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Biochemical and Genetic Analyses of a Novel γ-Cyclodextrin Glucanotransferase from an Alkalophilic *Bacillus clarkii* 7364

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On screening for microorganisms in soil obtained in Japan that produce large amounts of γ-cyclodextrin (γ-CD), we identified a novel alkalophilic bacterium, *Bacillus clarkii* 7364. The cyclodextrin glucanotransferase (CGTase) secreted into the culture medium by this bacterium was purified by affinity chromatography on a γ-CD–immobilized column, followed by chromatography on a gel filtration column. The enzyme converted 13.7% of pre-gelatinized potato starch (10% w/w per reaction mixture) into CDs, and the majority (79%) of the product CDs was of the γ form. This property is quite unique among known CGTases and thus we named this enzyme γ-CGTase. The N-terminal and internal amino acid sequences of γ-CGTase were determined and used to design PCR primers for amplification of the nucleotide sequence that encodes the γ-CGTase gene. The entire gene sequence amplified by PCR was determined and then cloned into *E. coli*. The recombinant enzyme synthesized by *E. coli* retained biochemical properties quite similar to those of the original one. Comparison of the deduced amino acid sequence of γ-CGTase with those of other known CGTases that have different product specificities revealed the importance of subsites –3 and –7 for the preferential γ-cyclization activity.

Key words: amino acid sequence, *Bacillus clarkii*, cyclic specificity, cyclodextrin, γ-CGTase.

Abbreviations: bp, base pair(s); Da, dalton(s); PCR, polymerase chain reaction(s); ORF, open reading frame; γ-CGTase, CGTase that produces γ-CD from starch as a main product; aa, amino acid(s); nt, nucleotide; BCG, bromocresol green. Enzyme: CGTase (cyclodextrin glucanotransferase; EC 2.4.1.19).

Since cyclodextrins (CDs) [cyclic α-(1,4)-linked oligosaccharides of 6, 7, or 8 glucose residues, respectively] can accommodate various organic molecules within the cavity of their ring structure made of D-glucose units, which leads to changes in the chemical and physical properties of the guest molecules, they are extensively utilized in industry for various purposes (*1*). These CDs are synthesized by cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19) from starch and related α-(1,4)-linked glucose polymers via a transglycosylation reaction (*2*). This enzyme belongs to the α-amylase family (family 13) of glycosyl hydrolases. Members of this family contain conserved catalytic residues and a conserved catalytic site architecture, but their activities vary (*3*). CGTase usually catalyzes four different reactions (cyclization, coupling, disproportionation, and hydrolysis). The mechanism underlying the cyclization reaction of CGTase has been studied in detail. The formation of CD starts with binding of CGTase to a linear maltooligosaccharide substrate, across multiple sugar binding subsites. This is followed by cleavage of an α-(1,4)-glycosidic bond resulting in an intermediate that is covalently bound to Asp 229 in subsite –1 (*4*). Subsequently, the non-reducing end of the substrate moves into the site of the covalent bond and is processed for the formation of a new intramolecular α-(1,4)-glycosidic bond. As separation of the different CDs is costly and time-consuming, a CGTase that predominantly produces one type of CD is of interest (*5*, *6*). Alfa- and β-CDs can be synthesized enzymatically on an industrial scale by using CGTases from various bacteria, such as *Bacillus macerans* (*7*), *B. circulans* 251 (*8*), and *Bacillus* sp. 290–3 (*5*). These CGTases convert starch into α- or β-CD as the main product but still give mixtures of CDs in different ratios. Although there is a demand for γ-CD, which can trap larger molecules that cannot be trapped by α- and β-CDs, in various industries, the industrial production of γ-CD is not yet practical due to the low yield of this substance with currently utilized CGTases (*2*, *9*). Several attempts have been made to change the properties of CGTases and to enhance the γ-CD production yield, but no satisfactory results have been obtained yet (*10*, *11*).

Therefore, in this study we cloned a novel CGTase gene from an alkalophilic bacterium, *Bacillus clarkii* 7364, whose protein product has the unique property of the production of a high proportion of γ-CD from pre-gelatinized potato starch. Comparison of the deduced amino acid sequence of this gene product with those of other known CGTases revealed the key amino acid residues that determine the product specificity.

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MATERIALS AND METHODS

Materials—Soluble starch was obtained from Nacalai Tesque (Kyoto). CDs were purchased from Nihon Shokuhin Kako (Tokyo). Glucoamylase from *Rhizopus niveus* and α-amylase (liquefying type) from *B. subtilis* were products of Seikagaku (Tokyo). All restriction and modification enzymes used for recombinant DNA manipulations were purchased from Takara Shuzo or Toyobo. All other chemicals were of analytical grade.

Bacteria and Culture Conditions—Bacillus clarkii 7364 (G-7364) was isolated from soil obtained in Japan. The bacteria were aerobically cultured in 300 ml of alkaline medium composed of 1% (w/v) dextrin, Pinedex 100 (Matsutani Kagaku), 1.5% fish extract, Cultibater No. 600 (Yaizu Suisan Kagakukogyo), 1% yeast extract, Asahi Supermeast AP-006 (Asahi Beer), 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, and 0.8% Na_2CO_3 at 37°C with continuous shaking. After 48 h incubation the culture was centrifuged at 12,000 rpm for 15 min and the supernatant was used for further purification of the γ-CGTase.

