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Purification and Properties of Cyclodextrin Glucanotransferase Synthesizing 2-*O*-α-D-Glucopyranosyl L-Ascorbic Acid from *Paenibacillus* sp. JB-13

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Abstract A Gram-positive bacterium (strain JB-13) that was isolated from soil as a producer of cyclodextrin glucanotransferase (CGTase) [EC 2.4.1.19] was identified as *Paenibacillus* sp. JB-13. This CGTase could catalyze the transglucosylation reaction from soluble starch to L-ascorbic acid (AA). A main product formed by this enzyme with α-glucosidase was identified as 2-*O*-α-D-glucopyranosyl L-ascorbic acid (AA-2G) by the HPLC profile and the elemental analysis. CGTase was purified to homogeneity using ammonium sulfate fractionation, ion-exchange chromatography on DEAE-Sephadex A-50, and gel chromatography on Sephacryl S-200HR. The molecular weight was determined to be 66,000 by both gel chromatography and SDS-PAGE. The isoelectric point of the purified enzyme was 5.3. The optimum pH and temperature was pH 7.0 and 45°C, respectively. The enzyme was stable in the range of pH 6–9 and at temperatures of 75°C or less in the presence of 15 mM $CaCl_2$. Hg^{2+}, Mn^{2+}, Ag^{+}, and Cu^{2+} all strongly inhibited the enzyme's activity.

Key words: Cyclodextrin glucanotransferase, 2-*O*-α-D-glucopyranosyl L-ascorbic acid, *Paenibacillus* sp. JB-13

Cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19) has an ability to convert starch into cyclodextrins (CDs) with closed-ring structures in which six or more glucose units are joined by α-1,4 glycosidic bonds [4]. Depending on the number of glucose units (six, seven, or eight), they are named α-, β-, or γ-CDs, respectively. In the presence of suitable acceptors, this enzyme also catalyzes an intermolecular transglycosylation in which glycosyl residues are transferred from CD or maltooligosaccharide to the acceptor [15, 31, 35, 44]. Transglycosylase activity of CGTase, which catalyzes a coupling reaction, has been widely utilized for the purpose of developing many modified compounds [11, 29, 31, 45].

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Recently, we found that CGTase from *Paenibacillus* sp. JB-13 produced 2-*O*-α-D-glucopyranosyl L-ascorbic acid (AA-2G) efficiently and regioselectively. α-Glucosidase and CGTase from microorganisms, animals and plants, rice seed [24], and mammals [25, 41] can catalyze to form AA-2G [1].

AA-2G is a novel vitamin C derivative synthesized from AA and maltose or oligosaccharide by the transglucosylation enzymes such as CGTase [1, 24, 25, 33, 41]. In contrast to AA, the glucose conjugate of AA shows a high resistance to thermal and oxidative degradations in an aqueous solution [1, 24, 25, 38, 39, 42]. The ascorbate moiety in this glucoside is fully protected from oxidation if the bond is not cleaved. However, AA-2G is hydrolyzed to AA and glucose by the action of mammalian α-glucosidase [25] that has biological activities such as AA *in vivo* [42] and in a tissue culture [23]. In addition, AA-2G acts as an effective antiscorbutic vitamin in guinea pig [36, 42] and stimulates collagen synthesis in human skin of fibroblasts *in vitro* [42]. So far, a number of 2-*O*-monosubstituted derivatives of AA, including its sulfate [20, 21], phosphate [21] and methyl ether [17], have been demonstrated to be stable *in vitro* and have shown nonreducing activity. Among them, ascorbic acid 2-*O*-sulfate (AA-2S) [3, 28, 30] and ascorbic acid 2-*O*-methyl ether (AA-2M) [5] were found as naturally occurring metabolites of AA. However, they were devoid of a substantial vitamin C activity in monkeys and guinea pigs [12, 18]. On the other hand, ascorbic acid 2-*O*-phosphate (AA-2P) exerted an antiscorbutic activity in monkeys and guinea pigs [19], although it had not been unequivocally identified as a metabolite of AA in these animals. Moreover, AA-2P did not enhance the synthesis of collagen more than AA-2G in cultured human skin fibroblasts. AA-2P was not persistent because AA was released very rapidly from AA-2P by hydrolysis and the hydrolyzed AA was unstable in the culture medium [13]. However, the rate of hydrolysis of AA-2G in the fibroblasts was more slow. These aspects of AA-

2G provide a further basis for its use as an effective pharmacological agent and/or as a promising food additive. Therefore, to efficiently and abundantly mass produce AA-2G, the first step is the purification and characterization of CGTase. In this study, we describe the purification and enzymatic properties of the CGTase from *Paenibacillus* sp. JB-13 for the production of AA-2G.

