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Purification and Characterization of Cyclodextrin Glucanotransferase from *Paenibacillus* sp. JK-12

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Abstract

Extracellular cyclodextrin glucanotransferase (CGTase) from *Paenibacillus* sp. JK-12 was purified through several purification steps consisting of ammonium sulfate precipitation and chromatographies on DEAE-sephadex A-50 and Mono QIM HR5/5. The purified CGTase exhibited a single band on SDS-PAGE and was estimated to be approximately 82 kDa. The isoelectric point of the enzyme was 7.2 as determined by isoelectric focusing. The CGTase from *Paenibacillus* sp. JK-12 had a transglucosylation activity at the C-2 position of L-ascorbic acid. The optimum pH and temperature for the CGTase activity were 8.0 and 50°C, respectively. The enzyme activity was stable from pH 6.0 to 9.0 and at temperatures up to 55°C at pH 8.0, having 80% residual activity. The activity of the CGTase was strongly resistant to metals such as Ag^{+} and Ba^{2+} but slightly inhibited by Hg^{+}, Ni^{2+} and Mg^{2+}. The enzymeproduced α-cyclodextrin (α-CD) and β-CD as the main products from starch, but not γ-CD.

Key words: transglucosylation, cyclodextrin glucanotransferase, *Paenibacillus* sp. JK-12, purification

INTRODUCTION

L-Ascorbic acid (AA) is an essential nutrient for humans as vitamin C, with obligatory roles in various physiological functions (1,2). However, AA is unstable under conditions such as exposure to heat, light, neutral pH and oxidative conditions (3,4). The instability of AA in aqueous solutions impairs its commercial production by cell culture fermentation. Therefore, a stable ascorbate derivative is needed for the production of AA for use as a supplement in foods, medicines and cosmetics.

In 1991, Tanaka et al. (5) discovered a nonreducible 2-*O*-α-D-glucopyranosyl L-ascorbic acid (AA-2G) formed by a regioselective transglucosylation with α-glucosidase and cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19). AA-2G in a neutral pH solution is very stable against heat, light and oxidation (6,7). AA-2G can be hydrolyzed to AA and glucose by the α-glucosidase bound to the intestinal mucosal membrane and has equivalent biological activities to AA *in vivo* and in tissue culture (7). Moreover, AA-2G has been demonstrated to exhibit antiscorbutic activity in guinea pigs (8,9) and facilitates collagen synthesis in human skin fibroblasts (10,11). Therefore, AA-2G can be used for the elucidation of other physiological functions of AA whose mechanism remains unclear. Several approaches to the synthesize AA-2G by enzymatic methods have been attempted, but, so far, little is known about the enzymatic transglucosylation of AA except by *Bacillus stearothermophilus* CGTase as reported by Aga et al. (12,13).

Numerous microorganisms were screened to identify species that glucosylate AA at the C-2 position specifically. We isolated two bacterial strains from soil, designated as *Paenibacillus* sp. JB-13 and *Paenibacillus* sp. JK-12, which produce extracellular CGTases with transglucosylation activity at the C-2 position of AA. We previously reported the purification and functional characterization of a CGTase from *Paenibacillus* sp. JB-13 (14). In this study, we describe the purification and characterization of *Paenibacillus* sp. JK-12 CGTase with transglucosylation activity at the C-2 position of AA.

MATERIALS AND METHODS

Chemicals

Soluble starch was purchased from Wako Pure Chemical Industries Co. Ltd. (Osaka, Japan). Glucoamylase from *Rhizopus* mold, rice seed α-glucosidase, ascorbate oxidase (ASOD, EC 1.10.3.3), sodium ascorbate, and other chemicals were obtained from Sigma Co. (St. Louis, MO, USA). DEAE-Sephadex A-50 and Mono QIM HR 5/5 columns were obtained from Pharmacia Fine Chemicals. (Uppsala, Sweden) AA-2G was kindly provided by professor Sakai,

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Microorganism and Cultivation

A bacterium strain producing CGTase with transglucosylation activity at the C-2 position of AA was isolated from soil, using a basal medium containing 1.0% soluble starch, 0.5% polypeptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 1.0% Na_2CO_3, 0.02% Congo Red, 0.01% methyl orange and 1.5% agar (pH 7.0). The isolated bacterium strain was identified as *Paenibacillus* sp. JK-12 by cultural, morphological, and biochemical characteristics, in addition to the analysis of its 16S rDNA sequence. The *Paenibacillus* sp. JK-12 was cultured in 500 mL Erlenmeyer flasks containing 100 mL of basal medium except without the Congo Red, methyl orange, and agar; at pH 7.0 and 37°C for 36 hrs.