Purification of γ-CGTase—The cell-free supernatant was concentrated to 62 ml using an ultrafiltration membrane PM-10 (Amicon). The concentrate was loaded onto a γ-CD–immobilized Sepharose 4B column (1.5ϕ × 300 mm) pre-equilibrated with 50 mM K-phosphate buffer (pH 6.5). The column was washed with 300 ml of the same buffer. The adsorbed proteins were eluted with 100 ml of 50 mM K-phosphate buffer (pH 6.5) containing 15 mg/ml γ-CD. The fractions containing γ-CGTase activity were collected and concentrated to 3 ml with PM-10. The concentrated enzyme fraction was gel-filtered by on a Superdex 200 column (HR16/60) equilibrated with 50 mM K-phosphate buffer (pH 6.8). The fractions containing γ-CGTase activity were collected and concentrated to 6.5 ml with PM-10. The protein concentration of the purified enzyme was determined by the method of Lowry *et al*. with crystalline bovine serum albumin as a standard (*12*). The purified enzyme was stored at 4°C until use.

Enzyme Activity Assay—Starch-degrading activity was measured by the blue value method of Fuwa (*13*) with the slight modification of the use of 0.1% soluble starch as the substrate. One unit of starch-degrading activity was defined as the amount of enzyme that gave a 10% decrease in absorbance at 700 nm per min. The γ-CD–forming activity was determined by the bromocresol green (BCG) method of Kato and Horikoshi (*14*) with some modifications, as follows. A reaction mixture comprising 450 μl of 1.5% (w/v) soluble starch in 25 mM Gly-NaCl-NaOH buffer (pH 10.0) and 50 μl of the enzyme was incubated at 40°C for 10 min. The reaction was terminated by the addition of 0.05 N HCl (500 μl), and then 100 μl of 5 mM BCG in 20% (v/v) ethanol was added. The reaction mixture was incubated at room temperature for 20 min to facilitate the formation of inclusion complexes between γ-CD and BCG. After incubation, 2 ml of 1 M acetate buffer (pH 4.2) containing 30 mM citric acid was added, and then the amount of γ-CD in the mixture was spectrophotometrically determined by measuring the absorbance at 630 nm. One unit of γ-CD–forming activity was defined as the amount of enzyme that produced 1 μmol of γ-CD per min under these conditions.

Determination of the Proportions of Product CDs—Five hundred microliters of the reaction mixture was heated in a boiling water bath for 10 min to inactivate the enzyme. To eliminate contaminating saccharides from the reaction mixture while leaving CDs intact, 500 μl of water, 1 ml of glucoamylase (70 U/ml), and 30 μl of α-amylase (420 U/ml) in 5 mM acetate buffer (pH 6.0) were added to the heated reaction mixture, and then the mixture was incubated at 50°C for 30 min. After heating the reaction mixture for 10 min, the insoluble materials were removed by passing the solution through a 0.45-μm membrane filter. The proportions of glucose units (α-, β-, and γ-) of the CDs in the reaction mixture were determined by HPLC. HPLC was performed using a Shimadzu LC-10A apparatus under the following conditions: column, Aminex HPX- 42A (8ϕ × 300 mm, Bio-Rad Laboratories); column temperature, 55°C; mobile phase, deionized water; flow rate, 0.5 ml/min. The CDs eluted from the column were detected with a refractive index detector (RID-10A refractometer, Shimadzu).

N-Terminal Amino Acid Sequence Determination of γ-CGTase—One hundred picomoles of the purified γ-CGTase was subjected to SDS–PAGE, and then the protein was transferred to a PVDF membrane. The membrane was stained with 0.2% CBB in a 40% methanol and 10% acetic acid solution, and then destained with a 60% methanol and 10% acetic acid solution. The protein band visualized was cut out and processed for Edman degradation with an HPG1005A Protein sequencing system (Takara).

PCR Amplification and Sequencing of the Gene Encoding γ-CGTase—Chromosomal DNA of *B. clarkii* 7364 was prepared by the method described by Declerk *et al*. (*15*). Primer 1 (5′-AAYGTIAAITAYGCIGARGARGT-3′) was designed based on the N-terminal aa sequence of the mature enzyme; SNATNDLSNVNYAEEVIYHIVT [from the 9th aa (N) to the 15th aa (E) of the mature enzyme]. Primer 2 (5′-GCRTCICCIGGYTTICCCATDCC3′) was designed based on the aa sequence of the internal region; ESGSAPVIGQVGPPMGKPGDA (the aa sequence underlined was used). Fifty microliters of the PCR reaction mixture had the following composition: 360 ng of *B. clarkii* 7364 genomic DNA, 50 pmol of primer 1, 50 pmol of primer 2, 1× Ex (Mg^{2+} free) Taq buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, and 1.25 units Takara Ex Taq. The following three rounds of temperature cycling were performed. (1st round) 94°C for 5 min; number of cycles, 1. (2nd round) 94°C for 1 min, 51°C for 2 min, 72°C for 3 min; number of cycles, 30. (3rd round) 72°C for 10 min; number of cycles, 1. Sequencing of the amplified fragment was performed by the chain termination method (*16*) with an automated DNA sequencer (Beckman Coulter, CEQ2000), and the results were analyzed using GENETYX-WIN software. A 1.5-kb amplified PCR fragment contained the aa sequences of VIYHIVT and ESGSAPVIGQVGP. To determine the remaining N- and C-terminal flanking regions of the amplified fragment, the *B. clarkii* 7364 genomic DNA was digested with S*ac* I, followed by treatment with T4 DNA ligase at 16°C under conditions that favored the formation of monomeric circles (*17*). Inverse PCR was performed with 240 ng of the above S*ac*I self-circularized DNA molecule as a template and 50 pmole of each of primer 3, 5′-GATCTGTTACAATATGATAAAT-3′, and pri-