MATERIALS AND METHODS

Materials

Soluble starch was purchased from Wako Pure Chemical Industries Co. Ltd. (Osaka). Glucoamylase from *Rhizopus* mold, rice seed α-glucosidase, ascorbate oxidase (ASOD, EC 1.10.3.3), sodium ascorbate, α-CD, β-CD, γ-CD, sodium dodecyl sulfate, ethylenediaminetetraacetic acid, and Sephacryl S-200HR were obtained from Sigma Co. (St. Louis, MO, U.S.A.). DEAE-Sephadex A-50 was obtained from Pharmacia Fine Chemicals. AA-2G was obtained from Professor T. Sakai at Kinki University. All other chemicals were of the analytical grade.

Bacterial Strain and Culture Conditions

The bacterium strain No. 13 with CGTase activity was isolated from soil and used throughout this study. The bacterium was identified as *Paenibacillus* sp. JB-13, as described under Results and Discussion. The medium used for the production of CGTase was composed of 1% soluble starch, 1% yeast extract, 1% Na_2CO_3, 0.1% K_2HPO_4, and 0.02% $MgSO_4 \cdot 7H_2O$. The culture was carried out at pH 7.0 and at 37°C for 48 h.

Assay of CGTase

CGTase activity was determined by the glucoamylase method that was introduced by Yu *et al.* [43], with some modifications. Using sucrose and α-CD as substrates, the coupling activity of CGTase was assayed by measuring the formation of glucose. The reaction mixture containing the enzyme, 5 mM of α-CD, and 25 mM of sucrose in 100 mM of phosphate buffer (pH 7.0) was incubated at 55°C for 1 h. The reaction was stopped by immersion in a boiling water bath for 5 min. Glucoamylase (10 units/ml) in 0.5 M of acetate buffer (pH 4.5) was added. After digestion with glucoamylase (30 min, 55°C), reducing sugar was measured by following the Somogyi-Nelson [27] method. One unit of the enzyme activity was defined as the amount of the enzyme produced as 1.0 μmol of glucose per min under the conditions employed.

Production and Detection of AA-2G

The reaction mixture was composed of 7% soluble starch, 3% sodium ascorbate, and 2,000 units/ml of CGTase in a total volume of 1.5 ml with 100 mM of acetate buffer (pH 6.0), and incubated at 55°C for 24 h. To hydrolyze AA-2-oligosaccharides produced by CGTase, glucoamylase (20 units/ml) was added to the reaction mixture of CGTase and incubated at 55°C, pH 4.5, for 24 h. Assays for AA and AA derivatives were carried out by HPLC. HPLC was performed in a μBondapak C_{18} (3.9×300 mm) column with the mobile phase of 0.1 M potassium phosphate-phosphoric acid (pH 2.0) at a flow rate of 0.5 ml/min.

Purification of CGTase

All operations were carried out at 4°C unless otherwise stated.

Step 1: Ammonium sulfate fractionation. Ammonium sulfate was added to 10% saturation level, and the precipitate was removed by centrifugation at 20,000 ×g for 30 min. Solid ammonium sulfate was further added to the supernatant to 50% saturation. This precipitate was collected by centrifugation and dissolved in 50 mM of Tris-HCl buffer (pH 8.0). The enzyme solution was dialyzed against 500 volumes of the same buffer for 24 h with three changes.

Step 2: DEAE-Sephadex A-50 column chromatography. The enzyme solution was applied to a DEAE-Sephadex A-50 column (1.5×15 cm) equilibrated with 50 mM of Tris-HCl buffer (pH 8.0). After the column was washed with the same buffer, the enzyme was eluted with a linear gradient formed from two buffer solutions containing 0 and 1.0 M of NaCl. The active fractions were pooled and concentrated by ultrafiltration (ZM 50 membrane; Amicon Co.).