Assay for CGTase activity

CGTase activity was determined by the glucoamylase method (15-17) with some modifications. Using sucrose and α-CD as substrates, the coupling activity of CGTase was analyzed by detecting the formation of glucose. The reaction mixture containing the enzyme, 5 mM α-CD and 25 mM sucrose in 100 mM phosphate buffer (pH 7.0) was incubated at 55°C for 1 hr. The reaction was stopped by boiling for 5 min. 10 units of glucoamylase in 0.5 M acetate buffer (pH 4.5) was added to the reaction mixture and incubated for 30 min at 55°C. The amount of reducing sugar produced was determined by the method of Somogyi-Nelson (18) under standard conditions. One unit of the enzyme was defined as the enzyme activity required to produce 1.0 μmole of reducing sugar per minute.

Purification of CGTase

All procedures were performed at 4°C. Solid ammonium sulfate was added to the culture supernatant to 10% saturation and left overnight. The supernatant was collected by centrifugation and brought to 70% saturation in the same manner. The precipitate was collected by centrifugation and dissolved in 50 mM phosphate buffer, pH 7.0, and dialyzed overnight against the same buffer. The dialyzed enzyme was applied to a DEAE-sephadex A-50 column (1.5 × 15 cm) equilibrated with 50 mM phosphate buffer (pH 7.0). The active fractions were eluted with the same buffer, pooled and concentrated by ultrafiltration using an Amicon ZM 500 membrane (Millipore Co.). The enzyme solution was then applied to a Mono QIM HR 5/5 column previously equilibrated with 20 mM Tris-HCl buffer (pH 8.0). After the column was washed with the same buffer, the CGTase was eluted with a linear gradient from 0 to 0.5 M NaCl. The active fractions were pooled, concentrated, and used as purified enzyme. The concentration of protein was determined by the method of Bradford (19), using bovine serum albumin as a standard.

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophresis (SDS-PAGE) was performed to check protein purity and to determine the molecular weight of the purified enzyme under denaturing conditions using 10% acrylamide gel, as described by Laemmli (20). After electrophoresis, the gel was stained with Coomassie brilliant blue R-250, and then destained in the same solution without the dye. The standard marker proteins were myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), fructose-6-phosphate kinase (84 kDa), albumin (66 kDa), glutamate dehydrogenase (55 kDa), ovalbumin (45 kDa), and glyceraldehyde-3-phosphate dehydrogenase (36 kDa).

Analytical methods

AA-2G and cyclodextrin produced by *Paenibacillus* sp. JK-12 CGTase were analyzed by HPLC (21). For the AA-2G, the reaction product was measured by HPLC using a Waters μ Bondapak C18 column (3.9 × 300 mm). The assay conditions were as follows: detection wavelength, 238 nm; mobile phase, 0.1 M KH_2PO_4-0.1 M H_3PO_4 (pH 2.0); flow rate, 0.5 mL/min. The amount of AA-2G was calculated on the basis of its standard curve of peak area. For the CD, the HPLC system was Waters 2680 equipped with a Carbohydrate column (Waters) and Waters RI detector. The flow rate of the eluent, a mixture of acetonitrile and water (65 : 35, v/v), was 1.0 mL/min.

Effect of pH on the CGTase activity and stability

To determine CGTase pH stability, the enzyme solution was added to 0.1 M sodium acetate (pH 3 ~ 6), potassium phosphate (pH 6 ~ 8), Tris-HCl (pH 8 ~ 10) and glycine (pH 10 ~ 12) buffers. After standing at 50°C for 30 min, the remaining CGTase activity was assayed as described above, and the pH of the sample with the highest activity was considered to be the optimal pH for CGTase.

Effect of temperature on the CGTase activity and stability

The optimum temperature for the enzyme activity was determined by assaying the CGTase activity at various temperatures, as described above, for 30 min and analyzing the residual activities.