mer 4, 5′-TTATTAGACGGTCAATCGTTA-3′, which had been designed from the terminal ends of the 1.5-kbp amplified fragment and other components as above. The following five rounds of temperature cycling were performed. (1st round) 94°C for 5 min; number of cycles, 1. (2nd round) 94°C for 0.5 min, 47°C for 0.5 min, 72°C for 0.5 min; number of cycles, 10. (3rd round) 94°C for 0.5 min, 50°C for 0.5 min, 72°C for 1 min; number of cycles, 10. (4th round) 94°C for 1 min, 50°C for 2 min, 72°C for 3 min; number of cycles, 10. (5th round) 72°C for 10 min; number of cycles, 1. An approximately 2-kbp amplified DNA fragment contained the remaining N- and C-terminal sequences. Full length PCR amplification of the γ-CGTase gene from *B. clarkii* 7364 was carried out with 360 ng of *B. clarkii* 7364 genomic DNA as a template and 50 pmole each of primer 5, 5′-GACTTGTACTAAGAC-AACCTTACG-3′, and primer 6, 5′-GCATCGGCTCTACT-CATTTCA-3′, other components being the same as above. The following three rounds of temperature cycling were performed. (1st round) 94°C for 5 min; number of cycles, 1. (2nd round) 94°C for 1 min, 55°C for 1.5 min, 72°C for 3 min; number of cycles, 32. (3rd round) 72°C for 10 min; number of cycles, 1.

Exogenous Expression of γ-CGTase in E. coli—The full-length PCR product was ligated into the pGEM-T vector and then used for the transformation of JM 109 *E. coli* cells. The transformed cells were grown in LB medium containing 50 μg/ml of ampicillin at 37°C for 16 h. Purification of the recombinant enzyme was performed according to the method used for the native one.

RESULTS AND DISCUSSION

Purification of γ-CGTase from Bacillus Clarkii 7364—Strain G-7364 was isolated during screening for microorganisms that produce γ-CD abundantly in a culture medium containing starch as the carbon source. The physiological and biochemical characteristics of strain G-7364 were determined according to the methods of Nielsen *et al*. (*18*) and Yumoto *et al*. (*19*). The organism grew better in an alkaline medium (pH 9–11) and no growth was observed at pH 7. The bacterium was found to be aerobic and motile, to produce spores located terminally, to be rod-shaped (0.6 to 0.8 by 3.0 to 5.0 μm), and to be gram-positive. It was positive for the production of catalase and oxidase, and the hydrolysis of starch, casein, gelatin, Tween 40, and Tween 60. It was negative for the formation of H_2S and indole, the production of urease, and growth in 10% (w/v) NaCl. The 16S rDNA gene sequence (*20*) of strain G-7364 was also compared with those of 10 type strains of *Bacillus* sp. and it exhibited the closest match with that from *Bacillus clarkii* (99.45%). Based on these findings, the bacterium was classified as *B. clarkii* (to be published in detail elsewhere), and strain G-7364 of this bacterium was therefore designated as *B. clarkii* 7364. The organism excreted into the medium an enzyme that preferentially produced γ-CD from starch. Figure 1 shows the courses of formation of CDs at pH 10.0 and 50°C. The enzyme produced α-, β-, and γ-CDs in the ratio of 1.9:1.0:10.8 from 10% (w/w) gelatinized potato starch on 48 h reaction. In the case of a substrate concentration of 1% (w/v), 20% total CDs were produced, but the ratio of α-, β-, and γ-CDs did not change. Furthermore, the addition of 20% (v/v) ethanol to the reaction mixture facilitated the formation of γ-CD, and about 23% γ-CD (α-CD, 0%; β-CD, 2%) was produced

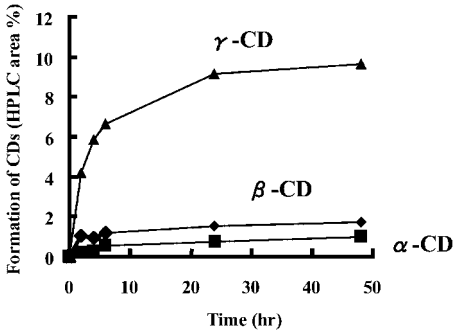

Fig. 1. **Courses of formation of CDs from gelatinized potato starch by γ-CGTase.** A reaction mixture containing 10% (w/w) gelatinized potato starch and crude γ-CGTase (0.75 U/g of dry starch) in 25 mM Gly-NaCl-NaOH buffer (pH 10.0) was incubated at 50°C. CDs formed in the mixture were determined by HPLC, as described under Materials and Methods. Closed triangles, γ-CD; closed diamonds, β-CD; closed squares, α-CD. From 100 ml of the reaction mixture, α-, β-CD and oligosaccharides were removed by crystallization and gel filtration. Finally, 175 mg of γ-CD was obtained. Its structure was confirmed by ^{13}C- and ^{1}H-NMR (data not shown).