Step 3: Sephacryl S-200HR column chromatography. The concentrated enzyme was applied to a Sephacryl S-200HR column (1.5×75 cm) previously equilibrated with 50 mM of potassium phosphate buffer (pH 7.0). Elution was carried out with the same buffer, and the active-peak fractions were pooled and concentrated by the ultrafiltration process. To eliminate any remaining contaminants, chromatography on Sephacryl S-200HR was repeated. The active fractions were combined and concentrated with an ultrafiltration membrane, and then used as the purified enzyme source.

Test for Homogeneity of the Enzyme

Native-polyacrylamide gel electrophoresis (PAGE) was performed using a 7% polyacrylamide gel with a pH 6.8 buffer system. SDS-PAGE with 10% polyacrylamide gel was performed by the procedure described by Laemmli [14]. The protein was stained with 2.5% Coomassie Brilliant Blue G-250.

Determination of Molecular Weight and Isoelectric Point

The molecular weight of CGTase was determined by gel filtration on a Sephacryl S-200HR column (1.5×75 cm). Blue dextran (MW 2,000,000), β-amylase from sweet potato (MW 200,000), alcohol dehydrogenase from yeast

(MW 150,000), bovine serum albumin (MW 66,000) and α-chymotrypsinogen (MW 25,000) were used as standard proteins. The subunit molecular weight was estimated by SDS-PAGE using a 10% polyacrylamide gel. Phosphorylase (MW 97,000), fructose-6-phosphate (MW 84,000), bovine serum albumin (MW 66,000), glutamic dehydrogenase (MW 55,000), ovalbumin (MW 45,000), and glyceraldehyde-3-phosphate dehydrogenase (MW 36,000) were used as standard markers (Sigma Chemical Co.). Isoelectric focusing was conducted with a 7.5% polyacrylamide gel containing 2.4% Ampholine (pH 3.0-10.0) for 5 h at a constant voltage of 200 V.

Kinetic Parameters

Kinetic parameters of the purified enzyme for CD synthesis, coupling, and CD opening were determined. CD synthesis activity was assayed using 0.5-10% (w/v) soluble starch as a substrate by measuring the production of CD by HPLC. Coupling activity of CGTase was assayed by following the standard method of CGTase that was mentioned in Materials and Methods, using 20 mM of sucrose, 1-20 mM α-CD, 1-20 mM β-CD, and 5-100 mM γ-CD. Ring opening activity of CGTase was assayed using α-, β-, and γ-CDs as substrates by measuring the increase in reducing power; the reaction mixture containing the enzyme, 1-35 mM α-, β-, and γ-CDs in 100 mM phosphate buffer (pH 7.0) and 20 units of glucoamylase was incubated at 45°C for 30 min. Reducing sugar was measured by the Somogyi-Nelson method [27]. Experimental data were fitted to a nonlinear least-squares program and also represented as Linweaver-Burk plots to yield K_m and V_{max} values.

Determination of Protein

The concentration of protein was determined by the method of Lowry [16] using bovine serum albumin as the standard. The protein concentration of column fractions was determined by measuring the absorbance at 280 nm.

Analysis of N-Terminal Amino Acid Sequence

The N-terminal sequence of the protein was determined using the automated Edman degradation with Perkinelmer Procise (U.S.A). The amino acid sequences were compared with others in the NCBI database by using the Blast program.

Results and Discussion

Identification of the Strain JB-13

The strain used in this work was a Gram positive, rod shaped, endospore-forming bacteria, catalase positive, and motility positive, but gas was not produced from glucose. Utilizations of glucose, sucrose, xylose, and mannitol were positive, but utilizations of inositol, arabinose, and inulin were negative. The strain hydrolyzed starch, gelatin, and casein and grew at pH 6.8 and 7% NaCl in nutrient broth as shown in Table 1. The TEM photo indicated a rod shape, as shown in Fig. 1. According to its morphological, cultural, and biochemical characteristics, the strain showed similarity to the *Bacillus* group, but a 90% similarity to *Paenibacillus campinasensis* was found by partial 16S rDNA sequence comparisons (data not shown). With these results and reference to *Bergey's Manual of Determinative Bacteriology*, 9th Edition and *Bergey's Manual of Systematic Bacteriology* Vol. 2, the strain used in this work was identified and tentatively named as *Paenibacillus* sp. JB-13.