Measurement of isoelectric point

The isoelectric point of the enzyme was determined by 5% polyacrylamide gel isoelectric focusing using 2.4% Ampholine (pH 3.5 ~ 10.0). Two identical gels were prepared, one for the calibration of the pH gradient and the other for detection of the enzyme. After electrofocusing at a constant voltage of 200 V per column for 2.5 hrs, the CGTase activity and pH of each slice were measured.

RESULTS AND DISCUSSION

Purification of CGTase

The crude extract of CGTase from *Paenibacillus* sp. JK-12 was sequentially purified by ammonium sulfate precipitation and two successive chromatographies using DEAE-Sephadex A-50 and Mono QIM HR 5/5. Elution profiles of the last two steps during chromatography are shown in Fig. 1 and Fig. 2, respectively. On the elution profile of Mono QIM HR 5/5 ion-exchange chromatography, the unique peak based on the activity coincided with a protein peak. Purity of each elute during the purification process was examined by electrophoresis on 10% SDS-PAGE. The active fraction from Mono QIM HR 5/5 ion-exchange chromatography showed a single protein band on SDS-PAGE. The purification procedures and results are summarized in Table 1. The CGTase was concentrated about 3.4 fold with a 5% recovery from the culture medium, yielding a purified CGPase enzyme with a specific activity of about

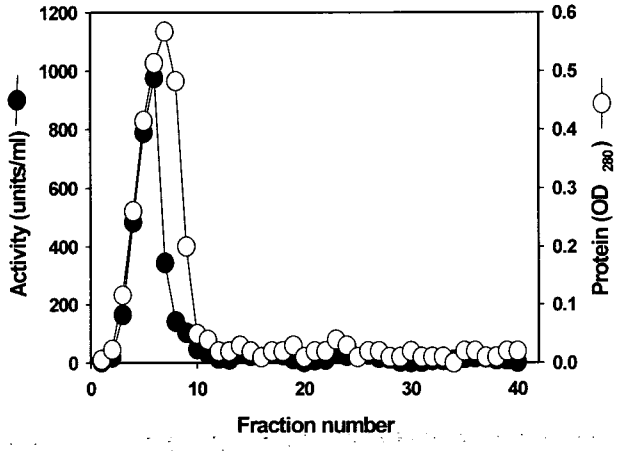

Fig. 1. DEAE-Sephadex A-50 column chromatography of CGTase from *Paenibacillus* sp. JK-12. Column size 1.5×15 cm, equilibrated with 50 mM potassium phsophate buffer (pH 7.0), fraction volume 5 mL, flow rate 0.25 mL/min.

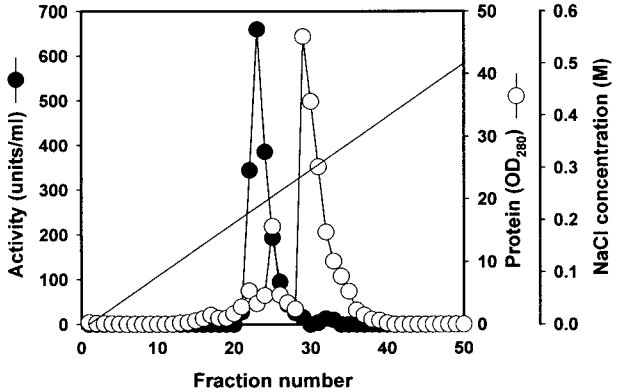

Fig. 2. Mono QTM HR 5/5 ion exchange chromatography of CGTase from *Paenibacillus* sp. JK-12. Equilibrated with 20 mM Tris-HCl buffer (pH 8.0), fraction column 0.2 mL, flow rate 0.5 mL/min, 0.5 M NaCl gradient.

Table 1. Purification of CGTase from *Paenibacillus* sp. JK-12

Step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Fold
Culture supernatant	41,360	868	48	100	1.0
$(NH_4)_2SO_4$ fractionation (10~70%)	30,601	406	75	74	1.6
DEAE-Sephadex A-50	10,496	101	104	25	2.2
Mono Q™ HR5/5	1,932	12	161	5	3.4

161 units/mg protein.

Molecular weight and isoelectric point of CGTase

As shown in Fig. 3, the molecular weight of the purified CGTase was determined to be approximately 82 kDa by SDS-PAGE. Typically, the molecular weights of CGTases from *Bacilli* are about 74 kDa, corresponding to 680+10 amino acid residues (33-35), and are highly conserved with 50~70% identity at the amino acids level. The molecular weight of the presently described enzyme is similar to that of the CGTases from *B. circulans* var. *alkalophilus* ATCC 21783 (36,37) and *B. ohbensis* sp. nov. C-1400 (32). The isoelectric point of the enzyme was estimated to be about pH 7.9.

