shown by open and closed symbols, respectively.

portant role in the structural integrity of the active site by interacting with the glucose O3 atom of the +2 glucose residue.³²⁾ Since the corresponding amino acid at subsite +2 in γ -CGTase is alanine, there might be few, if any, hydrogen-bonding interactions with the +2 glucose residue, perhaps resulting in a change in enzyme specificity. We replaced Ala223 at subsite +2 in Cgt with three basic amino acids, lysine, arginine and histidine³³⁾ and performed a γ -CD-forming activity assay at various pH values ranging from 4 to 11.5 (Fig. 1). The A223H and A223K mutants had higher activity at neutral pHs (pH 6–9) than that of Cgt, whereas the A223R mutant had higher activity in a broader pH range (pH 6–10.5). The maximum activity of the A223R mutant was 1.5-fold higher than Cgt. Although the activity profile vs pH of γ -CD-forming activity changed, especially at neutral pH, the optimum pH did not change, and remained around 10.0. On the other hand, the A223D and A223E mutants, obtained by replacing Ala223 with acidic amino acids, showed significant decreases in the activity at all pH values ranging from 4 to 11.5 (data not shown).

The production of CDs in reaction mixtures containing 10% soluble starch and a (mutant) CGTase (0.15 mg/g dry starch) incubated at pH 7.5 and 10.0 and at 50°C was analyzed (Fig. 2). The A223H, A223K and A223R mutants produced greater amounts of γ -CD at pH 7.5 than did Cgt. Both the A223K and A223R mutants produced approximately 8% γ -CD after 48 h of incubation. At this time, the mutants had almost the same production of β -CD as Cgt. In the case of Cgt and the A223H mutant, even when the reaction was prolonged and/or excessive amounts of CGTase were used at pH 7.5, the γ -CD production still reached a plateau at 8% (data not shown). At pH 10.0, all CGTases produced the maximum amount of 8% γ -CD after 8 h of incubation. Replacing Ala223 with basic amino acids did not change the maximum amounts of γ -CD but the rates of γ -CD formation were efficiently accelerated without any change in CD production specificity.

The change in γ -CD-forming activity of the A223H, A223K, and A223R mutants was thought to be due to the introduction of the amino group in the side chain of these basic amino acids positioned for interaction with the linear substrate and the protonation of the amino group. We constructed a theoretical structure of Cgt using homology modeling from the SWISS-MODEL protein-modeling server (<http://www.expasy.ch/swissmod/SWISS-MODEL.html>) with the crystal structures of CGTases (PDB accession codes 1CIU, 1A47, 1CYG, 1PAM and 1I75) as templates, and simulated the effects of the A223H, A223K and A223R mutations (Fig. 3). The maltonaose (G9) structure was derived from the complex structure of *B. circulans* 251 CGTase with G9 (PDB accession code 1CXK). The constructed models of Cgt and its mutants indicated that Ala223 at subsite +2 in Cgt was located apart from the glucose residues of linear oligosaccharides whereas the amino groups in the side chains of His223, Lys223 and Arg223 were located close to the glucose O3 atom of the +2 glucose residue. The distance between these amino acid residues positioned at 223 and the glucose residue of a linear oligosaccharide seems to correlate

Fig. 3. Stereoviews of the structural conformation around the amino acid residues positioned at 223 of Cgt (A), A223H (B), A223K (C) and A223R (D) complexed with maltonaose.

Cgt and its mutant are shown in gray and maltonaose in light gray. The glucose residues are numbered +2, +1 and -1 as their corresponding subsite numbering.

with the enhancement of γ -CD-forming activity. Meanwhile, the changes in the pH vs γ -CD-forming activity profiles of the A223H, A223K and A223R mutants are not explained in terms of changes in the distance between these amino acid residues positioned at 223 and the substrate. The changes in the pH profile might be explained by the protonation of the amino groups in the side chains of His223, Lys223 and Arg223. The pK_a values of these three basic amino acids, histidine, lysine, and arginine, are 6.0, 10.5 and 12.5, respectively. At pH 7.5, the amino group in the side chain of His223 is thought to slightly prefer the protonated form ($-NH_3^+$), and those of Lys223 and Arg223 are thought to predominantly prefer the protonated forms, although the experimental pK_a values for

His223, Lys223 and Arg223 in each mutant were not measurable. At pH 10.0, the amino group in the side chain of Arg223 is thought to predominantly prefer the protonated form. The results suggest that the high proportion of the protonated state of the amino groups in the side chains of these amino acids positioned at 223 corresponds to the observed enhancement of γ -CD-forming activity. The protonation state seems to enhance the hydrogen bonding between these basic amino acids at subsite +2 and the glucose residues of linear oligosaccharides. The enhancement of the interaction at subsite +2 may play an important role in guiding the substrate to reach subsite +1, hence increasing the γ -CD-forming activity and changing the pH profile of the activity.