Table 1. Taxonomical characteristics of isolated strain JB-13.

Contents			
Morphological characteristics			
Cell shape	Rod		
Cell size (μm)	0.5-1.0×1.5-2.2		
Gram stain	Positive		
Spore formation	Endospore forming		
Mortility	Mortile		
Cultural characteristics			
Colony shape	Round, convex		
Colony surface	Smooth to rough		
Colony color	Ivory		
Colony opacity	Opaque		
Biochemical characteristics		*Nutritional characteristics*	
Aerobic/anaerobic growth	+/-	Sucrose	+
Catalase	+	Glucose	+
Indole test	-	Inositol	-
Voges-Proskauer test	-	Inulin	-
Hydrolysis		Melibiose	+
Casein	+	Mannitol	+
Gelatin	+	Mannose	+
Starch	+	Maltose	+
Gas from Glucose	-	Galactose	-
Growth at pH		Lactose	+
6.8, nutrient broth	+	Raffinose	+
5.7, nutrient broth	-	Arabinose	-
Growth in NaCl		Xylose	+
2%	+	Fructose	+
5%	+	Trehalose	-
7%	+	Sorbitol	-
10%	-		

AA-2G Production with CGTase

AA-2G production catalyzed by CGTase was carried out at pH 6.0 and 55°C using soluble starch as a substrate. Figure 2 shows a typical HPLC profile. A peak of AA-2G was observed just after a peak of AA (Fig. 2-B), by comparing the retention time of authentic AA-2G. The AA-2G, heated for 30 min in boiling water, showed the same peak height to that of AA-2G without the heat treatment, which

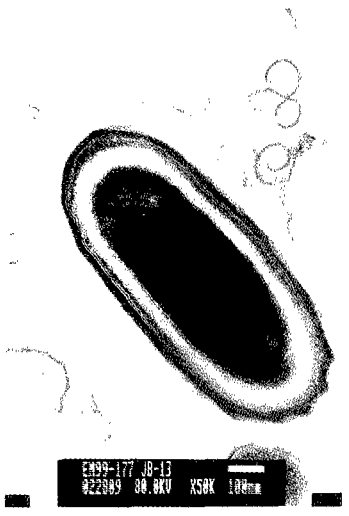

Fig. 1. Transmission electron microscope of the isolated strain, JB-13.

indicates heat stability of AA-2G (Fig. 2C). When the sample was treated with 5 units of ASOD at 25°C for 10 min, it was completely resistant to oxidation (Fig. 2D). Therefore, AA-2G is stable to ASOD-catalyzed oxidation, as reported earlier [25], while AA is susceptible to the enzymatic oxidation in water under aerobic condition. As AA-2G can be effectively hydrolyzed *in vitro* by rice seed α-glucosidase [24], the HPLC retention patterns before and after α-glucosidase hydrolysis of the sample were examined. After the treatment of the sample with 1 unit of rice seed α-glucosidase in 0.02 M of acetate buffer (pH 5.5) at 37°C for 30 min, two peaks corresponding to AA and glucose were produced, while the peak corresponding to AA-2G almost disappeared (Fig. 2E). Furthermore, after a mild acid hydrolysis with 1 N HCl at 100°C for 3 min, the peak height of AA increased, as shown in Fig. 2F, thus identifying the compound produced by *Paenibacillus* sp. JB-13 to be AA-2G by CGTase.

Purification of the Enzyme

The purification of the enzyme is summarized in Table 2. The enzyme was purified to homogeneity from the cultural supernatant of *Paenibacillus* sp. JB-13 by ammonium sulfate fractionation, DEAE-Sephadex A-50, and Sephacryl