Effect of pH and temperature on the enzyme activity

The enzyme activity was measured over the range of pH 3.0 to 12.0. As shown in Fig. 4A and 4B, the maximum activity was observed at pH 8.0. However, more than 80% of the enzyme activity was maintained over the range from pH 6.0 to 9.0.

pH and temperature stability

The effect of temperature on the enzyme activity was determined over the range of 25°C to 80°C. The optimal temperature was 50°C (Fig. 5A) and the enzyme remained stable up to 55°C (Fig. 5B).

Effect of metal ions and inhibitors

The effects of various metal ions and inhibitors on the

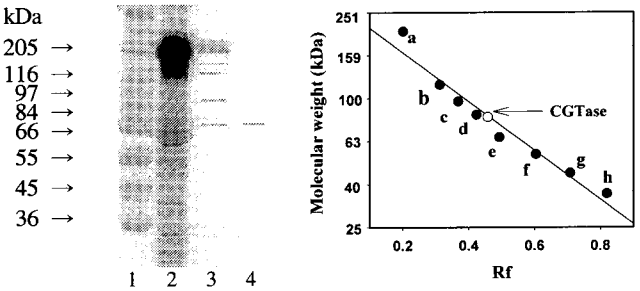

Fig. 3. Determination of the molecular weight of purified CGTase by SDS-PAGE. The conditions of SDS-PAGE are described in Materials and Methods. Lane 1: standard (a, 205 kDa; b, 116 kDa; c, 97 kDa; d, 84 kDa; e, 66 kDa; f, 55 kDa; g, 45 kDa; h, 36 kDa), Lane 2: ammonium sulfate 70%, Lane 3: DEAE-sephadex A-50, Lane 4: Mono Q™ HR 5/5.

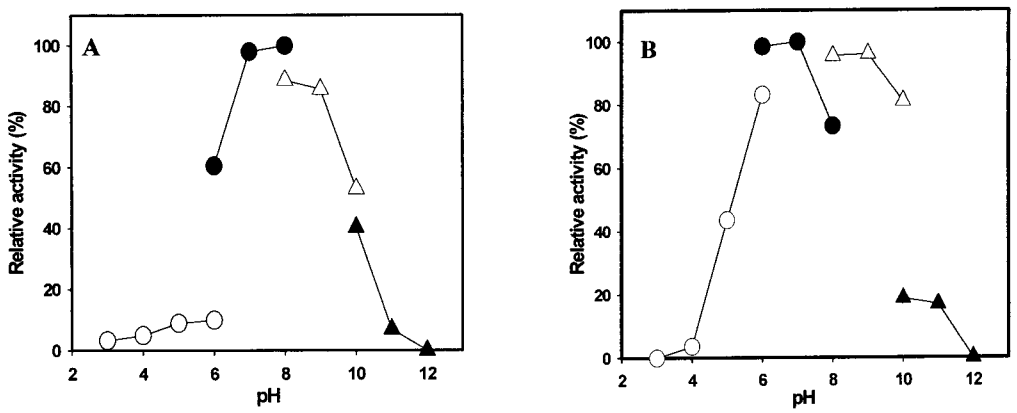

Fig. 4. Effects of pH on activity (A) and stability (B) of CGTase from *Paenibacillus* sp. JK-12. A: reaction mixtures with the enzyme solution, sucrose and α-CD in 0.1 M sodium acetate buffer (pH 3.0~6.0 -○-) or phosphate buffer (pH 6.0~8.0 -●-) or Tris-HCl buffer (pH 8.0~10.0 -△-) or glycine buffer (pH 10.0~12.0 -▲-) were incubated at 55°C for 1 hr. B: enzyme solution was incubated in 0.5 mL of 0.1 M sodium acetate buffer (pH 3.0~6.0 -○-) or phosphate buffer (pH 6.0~8.0 -●-) or Tris-HCl buffer (pH 8.0~10.0 -△-) or glycine buffer (pH 10.0~12.0 -▲-) at 50°C for 30 min and then the residual activity was measured.