Although the γ -CD-forming activity was enhanced without any change in CD production specificity by the replacement of Ala233 with the basic amino acids, the γ -CD production by Cgt and its mutants reached a plateau at 8%. This plateau in γ -CD production is thought to be caused by two phenomena involving γ -CD. One is the product inhibition by γ -CD and the other is the equilibrium between γ -CD production *via* the cyclization reaction and γ -CD degradation *via* the coupling reaction.^{34–37} It has been reported that the amino group in the side chain of lysine at subsite +2 in CGTase from *B. circulans* undergoes a hydrogen bonding interaction with the glucose residue of a linear oligosaccharide and has very little interaction with circular glucose residues contained in CDs.^{8,38} As the amino acid positioned at 223 seemed to hardly interact with γ -CD, the plateau in γ -CD production in Cgt and its mutants might remain constant.

Cloning of *cda* encoding Cda.

We determined the nucleotide sequence of the adjacent region of the *cgt* gene encoding Cgt to confirm whether *B. clarkii* 7364 has a gene cluster for the starch degradation pathway *via* CD. A new ORF, which was designated the *cda* gene, was found about 200 bases downstream of the termination codon of *cgt* through nucleotide sequence analysis.³¹ The *cda* consisted of 2001 bases and encoded 666 amino acids. At 10 bases upstream of the initial co-

don, a putative ribosome-binding site (Shine-Dalgarno sequence) with the sequence of 5'-AGGAGGA-3' was found. A homology search of the deduced amino acid sequence of the protein (Cda) encoded by *cda* through the BLAST WWW System (<http://blast.ddbj.nig.ac.jp/>) revealed that it had some similarity with α -amylases, such as α -amylase from *Halothermothrix orenii* H168 (UniProt No, Q2ADT7) and α -amylase 3 from *Dictyoglomus thermophilum* (P14899). The sequence of Cda contained four conserved regions commonly found in the α -amylase family of enzymes. These results implied that Cda was likely an amylolytic enzyme. Another ORF was not found within about 2000 bases in the upstream or downstream regions of the two genes.

Characterization of Cda.

Cda was successfully produced as a recombinant protein in *E. coli*. To determine its amylolytic characteristics, the hydrolysis activities of Cda toward various substrates were investigated (Table 2). Cda rapidly hydrolyzed γ -CD, whereas it slowly hydrolyzed α - and β -CD, maltooctase, amylose, amylopectin, and soluble starch. The hydrolysis activities toward these substrates were less than 4% of that toward γ -CD. Pullulan and glycogen were minimally hydrolyzed. Thus, Cda was a CDase that had high specificity for only γ -CD. Other CDases do not possess such a high specificity for only one CD like Cda (Table 3). The examinations of the effects of pH and temperature on the γ -CD-hydrolyzing activity revealed that

Table 2. Hydrolysis activities toward various substrates of Cda.

Substrate	Relative activity (%)
α -CD	0.1
β -CD	0.4
γ -CD	100
Maltooctase	1.8
Soluble starch	2.3
Amylose	3.3
Amylopectin	1.1
Pullulan	< 0.1
Glycogen	< 0.1

Table 3. Relative activities of CDases toward α -, β - and γ -CD, soluble starch and pullulan.

Origin	Relative ratio (α -CD: β -CD: γ -CD: SS ^a : Pul ^b)	Reference
<i>Bacillus clarkii</i> 7364	0.1: 0.4: 100: 2.3:<0.1	31)
<i>Bacillus</i> sp. I-5	34: 100: 79.1: 8.4: 3.0	46)
<i>Bacillus sphaericus</i>	44: 100: ND ^c : 2.9: 1.5	47)
<i>Bacillus</i> sp.	100: 65: 38: ND ^c : ND ^c	48)
<i>Clostridium thermohydrosulfuricum</i> 39E	100: 87: 29: 3: 5	21)
<i>Paenibacillus</i> sp. A11	100: 72: 57: 9: 3	24)
<i>Thermococcus</i> sp. strain B1001	55: 100: 33: ND ^c : ND ^c	25)
Metagenomic DNA from rumen fluid	95: 100: 80: 53: 33	49)

SS^a, Pul^b and ND^c indicate soluble starch, pullulan and no data, respectively.