Table 1. **Purification of γ-CGTase from *Bacillus clarkii* 7364 and *E.coli* JM109 harboring pGFT-01.**

Purification step	Total volume (ml)	Total protein (ml)	Enzyme activity		Specific activity (unit/mg of protein)
			Total units	Yield (%)	
Bacillus clarkii					
Crude	62	141.95	23.56	100	0.17
Affinity column	3.0	2.19	a	a	a
Gel filtration	6.5	1.97	10.66	45.2	5.41
***E. coli* JM109**					
Crude	10	125.50	108.7	100	0.87
Affinity column	3.0	17.30	a	a	a
Gel filtration	20	15.60	79.8	73.4	5.12

[a]The enzyme activity after affinity column chromatography was not determined because the purified enzyme preparation contained γ-CD, which is a competitive inhibitor of γ-CGTase (see "RESULTS AND DISCUSSION").

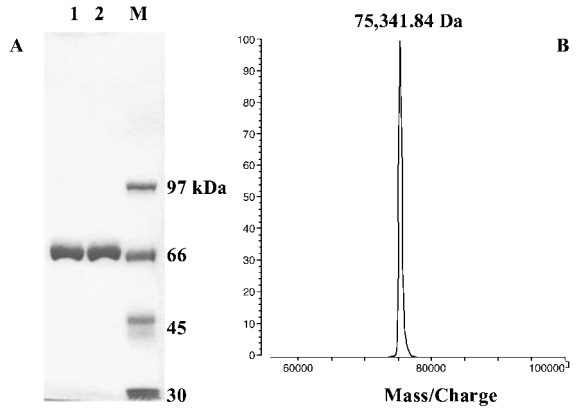

Fig. 2. **SDS–PAGE (A) and TOF-MS (B) of the purified γ-CGTase.** (A) SDS–PAGE: The purified enzyme (3.62 μg) was visualized by Coomassie Brilliant Blue staining for protein. Lanes 1 and 2 show the enzyme purified from *B. clarkii* 7364 and *E. coli* cells harboring pGEFT-01, respectively. Lane M shows molecular mass markers (calibration in kilodaltons). (B) TOF-MS: The purified enzyme (270 pmol) from *B. clarkii* 7364 was applied to an AXIMA-CFR TOF-MS system (Shimadzu, Kyoto). The number above the peak indicates the molecular mass (in daltons) of the enzyme.

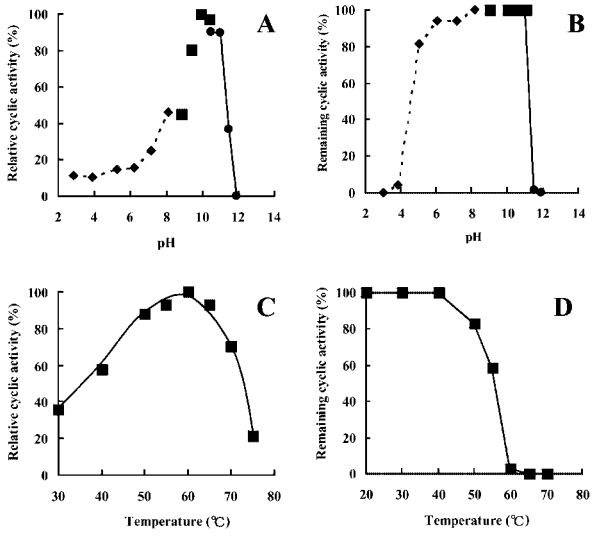

Fig. 3. **Effects of pH and temperature on the activity and stability of γ-CGTase.** (A) Effect of pH on the enzyme activity. Closed diamonds, enzyme activity when 1/4 ×McIlvaine buffer was used at pH 3–8; closed squares, enzyme activity when 25 mM Gly-NaCl-NaOH buffer was used at pH 8–10.5; closed circles, enzyme activity when 25 mM Na_2HPO_4-NaOH buffer was used at pH 10.5–11.9. (B) Effect of pH on the enzyme stability. The symbols (closed diamonds, closed squares and closed circles) have the same meaning as in (A). (C) Effect of temperature on the enzyme activity. (D) Effect of temperature on the enzyme stability. The experimental conditions for each reaction are given in the text.