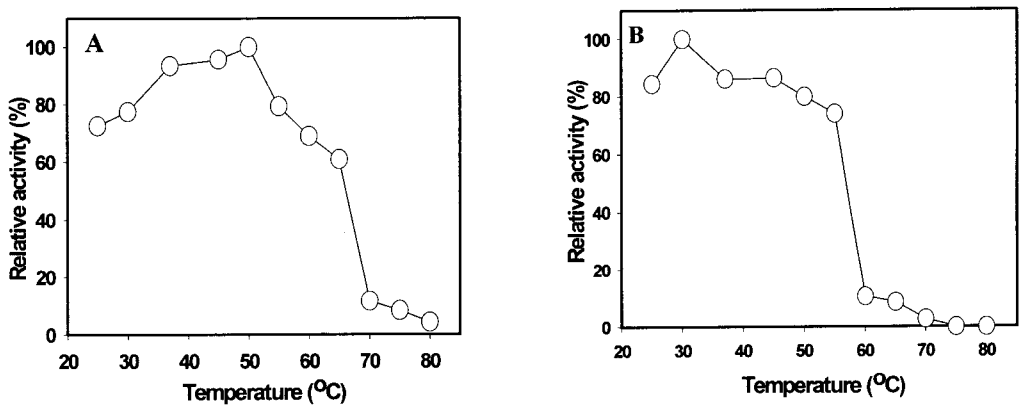

Fig. 5. Effects of temperature on activity (A) and stability (B) of CGTase from *Paenibacillus* sp. JK-12. A: reaction mixtures containing of the enzyme solution and sucrose and α-CD in 0.1 M phosphate buffer (pH 8.0) were incubated for 1 hr at various temperatures. B: enzyme solution was incubated in 0.1 M phosphate buffer (pH 8.0) for 30 min at various temperatures and the residual activity was measured.

enzyme activity were investigated (Table 2, 3). The enzymes was preincubated with various reagents for 1 hr at 50°C at the final concentration of 1 mM for metal ions and 10 mM inhibitors before the activities were determined. Enzyme activity was strongly inhibited by Ba^{2+}, Ag^{+}, Hg^{2+}, L-cysteine hydrochloride, SDS and PMSF, but was not greatly affected by other metal ions and inhibitors.

Prior efforts by most investigators have focused on the purification of bacterial CGTases and characterization of the purified enzymes (38). Typically, bacterial CGTases display the maximal activity at pH values of 4.5~7.0 and at temperature of 40~85°C. As mentioned above, and shown in Table 4, the characteristics of *Paenibacillus* sp. JK-12 CGTase are quite different from those of the previously reported CGTases, and from *Paenibacillus* sp. JB-13 CGTase (14).

AA-2G formation

As described previously (14), the reaction mixture for

Table 2. Effect of the metal ions on the activity of CGTase from *Paenibacillus* sp. JK-12

Metal ion	Relative activity (%)
None	100
$MnCl_2$	77.76
$MgCl_2$	62.36
$CoCl_2$	145.30
$CuSO_4$	159.74
$CaCl_2$	316.48
$FeSO_4$	88.50
$BaCl_2$	33.52
$ZnCl_2$	112.17
$NiCl_2$	66.11
$CdCl_2$	114.79
KCl	147.57
$AgNO_3$	16.11
$HgCl_2$	45.13

The enzyme was dialyzed with 50 mM potassium phosphate buffer (pH 7.0) containing 20 mM EDTA for 24 hrs at 4°C, then dialyzed against the same buffer without EDTA for 24 hrs at 4°C. The enzyme activity was measured in the standard reaction mixture in the presence of 1 mM metal ion for 1 hr at 50°C.

Table 3. Effect of some reagents on the activity of CGTase from *Paenibacillus* sp. JK-12

Reagents	Concentration (mM)	Relative activity (%)
None		100
Ammonium persulfate	10	121
L-Cysteine hydrochoride	10	19
N-Ethylmaleimide	10	106
β-Mercaptoethanol	10	111
SDS	10	16
NaN_3	10	113
Na_2HAsO_4	10	106
NaF	10	107
Sodium thioglycolate	10	106
Pentachlorophenol	10	103
1,10-Phenanthioline	10	101
Potassium permanganate	10	104
PMSF[1)]	10	10
Tris	10	112

[1)]Phenylmethylsulfonylfluoride.