Table 4. Hydrolysis activity and kinetic parameters of Cda.

Substrate	Hydrolysis activity (U/mg of protein)	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ mM ⁻¹)
γ -CD	1.92 $\pm$ 0.06	0.0085 $\pm$ 0.0015	3.1 $\pm$ 0.5	367 $\pm$ 8
Maltooctase	0.0346 $\pm$ 0.0011	0.067 $\pm$ 0.006	0.030 $\pm$ 0.004	0.45 $\pm$ 0.04

Cda exhibited the highest activity at pH 8.5 and 30°C. More than 90% of the enzyme activity was retained in the pH range of 8.0–10.0 after pH treatment for 1 day and at 4°C after temperature treatment at pH 8.5 for 15 min. Moreover, Cda was efficiently stabilized by CaCl₂. In the presence of CaCl₂, Cda was stable in the pH range of 7.0–10.5 and at temperatures below 30°C. Kinetic parameters of Cda for γ -CD and maltooctaose were determined by measuring the hydrolysis activities using various substrate concentrations (Table 4). Cda showed a lower K_m value and a higher k_{cat} value for γ -CD than those for maltooctaose. The k_{cat}/K_m value for γ -CD was nearly 800-fold higher than that for maltooctaose, indicating that Cda possessed extremely high specificity for the cyclic substrate. The molecular mass of Cda was estimated to be 979 kDa by gel filtration chromatography. The calculated molecular mass of Cda was 77.8 kDa. This indicates that Cda forms a dodecamer.

Analysis of reaction product composition.

γ -CD and various maltooligosaccharides were used as substrates to investigate the reaction pattern of Cda. γ -CD was hydrolyzed to maltooctaose in the initial stage of the reaction; however, the maltooctaose formed was very slowly hydrolyzed (Fig. 4). Maltooctaose was split mainly into maltohexaose and maltose, and small amounts of maltotriose, maltotetraose and maltopentaose were also observed. Taking advantage of this feature of the hydrolysis reaction, Cda is thought to be industrially applicable to efficiently produce highly pure maltooctaose from γ -CD. Cda also produced transglycosylation products besides hydrolysates from linear oligosaccharides in the initial stage of the reaction (Fig. 5). Through the transglycosylation reaction, maltononaose, maltooctaose and maltohexaose were produced from maltohexaose, maltopentaose and maltotetraose, respectively. The reaction products containing transglycosylation products were completely hydrolyzed into maltotriose and maltose by β -amylase from soybean (data not shown). This indicated that the transglycosylation product was a linear molecule linked by only α -(1,4)-linkages and Cda newly formed α -(1,4)-

Fig. 4. Courses of reaction products from γ -CD by Cda.

A reaction mixture containing 1% (w/v) γ -CD, Cda (5 U/g of γ -CD) and 1 mM CaCl₂ in 25 mM HEPES-NaOH buffer (pH 8.5) was incubated at 30°C. The proportion of reaction products was analyzed by HPLC. ○, γ -CD; ●, maltooctaose; ▲, maltohexaose; ◇, the total amount of maltopentaose, maltotetraose and maltotriose; ■, maltose.

Fig. 5. HPLC analysis of the reaction products from maltohexaose (A), maltopentaose (B) and maltotetraose (C) by Cda.

A reaction mixture containing 1% (w/v) maltooligosaccharide, Cda (50 U/g of maltooligosaccharide) and 1 mM CaCl₂ in 25 mM HEPES-NaOH buffer (pH 8.5) was incubated at 30°C for 2 h. The proportion of reaction products was analyzed by HPLC. The numbers above the peaks indicate the degree of polymerization of each maltooligosaccharide.

linkages through transglycosylation. It seems that Cda tends to transfer a maltotriose moiety from the substrates to an acceptor and since maltotetraose is too short for Cda to transfer a maltotriose moiety to an acceptor, Cda may transfer a maltose moiety from maltotetraose instead. Several CDases have a transglycosylation activity as well as hydrolysis activity.^{20,39)} These enzymes transfer a glucose and a maltose moiety from the substrates to an acceptor. There have been no other reports of a CDase which is capable of transferring maltotriose and thus, this transglycosylation profile of Cda is a novel feature in CDases.