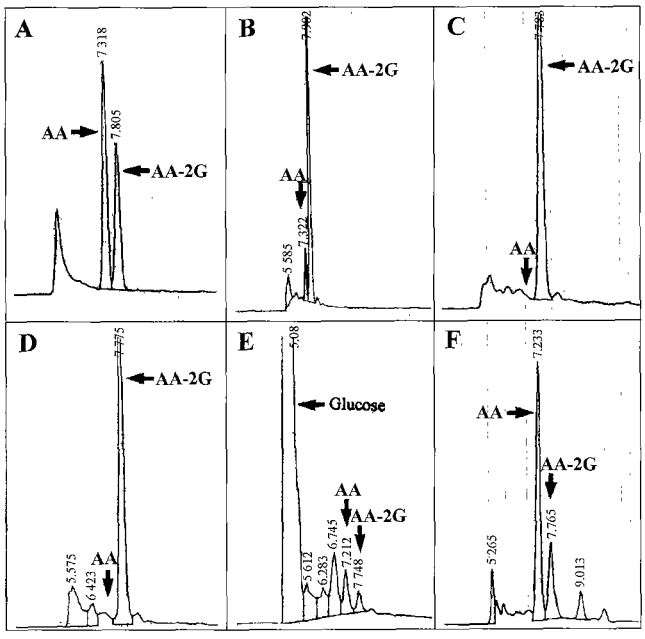

Fig. 2. HPLC profiles of transglucosylation products by CGTase from *Paenibacillus* sp. JB-13.

The reaction mixture consisting of 3% AA, 7% soluble starch, and 2,000 units/ml of CGTase was incubated at 55°C, pH 6.0, for 24 h. AA-2-oligosaccharides were hydrolyzed by 20 units/ml of glucoamylase. A. AA and AA-2G standard; B. Reaction product; C. Heat treatment (100°C, 30 min); D. Ascorbate oxidase treatment (25°C, 10 min); E. α-Glucosidase treatment (37°C, pH 5.5, 30 min); F. 1 N HCl treatment (100°C, 3 min).

S-200HR column chromatographies. The elution patterns on the DEAE-Sephadex A-50 and Sephacryl S-200HR column chromatographies are shown in Figs. 3 and 4, respectively. The elution profile on the final 2nd Sephacryl S-200HR column chromatography is shown in Fig. 5. The purified enzyme was homogeneous, evidenced by a single band on SDS/PAGE and native PAGE (Fig. 6) with a molecular weight of 66,000. By these purification procedures, the enzyme was purified 305-fold with a yield of 14.5%.

N-Terminal Amino Acid Sequence

The N-terminal amino acid sequence of the purified JB-13 CGTase was compared to those of CGTase from various sources, as shown in Fig. 7. The sequence of JB-13

Table 2. Purification of the CGTase from *Paenibacillus* sp. JB-13.

Step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Fold
Crude extract	347,050.00	9,040.00	38.39	100.00	1.00
$(NH_4)_2SO_4$ fractionation (10-50%)	252,959.29	312.96	808.28	72.89	21.05
DEAE-Sephadex A-50	239,272.55	186.19	1,285.10	68.94	33.47
1st Sephacryl S-200HR	76,464.37	8.77	8,718.86	22.03	227.11
2st Sephacryl S-200HR	50,638.92	4.32	11,721.97	14.59	305.34

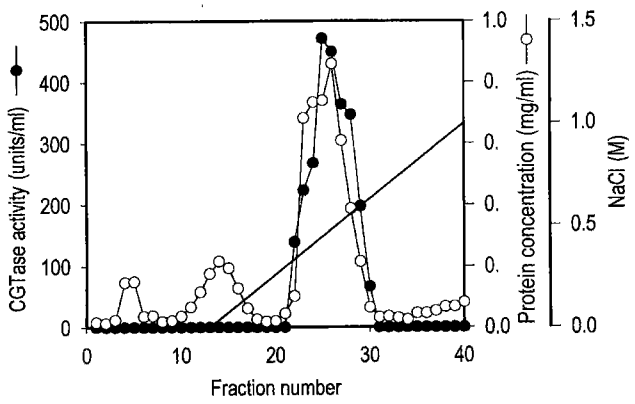

Fig. 3. DEAE-Sephadex A-50 column chromatography of CGTase from *Paenibacillus* sp. JB-13.
Column size 1.5×15 cm; equilibrated with 50 mM Tris/HCl buffer (pH 5.0); eluted with 0–1.0 M NaCl linear gradient in the same buffer; fraction volume 7 ml; flow rate 0.6 ml/min.