from 1% gelatinized potato starch (data not shown). These results strongly suggested that the CGTase from *B. clarkii* 7364 is a novel type of enzyme that produces γ-CD as the main product from starch on industrial γ-CD production (*2*, *9–10*). The practical interest in the enzymatic formation of γ-CD from starch prompted us to perform more detailed studies on the enzyme. Table 1 summarizes the overall purification steps. In the case of enzyme purification with a culture filtrate of *B. clarkii* 7364, the specific γ-CD–forming activity increased 32-fold. The purified enzyme exhibited specific activity of 5.41 units per mg of protein, with a final yield of 45%. The purified enzyme gave a single protein band on both SDS–PAGE and isoelectric focusing. The molecular mass of the purified enzyme was determined to be 68 kDa and 75,341 Da by SDS–PAGE and TOF-MS, respectively (Fig. 2). The isoelectric point of the purified enzyme was judged to be 3.98. The N-terminal aa sequence of the purified enzyme was SNATNDLSNVNYAEEVIYHIVT.

The Effects of pH and Temperature on the Enzyme Activity—The effects of pH and temperature on the γ-CD–forming activity of the purified enzyme were studied under the standard assay conditions at 40°C for 10 min with buffers of various pHs and different reaction temperatures (pH 10.0). The enzyme showed a single maximum at pH 10.5–11.0, and at 60°C (Fig. 3, A and C). The stability of the enzyme with changes in pH and temperature was also examined. The pH values of solutions containing equal amounts (10 μl) of the purified enzyme (9 μg protein) were adjusted to the range of 3.0 to 12.0 by adding 90 μl of various buffers (pH 3.0–8.0, 1/4 ×McIlvaine buffer; pH 8.0–10.5, 25 mM Gly-NaCl-NaOH buffer; pH 10.5–11.9, 25 mM Na_2HPO_4–NaOH buffer). Each buffered enzyme solution was kept at 4°C for 24 h, and then the pH of each solution was adjusted to 10.0 by the addition of 100 μl of 25 mM Gly-NaCl-NaOH buffer (pH 10.0). The remaining enzyme activity was then measured under the standard assay conditions. The enzyme was completely stable over the pH range of 6.0–11.0 at 4°C for 24 h (Fig. 3B). The purified enzyme (9 μg protein) in 100 μl of 25 mM Gly- NaCl-NaOH buffer (pH 10.0) was heated at various temperatures for 15 min and then cooled immediately in an ice bath. The remaining enzyme activity was then measured with 50 μl of each enzyme solution by means of a standard assay at 40°C for 10 min. Gamma-CGTase retained its original activity upon heating to a temperature below 40°C (Fig. 3D).

Cloning and Determination of the Nucleotide Sequence of the γ-CGTase Gene—To understand the unique features of the γ-CGTase, we cloned and sequenced the enzyme gene. By sequencing the amplified DNA fragment, we identified the entire nucleotide sequence of the γ-CGTase gene and flanking region (the original nucleotide sequence reported in this paper has been deposited in the GenBank, EMBL, and DDBJ databases under the accession No. AB082929). Analysis of this sequence revealed the presence of a unique ORF starting with a GTG codon at nt position 321 bp and encoding a TAA stop codon at nt position 2,427 bp. At seven bp upstream of the initiation codon, there was a putative RBS with the sequence of 5′-AGGAGG-3′ exhibiting good complementarity to the 3′-end of *B. subtilis* 16S rRNA (*21*). Upstream of the RBS, there was a putative sequence of 5′-TTGACA-3′ as a potential –35 region and 5′-TACAAT-3′ as a potential –10 region separated by 17 bp. A long inverted-repeat sequence was found at 28 bp downstream of the termination codon in the ORF. The ORF encoded a protein of 674 aa residues (not including a signal sequence) with a predicted molecular mass of 75,311 Da. The N-terminal aa sequence of the purified γ-CGTase