Comparison of the amino acid sequence of Cda with those of other CD-degrading enzymes.

A comparison of the entire amino acid sequence of Cda to those of other CDases revealed that the sequence of Cda had the highest identity with that of CDase from alkaliphilic *Bacillus* sp. I-5 (31% identity). Figure 6 shows the amino acid sequence alignment of Cda and three typical CD-degrading enzymes, CDase from alkaliphilic *Bacillus* sp. I-5, maltogenic amylase from *Thermus* strain IM6501, and neopullulanase from *Geobacillus stearothermophilus*. The amino acid sequence of Cda was significantly different from those of these typical CD-degrading enzymes. About 70 amino acids in the N-terminal region of the typical CD-degrading enzymes were absent in Cda. Enzymes capable of degrading CDs, such as CDase, maltogenic amylase, and neopullulanase all possess an N-domain which is involved in dimerization.^{14,40–42)} The dimer formation shapes a deep and narrow active site cleft which contributes to the preference of CDs. Cda can also

Fig. 6. Sequence alignment of CD-degrading enzymes.

Cda, I5CD, ThMA and NPU indicate Cda from *B. clarkii* 7364 (AB432985), CDase from alkaliphilic *Bacillus* sp. I-5 (AA092925), maltogenic amylase from *Thermus* strain IM6501 (AAC15072) and neopullulanase from *Geobacillus stearothermophilus* (AAK15003), respectively. The catalytic residues, two Asp residues and one Glu residue are given in white with a black background. The four conserved regions of α -amylase family enzymes are shown below the sequences.

form a dodecamer; however, the N-domain is absent in this case. Additionally, a deleted region (Gly81–Ile82), several gap regions (Ser193–Ile201, Asp254–Ile259, Pro 292–Tyr293, Gln340–Phe342 and Gly415–Gly446), and an extra sequence (Val551–Gln666) were found in Cda. The extra sequence in the C-terminal region was composed of more than 100 amino acids. A homology search of the extra sequence through the BLAST WWW System revealed that it was homologous to the C-terminal region of isocyclomaltooligosaccharide glucanotransferase from *Bacillus circulans* (34% identity).⁴³ However, the function of this region is not clear. The long extra sequence might contribute to the oligomerization of Cda through a new mechanism. Moreover, this new mechanism of oligomerization might affect the high specificity for γ -CD of Cda.