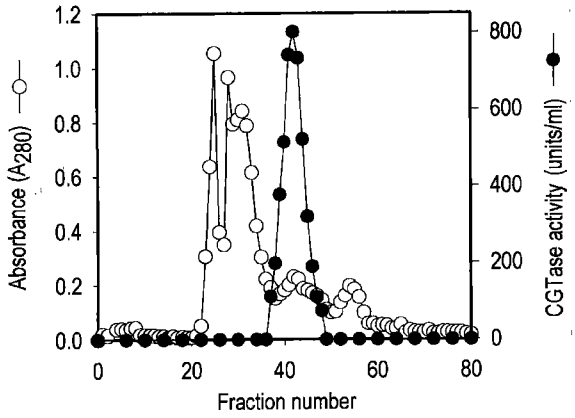

Fig. 4. Sephacryl S-200HR column chromatography of CGTase from *Paenibacillus* sp. JB-13.
Column size 1.5×75 cm; equilibrated with 50 mM potassium phosphate buffer (pH 5.0); eluted with the same buffer; fraction volume 2 ml; flow rate 0.25 ml/min.

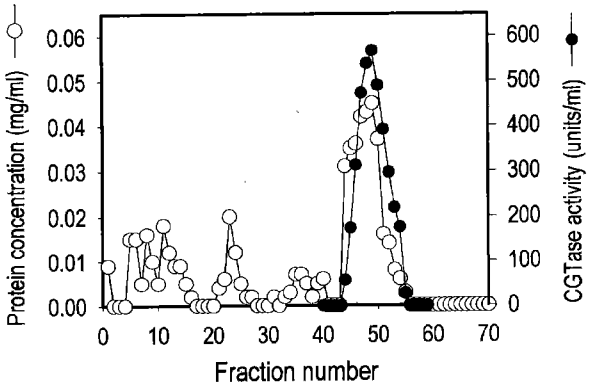

Fig. 5. 2nd-Sepharcryl S-200HR column chromatography of the partially purified CGTase from *Paenibacillus* sp. JB-13.
Column size 1.5×7.5 cm; equilibrated with 50 mM potassium phosphate buffer (pH 7.0); eluted with the same buffer; fraction volume 2 ml; flow rate 0.25 ml/min.

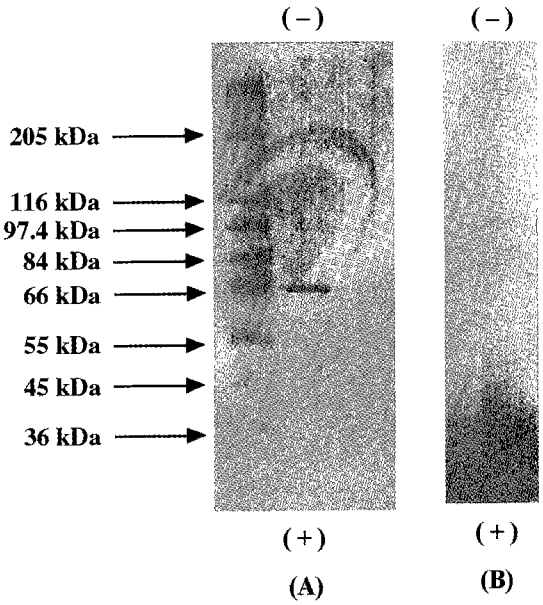

Fig. 6. Electrophoretic analysis of the purified CGTase from *Paenibacillus* sp. JB-13.
(A) SDS-PAGE (10% gel); (B) Native-PAGE (7% gel).

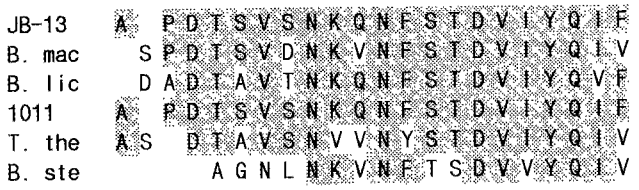

Fig. 7. Comparison of the N-terminal sequences between JB-13 CGTase and other CGTases.
The highly homologous regions are shaded. Abbreviations: JB-13, *Paenibacillus* sp. JB-13; B. mac, *Bacillus macerans* [31]; B. lic, *B. licheniformis* [33]; 1011, alkalophilic *Bacillus* sp. 1011 [31]; T. the, *Thermoanaerobacterium thermosulfurigenes* [33]; B. ste, *B. stearothermophilus* [33].