AA-2G formation was composed of 3% AA, 7% soluble starch and 500 units/mL (pH 5.5), and incubated at 55°C for 48 hrs. To hydrolyze AA-2-oligoglucosides (AA-2Gs) produced by CGTase from *Paenibacillus* sp. JK-12, 20 units of glucoamylase was added to the reaction mixture and incubated at the same temperature for 24 hrs.

Typical chromatograms of the reaction mixtures with *Paenibacillus* sp. JK-12 CGTase are shown in Fig. 6. Peak 2 in Fig. 6a coincided with the authentic AA-2G retention time. AA-2G, in contact to AA and AA-6G, is characterized by its high stability and nonreducibility (6,7). Further evidence was obtained from its HPLC retention behavior before and after heat treatment and ascorbate oxidase-catalyzed oxidation. The compound heated for 30 min in boiling water showed the same retention characteristics, demonstrating its heat stability, as the standard compound, AA-2G (Fig. 6b). After treatment with ascorbate oxidase (5 units) at 25°C for 10 min, the peak of AA-2G remained

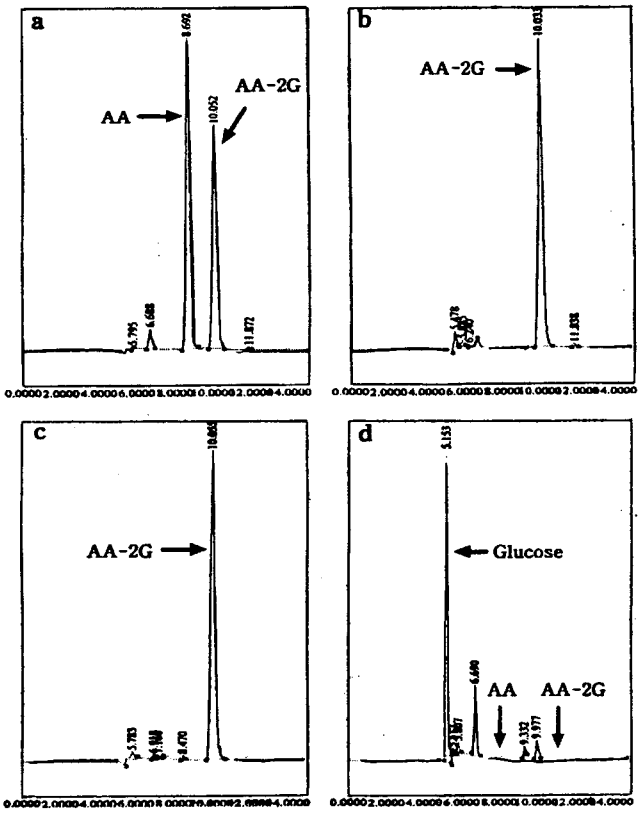

Fig. 6. HPLC chromatograms of the reaction mixture obtained from transglycosylation reaction of CGTase using AA as the glucosyl acceptor and soluble starch as the glucosyl donor. a: reaction product by the CGTase from *Paenibacillus* sp. JK-12, b: heat treatment (100°C, 30 min), c: ascorbate oxidase treatment (5 units, 25°C, 10 min), d: α-glucosidase treatment (1 unit, 37°C, 30 min).

Table 4. Characteristics of bacterial CGTases

Producer	Optimum conditions		Stability		Molecular weight	CD mainly produced	pI	Ref.
	pH	Temp. (°C)	pH	Temp. (°C)				
Paenibacillus sp. JB-13	7.0	45	6.0~9.0	55	66,000	β-CD	5.3	14
***Paenibacillus* sp. JK-12**	**8.0**	**50**	**6.0~9.0**	**55**	**82,000**	**β-CD**	**7.9**	**This study**
Bacillus firmus	6.0	50	6.0~6.5	60	77,000	β-CD	6.2	22
Brevibacterium sp. No 9605	10.0	45	6.0~8.0	50	75,000	γ-CD	2.8	23
Paenibacillus sp. F8	7.5	50	6.0~8.0	50	72,000	β-CD	–	24
B. circulans	6.0	55	6.0~9.5	55	200,000	β-CD	8.5, 8.8	25, 26
B. coagulans	6.0	70	5.5~9.5	70	65,000	β-CD	4.6	27
B. macerans	5.5	55	6.5~8.5	50	68,000	α-CD	4.7	25, 26
B. megaterium	5.0~5.7	55	7.0~10.0	55	66,000	β-CD	6.1, 6.8	25
B. ohbensis	5.5	60	6.5~9.5	55	35,000	β-CD	4.0	26
B. stearothermophilus	6.0	70	7.0~9.2	50	68,000	β-CD	4.5	25, 28
B. subtilis	8.0	65	6.0~8.0	50	64,000	γ-CD	7.1	29
Bacillus sp. AL-6	7.0~10.0	60	5.0~8.0	40	74,000	γ-CD	3~4	30
Thermoanaerobacterium thermosulfurigenes EM1	4.5~7.0	80~85	–	70	68,000	β-CD	–	31
B. ohbensis sp. nov. C-1400	5.0	55	6.5~10.0	55	80,000	β-CD	–	32