REFERENCES

- 1) C. Vauton and M. Hutin: The use of cyclodextrins in various industries. in *Cyclodextrins and Their Industrial Uses*. D. Duchêne, ed., de Santé, Paris, pp. 297–350 (1991).
- 2) B.A. van der Veen, J.C.M. Uitdehaag, B.W. Dijkstra and L. Dijkhuizen: Engineering of cyclodextrin glycosyltransferase reaction and product specificity. *Biochim. Biophys. Acta*, **1543**, 336–360 (2000).
- 3) B. Henrissat: A classification of glycosyl hydrolases based on amino-acid sequence similarities. *Biochem. J.*, **280**, 309–316 (1991).
- 4) B. Henrissat and A. Bairoch: New families in the classification of glycosyl hydrolases based on amino-acid sequence similarities. *Biochem. J.*, **293**, 781–788 (1993).
- 5) B. Henrissat and A. Bairoch: Updating the sequence-based classification of glycosyl hydrolases. *Biochem. J.*, **316**, 695–696 (1996).
- 6) H. Leemhuis, R.M. Kelly and L. Dijkhuizen: Engineering of cyclodextrin glucanotransferases and the impact for biotechnological applications. *Appl. Microbiol. Biotechnol.*, **85**, 823–835 (2010).
- 7) J.C. Uitdehaag, G.J. van Alebeek, B.A. van Der Veen, L. Dijkhuizen and B.W. Dijkstra: Structures of maltohexaose and maltoheptaose bound at the donor sites of cyclodextrin glycosyltransferase give insight into the mechanisms of transglycosylation activity and cyclodextrin size specificity. *Biochemistry*, **39**, 7772–7780 (2000).
- 8) J.C.M. Uitdehaag, K.H. Kalk, B.A. van der Veen, L. Dijkhuizen and B.W. Dijkstra: The cyclization mechanism of cyclodextrin glycosyltransferase (CGTase) as revealed by a γ -cyclodextrin-CGTase complex at 1.8-Å resolution. *J. Biol. Chem.*, **274**, 34868–34876 (1999).
- 9) B. Strokopytov, R.M. Knegt, D. Penninga, H.J. Rozeboom, K.H. Kalk, L. Dijkhuizen and B.W. Dijkstra: Structure of cyclodextrin glycosyltransferase complexed with a maltononaose inhibitor at 2.6 Å resolution. Implications for product specificity. *Biochemistry*, **35**, 4241–4249 (1996).
- 10) K.A. Sin, A. Nakamura, H. Masaki, Y. Matsuura and T. Uozumi: Replacement of an amino acid residue of cyclodextrin glucanotransferase of *Bacillus ohbensis* doubles the production of γ -cyclodextrin. *J. Biotechnol.*, **32**, 283–288 (1994).
- 11) G. Parsiegla, A.K. Schmidt and G.E. Schulz: Substrate binding to a cyclodextrin glycosyltransferase and mutations increasing the gamma-cyclodextrin production. *Eur. J. Biochem.*, **255**, 710–717 (1998).
- 12) T. Kometani, Y. Terada, T. Nishimura, H. Takii and S. Okada: Transglycosylation to hesperidin by cyclodextrin glucanotransferase from an alkaliphilic *Bacillus* species in alkaline pH and properties of hesperidin glycosides. *Biosci. Biotechnol. Biochem.*, **58**, 1990–1994 (1994).
- 13) V. Jaitak, V.K. Kaul, Bandna, N. Kumar, B. Singh, L.S. Savergave, V.V. Jogdand and S. Nene: Simple and efficient enzymatic transglycosylation of stevioside by β -cyclodextrin glucanotransferase from *Bacillus firmus*. *Biotechnol. Lett.*, **31**, 1415–1420 (2009).
- 14) K.H. Park, T.J. Kim, T.K. Cheong, J.W. Kim, B.H. Oh and B. Svensson: Structure, specificity and function of cyclomalto-dextrinase, a multispecific enzyme of the α -amylase family. *Biochim. Biophys. Acta*, **1478**, 165–185 (2000).
- 15) A. Hulsman, R. Lurz, F. Scheffel and F. Schneider: Maltose and maltodextrin transport in the thermoacidophilic gram-positive bacterium *Alicyclobacillus acidocaldarius* is mediated by a high-affinity transport system that includes a maltose binding protein tolerant to low pH. *J. Bacteriol.*, **182**, 6292–6301 (2000).
- 16) P. Turner, A. Labes, O.H. Fridjonsson, G.O. Hreggvidsson, P. Schonheit, J.K. Kristjansson, O. Holst and E.N. Karlsson: Two novel cyclodextrin-degrading enzymes isolated from thermophilic bacteria have similar domain structures but differ in oligomeric state and activity profile. *J. Biosci. Bioeng.*, **100**, 380–390 (2005).