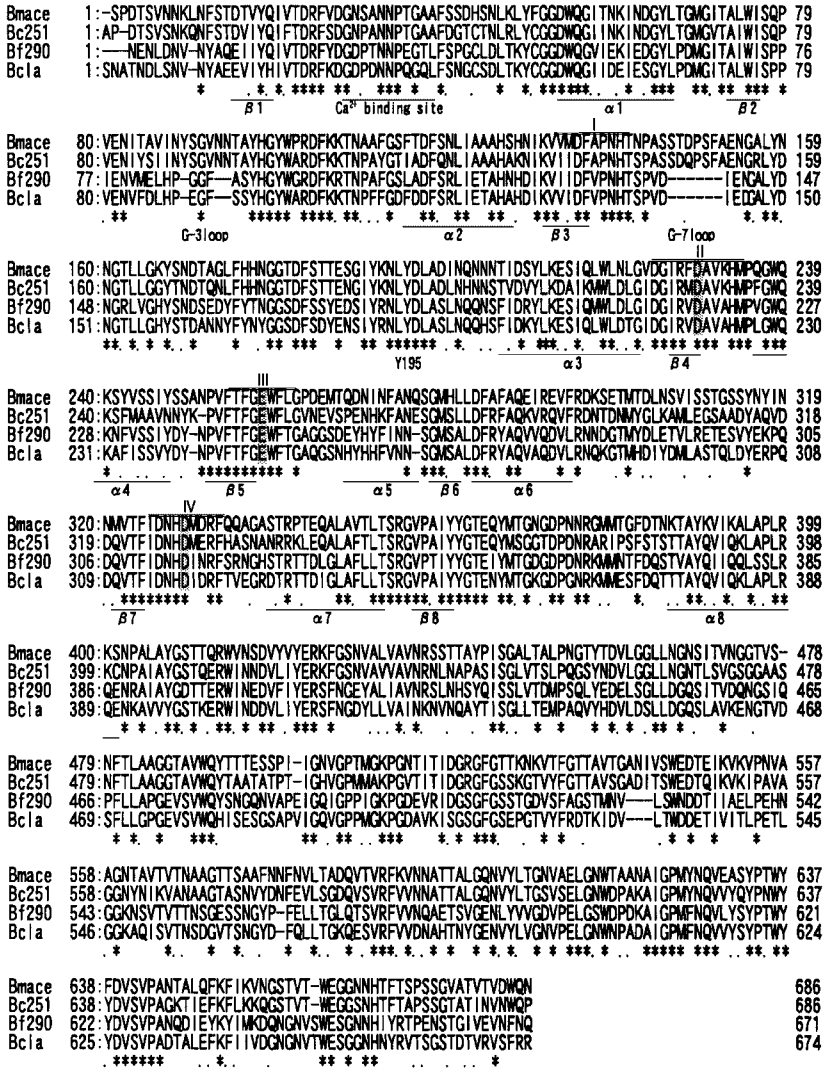

Fig. 4. **Alignment of aa sequences of typical α-, β-, β/γ-CGTases and the γ-CGTase from *Bacillus clarkii* 7364.** In each case the numbering starts after the respective signal sequence. Bmace, Bc251, Bf290, and Bcla indicate the aa sequences of α-CGTase from *B. macerans* (7), b-CGTase from *B. circulans* 251 (8), β/γ-CGTase from *Bacillus* sp. 290–3 (5), and γ-CGTase from *B. clarkii* 7364, respectively. The aa residues indicated by asterisks are completely conserved in all types of CGTase. The aa residues indicated by dots are conserved in three types of CGTase. Dashes denote deleted aa. The locations of α-helices (α1–α8), β-sheets (β1–β8), loops at subsites –3 and –7, and the Ca^{2+} binding site in the structure of Bc251 (8) are shown under the sequences. The four conserved sequences of α-amylase family enzymes are shown above the sequences.

could be matched with the aa residues (S_{29} to T_{50}) of the deduced aa sequence. The fact that aa residues V_1 to A_{28} are not present in the mature enzyme indicates that this peptide may act as a signal sequence and that they are cleaved off during secretion. The deduced molecular mass of the mature enzyme (75,311 Da) correlated well with the molecular mass determined by TOF-MS (75,341 Da) for the purified enzyme protein (Fig. 2). To express the complete gene of γ-CGTase in *E. coli* JM109 cells, a DNA fragment containing the ORF, stop codon, promoter region, and termination signal of γ-CGTase from *B. clarkii* 7346 was amplified by PCR with genomic DNA and suitable primers. The synthesized primers were 5′-GACTTGTACTAAGACAACCTTACG-3′ and 5′-GCATC-GGCTCTACTCATTCA-3′, which had been designed from sequences 237 bp upstream of the initiation codon and 77 bp downstream of the termination codon, respectively. The PCR product (2,530 bp) was ligated with pGEM-T

Table 2. **Comparison of the properties of CGTases from various *Bacillus* sp.**

	Bcla	Recombinant	Bmace	Bmega	Bf290	Brev
Isoelectric point	3.98	3.98	4.6	–	4.1	–
Optimum pH	10.5–11.0	10.5–11.0	5.2–5.7	5.0–5.7	6.0–8.0	10
Optimum temp. (°C)	60	60	55	55	50	45
pH stability	6.0–11.0	6.0–11.0	8.0–10.0	7.0–10.0	6.0–8.0	6.0–8.0
Thermal stability (°C)	40	40	55	55	–	30
Molecular weight (Da)	68,000	68,000	65,000	75,000	75,000	75,000
Main product	γ	γ	α	β	β/γ	β/γ

Bmace, Bmega, Bf290, Brev, and Bcla indicate α-CGTase from *B. macerans* (7), β-CGTase from *B. megaterium* (29), β/γ-CGTase from *Bacillus* sp. 290–3 (5), *Brevibacterium* sp. (28), and *B. clarkii* 7364, respectively. "Recombinant" indicates the enzyme purified using a periplasmic fraction of *E. coli* JM109 cells harboring pGEFT-01.