completely stable, as shown in the Fig. 6c, whereas AA was completely degraded by the same treatment. Also, AA-2G is easily hydrolyzed to AA and glucose by the action of rice seed α-glucosidase (4,39). Following hydrolysis with α-glucosidase at 37°C for 30 min, the hydrolysate exhibited two new peaks, which were a little faster than AA-2G, and corresponded to glucose and AA, respectively (Fig. 6d). These results provide unequivocal evidence that the compound detected in the reaction mixture is AA-2G.

Formation of cyclodextrins from various substrates

Table 5 shows the yields of α-, β-, and γ-CD from various α-glucans following digestion with CGTase from *Paenibacillus* sp. JK-12. The enzyme produced mainly β-CD, with yields of 0.52~2.11 g/L from various starches, amylopectin and amylose. α-CD was produced in yields of 0.45~1.48 g/L, but no γ-CD was produced. The maximum level of total cyclodextrins (CDs) obtained from soluble starch was 3.59 g/L in the ratio of 1.5 : 2.1 for α: β-CD. *B. subtilis* CGTase digestion produced γ-CD and maltooligosaccharides from soluble starch but not α- or β-CD (29). The CGTases from *Bacillus* sp. AL-6 (30) and *Brevibacterium* sp. No. 9605 (23) produced γ-CD as the main product and β-CD as a minor product from starch, but not α-CD. Digestion with *Paenibacillus* sp. JB-13 CGTase produced mainly β-CD, and a small amount of α-CD, but not γ-CD (14).

Table 6 and 7 show the effects of pH and temperatureon the CDs formation of CGTase. The enzyme produced CDs from soluble starch over a broad pH range from 4 to 10 at 37°C. The maximum yield of CDs was obtained at pH 6.0. The enzyme reaction was performed in 0.01 M buffer (pH 6.0) at various temperatures for 24 hrs. The optimum temperature for CDs formation was 37°C, and was unable to produce CDs at temperatures above 70°C.

Table 5. Substrate specificities for CD formation

Substrates (1%)	CD formed, g/L			Conversion yield (%)
	α-CD	β-CD	Total CD	
Soluble starch	1.48	2.11	3.59	35.9
Corn starch	1.25	0.52	1.77	17.7
Potato starch	0.83	1.27	2.10	21.0
Dextrin	0	0	0	0
Amylose	0.83	1.31	2.14	21.4
Amylopectin	0.45	0.72	1.17	11.7

Table 6. Effect of pH for CD formation

pH	CD formed, g/L			Conversion yield (%)
	α-CD	β-CD	Total CD	
3	–	0.07	0.07	0.7
4	0.03	1.99	2.02	20.2
5	1.89	2.45	4.34	43.4
6	1.69	2.82	4.51	45.1
7	1.21	1.82	3.03	30.3
8	0.91	1.37	2.28	22.8
9	0.86	0.67	1.53	15.3
10	0.44	1.11	1.55	15.5
11	–	1.22	1.22	12.2
12	–	0.02	0.02	0.2

Table 7. Effect of temperature for CD formation

Temperature (°C)	CD formed, g/L			Conversion yield (%)
	α-CD	β-CD	Total CD	
25	1.38	2.14	3.52	35.2
30	1.20	1.75	2.95	29.5
37	1.35	1.89	3.24	32.4
45	0.74	0.40	1.14	11.4
50	0.88	0.58	1.46	14.6
55	0.80	0.27	1.07	10.7
60	0.34	0.20	0.54	5.4
65	0.21	0.16	0.37	3.7
70	0.09	0.06	0.15	1.5

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