- 17) S. Kitahata, M. Taniguchi, S.D. Beltran, T. Sugimoto and S. Okada: Purification and some properties of cyclodextrinase from *Bacillus coagulans*. *Agric. Biol. Chem.*, **47**, 1441–1447 (1983).
- 18) T. Oguma, A. Matsuyama, M. Kikuchi and E. Nakano: Cloning and sequence analysis of the cyclomaltodextrinase gene from *Bacillus sphaericus* and expression in *Escherichia coli* cells. *Appl. Microbiol. Biotechnol.*, **39**, 197–203 (1993).
- 19) K. Ohdan, T. Kuriki, H. Takata and S. Okada: Cloning of the cyclodextrin glucanotransferase gene from alkalophilic *Bacillus* sp. A2–5a and analysis of the raw starch-binding domain. *Appl. Microbiol. Biotechnol.*, **53**, 430–434 (2000).
- 20) T.J. Kim, J.H. Shin, J.H. Oh, M.J. Kim, S.B. Lee, S. Ryu, K. Kwon, J.W. Kim, E.H. Choi, J.F. Robyt and K.H. Park: Analysis of the gene encoding cyclomaltodextrinase from alkalophilic *Bacillus* sp. I-5 and characterization of enzymatic properties. *Arch. Biochem. Biophys.*, **353**, 221–227 (1998).
- 21) S.M. Podkovyrov and J.G. Zeikus: Structure of the gene encoding cyclomaltodextrinase from *Clostridium thermohydrosulfuricum* 39E and characterization of the enzyme purified from *Escherichia coli*. *J. Bacteriol.*, **174**, 5400–5405 (1992).
- 22) H.B. Fritzsche, T. Schwede and G.E. Schulz: Covalent and three-dimensional structure of the cyclodextrinase from *Flavobacterium* sp. no. 92. *Eur. J. Biochem.*, **270**, 2332–2341 (2003).
- 23) G. Fiedler, M. Pajatsch and A. Böck: Genetics of a novel starch utilisation pathway present in *Klebsiella oxytoca*. *J. Mol. Biol.*, **256**, 279–291 (1996).
- 24) J. Kaulpiboon and P. Pongsawasdi: Expression of cyclodextrinase gene from *Paenibacillus* sp. A11 in *Escherichia coli* and characterization of the purified cyclodextrinase. *J. Biochem. Mol. Biol.*, **37**, 408–415 (2004).
- 25) Y. Hashimoto, T. Yamamoto, S. Fujiwara, M. Takagi and T. Imanaka: Extracellular synthesis, specific recognition, and intracellular degradation of cyclomaltodextrins by the hyperthermophilic archaeon *Thermococcus* sp. strain B1001. *J. Bacteriol.*, **183**, 5050–5057 (2001).
- 26) Y. Matsuura, M. Kusunoki, W. Harada and M. Kakudo: Structure and possible catalytic residues of Taka-amylase A. *J. Biochem.*, **95**, 697–702 (1984).
- 27) H.S. Lee, M.S. Kim, H.S. Cho, J. I. Kim, T.J. Kim, J.H. Choi, C. Park, H.S. Lee, B.H. Oh and K.H. Park: Cyclomaltodextrinase, neopullulanase, and maltogenic amylase are nearly indistinguishable from each other. *J. Biol. Chem.*, **277**, 21891–21897 (2002).
- 28) J.S. Kim, S.S. Cha, H.J. Kim, T.J. Kim, N.C. Ha, S.T. Oh, H. S. Cho, M.J. Cho, M.J. Kim, H.S. Lee, J.W. Kim, K.Y. Choi, K.H. Park and B.H. Oh: Crystal structure of a maltogenic amylase provides insights into a catalytic versatility. *J. Biol. Chem.*, **274**, 26279–26286 (1999).
- 29) H. Hondoh, T. Kuriki and Y. Matsuura: Three-dimensional structure and substrate binding of *Bacillus stearothermophilus* neopullulanase. *J. Mol. Biol.*, **326**, 177–188 (2003).
- 30) M. Takada, Y. Nakagawa and M. Yamamoto: Biochemical and genetic analyses of a novel γ -cyclodextrin glucanotransferase from an alkalophilic *Bacillus clarkii* 7364. *J. Biochem.*, **133**, 317–324 (2003).
- 31) Y. Nakagawa, W. Saburi, M. Takada, Y. Hatada and K. Horikoshi: Gene cloning and enzymatic characteristics of a novel γ -cyclodextrin-specific cyclodextrinase from alkalophilic *Bacillus clarkii* 7364. *Biochim. Biophys. Acta*, **1784**, 2004–11 (2008).
- 32) B.A. van der Veen, H. Leemhuis, S. Kralj, J.C.M. Utidehaag, B.W. Dijkstra and L. Dijkhuizen: Hydrophobic amino acid residues in the acceptor binding site are main determinants for reaction mechanism and specificity of cyclodextrin-glucosyltransferase. *J. Biol. Chem.*, **276**, 44557–44562 (2001).
- 33) Y. Nakagawa, M. Takada, K. Ogawa, Y. Hatada and K. Horikoshi: Site-directed mutations in Alanine 223 and Glycine 255 in the acceptor site of γ -Cyclodextrin glucanotransferase from alkalophilic *Bacillus clarkii* 7364 affect cyclodextrin production. *J. Biochem.*, **140**, 329–36 (2006).