Table 3. **Comparison of the aa residues around the active center in the four types of CGTase.**

Residue No.	Residue in α-CGTase	Residue in β-CGTase	Residue in β/γ-CGTase	Residue in γ-CGTase	Function in CGTase
Subsite +2					
183	F	**F**	F	F	Cyclization
232	K	K	A	A	Disproportionation
259	F	**F/Y**	F	F	Cyclization and disproportionation
Subsite +1					
194	L	L	L	L	Cyclization
230	A	A	A	A	–
233	H	H	H	H	General activity
Subsite –1					
The catalytic residues (**D229, E257, and D328**) are completely conserved.					
Subsite –2					
98	H	H	H	H	General activity
101	W	W	W	W	–
375	R	R	R	R	–
Subsite –3					
89	Y	Y	–	–	Cyclization and specificity
87–93	INYSGVN	INYSGVN	HP-GGF-	HP-GGF-	Cyclization and specificity
371	D	D	D	D	Cyclization
196	D	**D**	D	D	Cyclization
47	K	R	T	T	Cyclization
Subsites –4 and –5					No side chain contacts
Subsite –6					
167	Y	**Y**	Y	Y	–
179–180	GG	**GG**	GG	GG	–
193	N	**N**	N	N	Cyclization
Subsite –7					
145–152	SSTDPSFA	SSDQPSFA	D – - - - - I	D – - - - - I	Specificity
Central					
195	Y	**Y/F**	Y	Y	Cyclization
Ca^{2+} binding site					
32–36	NNPTG	NNPTG	NNPEG	NNPQG	Ca^{2+} binding

Reproduced from Uitdehaag, J.C.M. *et al*. (*22*), with some modifications. Boldface type indicates residues that are conserved in 20 CGTase sequences, but not in α-amylases (*22*). Dashes denote deletions. The underlined residues and sequences are very specific in CGTases that produce γ-CD primarily. α-, β-, β/γ-, and γ-CGTase indicate the CGTases from *B. macerans* (*7*), *B. circulans* 251 (*8*), *Bacillus* sp. 290–3 (*5*), and *B. clarkii* 7364, respectively.

(Promega), the resulting plasmid being designated as pGEFT-01. *E. coli* JM109 cells were then transformed with pGEFT-01.

To produce the recombinant enzyme, transformed *E. coli* JM109 cells were grown in LB medium containing 50 μg/ml of ampicillin at 37°C for 16 h. The enzyme was purified using a periplasmic fraction of *E. coli* JM109 cells harboring pGEFT-01. The enzyme purification scheme described under "MATERIALS AND METHODS" resulted in a final recovery of 73% of total activity. The purified recombinant enzyme gave a single protein band on SDS–PAGE, and exhibited specific activity of 5.12 units per mg of protein (Table 1 and Fig. 2A). The molecular weight, isoelectric point, N-terminal aa sequence and other enzymatic properties of the enzyme from *E. coli* were all identical to those of the enzyme from *B. clarkii* 7364 (Fig. 2A and Table 2). The purified enzyme (0.75 U/g of dry starch) produced a final amount of 10% γ-CD from 10% (w/v) sol-

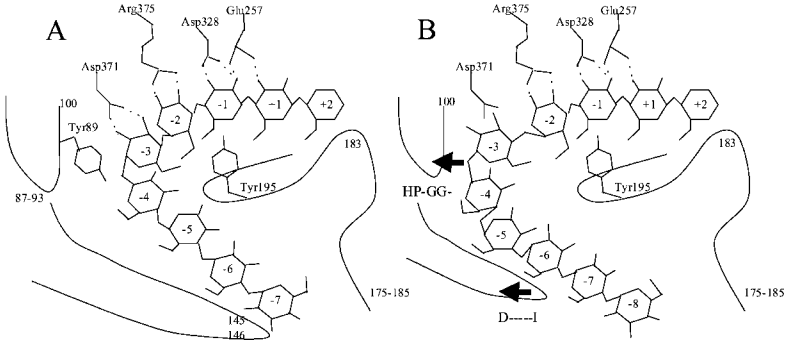

Fig. 5. **Schematic structure of the substrate binding sites of CGTase.** (A) β-CGTase: Reproduced from Uitdehagg *et al*. (2, 4), with modification. (B) γ-CGTase from *B. clarkii* 7364: The deduced structure of the substrate binding sites of γ-CGTase based on alignment of the aa sequences of β- and γ-CGTases, as shown in Table 3.

uble starch at pH 10 and 50°C on 48 h incubation, the same as in the case of the native enzyme (data not shown).