CGTase showed a significant similarity to those of known CGTases, suggesting that archaeal CGTase has an evolutionary relationship to bacterial CGTase.

Effect of pH on Activity and Stability of CGTase

The optimum pH for CGTase was determined by using acetate buffer (pH 3 to 5), potassium phosphate buffer (pH 5 to 8), glycine-NaOH buffer (pH 8 to 10), and Na_2HPO_4-NaOH buffer (pH 10 to 12) under the standard assay conditions. As shown in Fig. 8A, the enzyme was the most active at pH 7.0. The effects of pH on the stability of the enzyme are shown in Fig. 8B. After being incubated at various pHs at 37°C for 1 h, the residual activity was assayed at pH 7.0. The enzyme was stable between pH 6 and 9.

Effect of Temperature on Activity and Stability

The effect of temperature on enzyme activity was evaluated by the standard assay using the temperature range of 25–

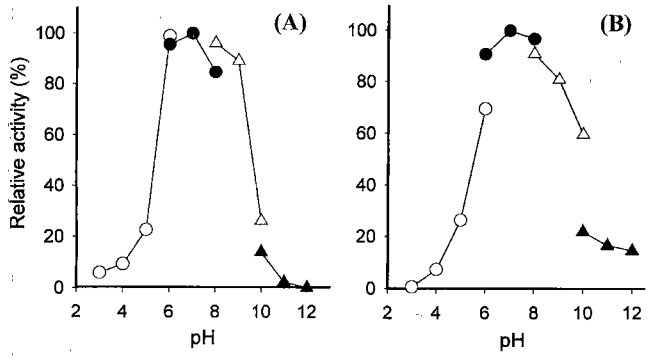

Fig. 8. Effects of pH on activity and stability of CGTase purified from *Paenibacillus* sp. JB-13.
(A) Effect of pH on activity; (B) Effect of pH on stability; the enzyme in 100 mM buffer having various pH values was kept for 1 h at 37°C. The experimental details are described in the text. The values are shown as relative activity. The buffer system used for the different pH ranges were as follows; △: Acetate buffer; ▲: Potassium phosphate buffer; ○: Glycine-NaOH buffer; ●: Na_2HPO_4-NaOH buffer.

80°C. The optimum temperature was 45°C in the absence or presence of 15 mM $CaCl_2$ (Fig. 9A). To check the thermal stability, the enzyme (in 100 mM phosphate buffer, pH 7.0) was incubated at various temperatures for 30 min, and the remaining activity was assayed by the standard method. The enzyme was stable at 55°C without 15 mM of $CaCl_2$ and at 75°C in the presence of 15 mM $CaCl_2$ (Fig. 9B).

The addition of Ca^{2+} ions increased the heat stability of *Paenibacillus* sp. JB-13 CGTase. It was reported that the presence of substrate, product, or calcium ions enhanced the stability of CGTases [6]. Activities of CGTases from *Brevibacterium* [22] and *Bacillus coagulans* [2] were also reported to increase in the presence of calcium ions, however, no effect of calcium ions on the temperature stability of CGTase from *B. firmus* was reported [8].

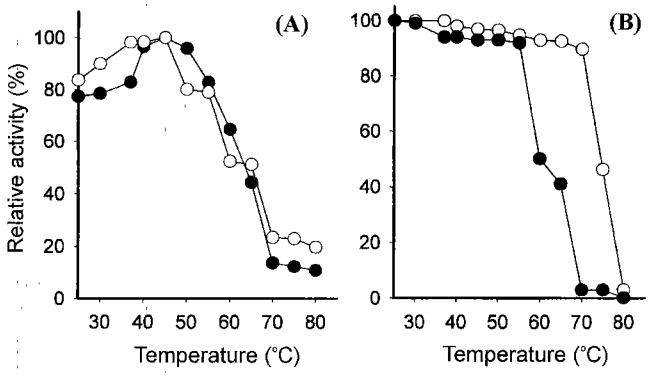

Fig. 9. Effect of temperature on activity and stability of the CGTase from *Paenibacillus* sp. JB-13.
(A) Effect of temperature on activity in the presence (○) or absence (●) of 15 mM $CaCl_2$. (B) Effect of temperature on stability in the presence or absence of $CaCl_2$; The enzyme in 100 mM phosphate buffer (pH 7.0) containing 15 mM $CaCl_2$ (○) or control (●) was incubated at various temperatures for 30 min. The values were shown as relative activity.