- 34) K. Tomita, T. Tanaka, Y. Fujita and K. Nakanishi: Some factors affecting the formation of γ -cyclodextrin using cyclodextrin glycosyltransferase from *Bacillus* sp. AL-6. *J. Ferment. Bioeng.*, **70**, 190–192 (1990).
- 35) D. Penninga, B.A. van der Veen, R.M.A. Knegtel, S.A.F.T. van Hijum, H.J. Rozeboom, K.H. Kalk, B.W. Dijkstra and L. Dijkhuizen: The raw starch binding domain of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251. *J. Biol. Chem.*, **271**, 32777–32784 (1996).
- 36) B.A. van der Veen, J.C.M. Utidehaag, B.W. Dijkstra and L. Dijkhuizen: The role of arginine 47 in the cyclization and coupling reactions of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251 implications for product inhibition and product specificity. *Eur. J. Biochem.*, **267**, 3432–3441 (2000).
- 37) G. Matioi, G.M. Zanin and F.F. De Moraes: Influence of substrate and product concentrations on the production of cyclodextrins by CGTase of *Bacillus firmus*, strain no. 37. *Appl. Biochem. Biotechnol.*, **98–100**, 947–961 (2002).
- 38) A.K. Schmidt, S. Cottaz, H. Driguez and G.E. Schulz: Structure of cyclodextrin glycosyltransferase complexed with a derivative of its main product β -cyclodextrin. *Biochemistry*, **37**, 5909–5915 (1998).
- 39) H. Bender: Studies of the transglycosylation reaction catalysed by the decycling maltodextrinase of *Flavobacterium* sp. no. 92 with malto-oligosaccharides and cyclodextrins. *Carbohydr. Res.*, **263**, 123–135 (1994).
- 40) H.S. Lee, M.S. Kim, H.S. Cho, J.I. Kim, T.J. Kim, J.H. Choi, C. Park, H.S. Lee, B.H. Oh and K.H. Park: Cyclomaltodextrinase, neopullulanase, and maltogenic amylase are nearly indistinguishable from each other. *J. Biol. Chem.*, **277**, 21891–21897 (2002).
- 41) J.S. Kim, S.S. Cha, H.J. Kim, T.J. Kim, N.C. Ha, S.T. Oh, H. S. Cho, M.J. Cho, M.J. Kim, H.S. Lee, J.W. Kim, K.Y. Choi, K.H. Park and B.H. Oh: Crystal structure of a maltogenic amylase provides insights into a catalytic versatility. *J. Biol. Chem.*, **274**, 26279–26286 (1999).
- 42) H. Hondoh, T. Kuriki and Y. Matsuura: Three-dimensional structure and substrate binding of *Bacillus stearothermophilus* neopullulanase. *J. Mol. Biol.*, **326**, 177–188 (2003).
- 43) H. Watanabe, T. Nishimoto, M. Kubota, H. Chaen and S. Fukuda: Cloning, sequencing, and expression of the genes encoding an isocyclomaltotooligosaccharide glucanotransferase and an α -amylase from a *Bacillus circulans* Strain. *Biosci. Biotechnol. Biochem.*, **70**, 2690–2702 (2006).
- 44) T. Takano, M. Fukuda, M. Monma, S. Kobayashi, K. Kainuma and K. Yamane: Molecular cloning, DNA nucleotide sequencing, and expression in *Bacillus subtilis* cells of the *Bacillus macerans* cyclodextrin glucanotransferase gene. *J. Bacteriol.*, **166**, 1118–1122 (1986).
- 45) K. Hirano, T. Ishihara, S. Ogasawara, H. Maeda, K. Abe, T. Nakajima and Y. Yamagata: Molecular cloning and characterization of a novel γ -CGTase from alkalophilic *Bacillus* sp. *Appl. Microbiol. Biotechnol.*, **70**, 193–201 (2006).
- 46) M.J. Kim, W.S. Park, H.S. Lee, T.J. Kim, J.H. Shin, S.H. Yoo, T.K. Cheong, S. Ryu, J.C. Kim, J.W. Kim, T.W. Moon, J.F. Robyt and K.H. Park: Kinetics and inhibition of cyclomaltodextrinase from alkalophilic *Bacillus* sp. I-5. *Arch. Biochem. Biophys.*, **373**, 110–115 (2000).
- 47) T. Oguma, M. Kikuchi and K. Mizusawa: Purification and some properties of cyclodextrin-hydrolyzing enzyme from *Bacillus sphaericus*. *Biochim. Biophys. Acta*, **1036**, 1–5 (1990).
- 48) A. Yoshida, Y. Iwasaki, T. Akiba and K. Horikoshi: Purification of properties of cyclodextrinase from alkalophilic *Bacillus* sp. *J. Ferment. Bioeng.*, **71**, 226–229 (1991).
- 49) M. Ferrer, A. Belouqui, O.V. Golyshina, F.J. Plou, A. Neef, T. N. Hernikova, L. Fernández-Arrojo, I. Ghazi, A. Ballesteros, K. Elborough, K.N. Timmis and P.N. Golyshin: Biochemical and structural features of a novel cyclodextrinase from cow rumen metagenome. *Biotechnol. J.*, **2**, 207–213 (2007).