Comparison of the aa Sequence of the γ-CGTase from B. Clarkii 7364 with Those of Other Typical CGTases—Figure 4 shows the aa sequence alignments between γ-CGTase from *B. clarkii* 7364 and other typical CGTases. The deduced aa sequence of the mature enzyme showed moderate homology (~70%) to those of CGTases from *B. macerans* (α-CGTase), *B. circulans* 251 (β-CGTase), and *Bacillus* sp. 290–3 (γ/β-CGTase). Furthermore, four highly conserved regions designated as regions I, II, III, and IV in α-amylase family enzymes (*3*) were found in all CGTases, as shown in Fig. 4. This suggests that the catalytic domain of our γ-CGTase is folded into a $(\beta/\alpha)_8$-barrel structures and the above four conserved regions in this catalytic domain may constitute the active center of the enzyme. On the basis of the results of many studies on the X-ray crystallographic structures of CGTases with their inhibitors, substrates or products, it has been proposed that the active center of CGTase has a tandem subsite architecture in the substrate binding groove and that it contains at least nine sugar- binding subsites designated, from the nonreducing end to the reducing end, as –7 through +2 (*2*, *4*, *22–23*). Several aa residues involved in the binding of maltononaose or γ-CD with β-CGTase from *B. circulans* 251 have been revealed (*22–23*). To identify the aa residues of γ-CGTase from *B. clarkii* 7364 involved in γ-cyclization specificity, the aa residues of β-CGTase involved in the binding of maltononaose or γ-CD were compared with those of α-, β/γ-, and -CGTases, as shown in Table 3. Tyrosine-195 (*B. circulans* 251 CGTase numbering) is located in a dominant position at the center of the active site cleft in many CGTases, and a mechanism for the α-(1,4)-glucan chain folding around this residue has been proposed (*23*). Thus, Tyr-195 may play an important role in the cyclization of CGTases (*2*). Attempts have been made to change the product specificity of CD by substituting this aa residue with another aa residue, but the such mutations (*8*, *10*, *11*, *24–26*) did not influence the preferred CD size. Furthermore, the fact that every aa residue in native α-, β-, β/γ-, and γ-CGTases at this position is Tyr indicates that this residue is not involved in the product specificity of CGTases. Since all aa residues except for Lys-232 forming subsites +2, +1, –1, –2, –4, –5, and –6 are conserved in all CGTases, these residues are also not thought to be involved in the product specificity of native CGTases. The first region that may be involved in product specificity is found at subsite –7. The aa sequences in this region (145–152) of α- and β-CGTases, which are located in a loop at the start of the B-domain, are SSTDPSFA and SSDQPSFA, respectively. β/γ- and γ-CGTases completely lack the six aa residues in this region (D–I). This indicates that it may be necessary for a higher level of γ-cyclization activity to have more space for the bound glucosyl chain at subsite –7 (Fig. 5 and Table 3). This hypothesis is supported by the finding that a deletion mutant [Δ(145–151)D] of β-CGTase from *B. circulans* strain 8 yields a greater amount of γ-CD than that yielded by the native enzyme (*25*). The second region that may be involved in product specificity is found at subsite –3. It has been reported that both Asp-196 and Asp-371 stabilize linear sugar chains at this subsite, on the other hand, cyclic compounds are stabilized by both Arg-47 and Asp-371 at this subsite (*22*). Of these three aa residues, both Asp-196 and Asp-371 are completely conserved in the four types of CGTases, as shown in Table 3. On the other hand, Arg-47 (β-CGTase) is replaced by Lys in α-CGTase. It is interesting that Thr is found at this position in both β/γ- and γ-CGTases, which primarily form γ-CD. Another specific region and aa residue at subsite –3 are loop 87–93 and Asn-94. These residues show hydrophobic interactions with glucose bound at this subsite and are remarkably different in the four types of CGTase. Both α- and β-CGTases have the sequence INYSGVN(N), and the stretch sequence of HPGGF- is found in γ- and β/γ-CGTases (Fig. 4 and Table 3). The fact that the loops in γ- and β/γ-CGTases are shorter than those in other types of CGTase indicates that more space for the bound glucosyl chain in this region is needed for a higher level of γ-cyclization activity, as in the case of subsite –7, as shown in Fig. 5.

Although many kinds of organisms producing extracellular CGTases have been reported, the optimum pHs are 5–8 (*5*, *6*, *27–29*) and the main products are α- or β-CDs (Table 2). These results indicate that a high level of γ-cyclization activity might depend on the structure of the substrate at alkaline pH. Another specific property of γ-CGTase is its CD productivity. As shown in Fig. 1, the enzyme produced CDs (α-, β- and, γ-CD) in a total yield of about 13% from 10% (w/w) gelatinized potato starch. CGTases used for the industrial production of CDs generally produce more than 30% CDs in total amount (*6*, *27–29*). Compared with these enzymes, the yield of total CDs with γ-CGTase is remarkably low. Our γ-CGTase cannot hydrolyze γ-CD and is inhibited by γ-CD itself. The K_i value for γ-CD as to the starch-degrading activity of γ-CGTase was 0.86 mM, and the inhibition type was competitive (data not shown). These results suggest that the reason for the low yield of total CDs might be product inhibition. It has also been reported that the productivity of γ-CD increased on the addition of ethanol in the cases of β/γ-CGTases (*26*, *28*), as well as our enzyme. We ascribe these increases in productivity to the inhibition of E-P (enzyme-product) complex formation with increasing hydrophobic atmosphere in the reaction solution.

Dijkhuizen *et al*. provided detailed insights into the product specificity of CGTases (*2*, *4*, *8*, *22–23*), and pointed out the necessity for construction of mutant enzymes to clarify the mechanism underlying the size specificity of the enzyme. We believe that the γ-CGTase from *B. clarkii* 7364 will provide information that will be useful for the construction of recombinant enzymes.

The original nucleotide sequence reported in this paper has been deposited in the GenBank, EMBL, and DDBJ databases under accession no. AB082929. We wish to thank Dr. G. Okada, professor emeritus of Shizuoka University, and Dr. Y. Michikawa, National Institute of Radiological Sciences, for their critical reading of the manuscript, and the staff of the Genomic Research Department of Shimadzu Co., Ltd., for the TOF-MS analysis.

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