***Bacillus clarkii* 7364 株が生産する γ -Cyclodextrin に高い特異性を有する二つの酵素**

中川佳紀¹, 佐分利亘¹, 山本 健¹, 高田正保¹
小川浩一¹, 山本幹男¹, 秦田勇二², 中村信之²

堀越弘毅²

¹ 日本食品化工株式会社

(417-8530 富士市田島 30)

² 独立行政法人海洋研究開発機構

(237-0061 横須賀市夏島町 2-15)

好アルカリ性細菌 *Bacillus clarkii* 7364 株が菌体外に分泌する cyclodextrin glucanotransferase (CGTase) は、澱粉を基質としたとき、 γ -cyclodextrin (γ -CD) を優先的に生成する γ -CGTase である。また、本酵素 (Cgt) をコードする遺伝子の約 200 bases 下流には、cyclodextrinase (Cda) をコードする遺伝子が存在する。本研究では、Cgt および Cda の機能解析を行った。Cgt は至適 pH 10.0 の好アルカリ酵素であった。Cgt を 1% (w/v) 可溶性澱粉に作用させると、CD 組成は α -CD<0.1%, β -CD 3.5% および γ -CD 27.5% となった。Cgt および他起源の CGTase のアミノ酸配列を比較したところ、Cgt ではサブサイト+3, +2, -3 および-7 においてアミノ酸の欠失および相違が認められた。サブサイト+2 に位置する Ala223 を三つの塩基性アミノ酸 (His, Lys および Arg) に置換した変異体は、いずれも中性 pH での γ -CD 合成比活性が向上した。これは、Ala223 の塩基性アミノ酸への置換により、プロトン化した側鎖のアミノ基が基質と相互作用を示すことによるものと推察される。一方、Cda は γ -CD のみに高い加水分解活性を示す cyclodextrinase であった。さらに、Cda はマルトリオース単位で糖転移させる活性を保持していた。Cda および他起源の CD 分解酵素のアミノ酸配列を比較したところ、Cda では他起源の CD 分解酵素が保持する N ドメインが欠失し、C 末端側が 100 アミノ酸以上長かった。CD 分解酵素の N ドメインは二量体形成に寄与するが、Cda は N ドメインを欠失しているにもかかわらず、12 量体を形成していた。これらの特徴は他起源の CD 分解酵素では認められず、Cda は新奇的な CD 分解酵素であることが示唆される。

〔質問〕

江崎グリコ 高田

Cgt の CD 合成以外の活性 (不均化活性, カップリング活性等) について調べておられたら教えて下さい。

〔答〕

Cgt は、不均化活性およびカップリング活性ともに、その活性を有していることは確認しておりますが、各反応における kinetics parameter は測定していません。

〔質問〕

林原生化研 西本

Cda の温度安定性が低いことと γ -CD の代謝に関する制御に関して何か考察できることはあるのでしょうか？

〔答〕

菌体内酵素である Cda の温度安定性は、菌体外酵素である Cgt より劣りますが、*B. clarkii* 7364 株の至適培養温度 30-40°C において、Cda が示す温度安定性でも菌体内で機能するのには十分なのかもしれません。

〔質問〕

築野食品工業 橋本

Cda が 2 糖単位で加水分解し、3 糖単位で糖転移するメカニズムを説明して下さい。

〔答〕

メカニズムは不明です。立体構造解析等がメカニズム解明の糸口となると考えます。

〔質問〕

大阪市工研 中野

Cda が 12 量体を形成しているということと γ -CD に選択性が高いということに、何か関連はあるのでしょうか。もしお考えがあれば教えて下さい。

〔答〕

12 量体の形成と γ -CD への高い選択性との関連は不明です。上記の糖転移活性と同じく、立体構造解析等がメカニズム解明の糸口となると考えます。