Table 3. Effect of metal ions on the activity of CGTase from *Paenibacillus* sp. JB-13.

Metal ions	Relative activity (%)		
	0.1 mM	1.0 mM	10 mM
$MnCl_2$	85.7	68.2	27.1
$FeSO_4$	97.7	87.2	67.4
KCl	102.6	100.4	96.2
$MgCl_2$	103.6	97.9	143.1
$CdCl_2$	101.1	106.2	93.0
$BaCl_2$	99.8	113.9	117.1
$HgCl_2$	102.6	100.2	0
$ZnCl_2$	101.9	105.8	66.1
$AgNO_3$	97.4	83.8	14.9
$CoCl_2$	86.6	97.6	146.1
$CuSO_4$	98.1	98.7	4.7
$CaCl_2$	107.0	114.7	171.4
$NiCl_2$	106.4	99.4	155.0
None	100.0	100.0	100.0

The enzyme was dialyzed against 100 mM potassium phosphate buffer, pH 7.0, containing 20 mM EDTA, for 24 h with several changes, and then further dialyzed against 100 mM potassium phosphate buffer (pH 7.0), for 24 h with several changes. The enzyme was preincubated with various metal ions for 30 min at 37°C. After incubation, the mixture was subjected to CGTase assay.

Effect of Metal Ions and Inhibitors

The enzyme was incubated with various metal ions in 100 mM of potassium phosphate buffer (pH 7.0) at 37°C for 30 min, and the remaining activity was measured under the standard assay conditions. As shown in Table 3, Mg^{2+}, Ba^{2+}, Co^{2+}, Ca^{2+}, and Ni^{2+} promoted the enzyme activity. The enzyme activity

Table 4. Effect of inhibitors on the activity of CGTase from *Paenibacillus* sp. JB-13.

Reagents	Relative activity (%)		
	0.1 mM	1.0 mM	10 mM
None	100	100	100
Ammonium persulfate	88	97	53
L-Cysteine hydrochloride	102	104	65
N-Ethylmaleimide	107	103	80
Ethylenediaminetetraacetic acid	104	86	75
β-Mercaptoethanol	99	103	87
Sodium dodecyl sulfate	95	83	10
NaN_3	96	98	66
NaCN	104	102	83
Na_2HAsO_4	98	98	82
NaF	100	97	83
Sodium thioglycolate	98	100	82
Pentachlorophenol	110	101	78
Phenanthroline	108	104	77
Potassium permanganate	73	0	0
PMSF	98	97	83

The enzyme was preincubated with various inhibitors for 30 min at 37°C. After incubation, the mixture was subjected to CGTase assay.

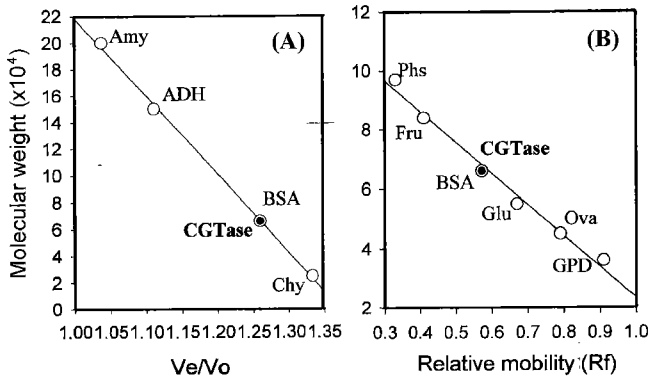

Fig. 10. Determination of molecular weight of CGTase by Sephacryl S-200HR gel filtration (A) and SDS-PAGE (B).
In (A), the standards are β-Amylase (Amy), MW 200,000; Alcohol dehydrogenase (ADH), MW 150,000; bovine serum albumin (BSA), MW 66,000; α-Chymotrypsinogen A (Chy), 25,000; Void volume (Vo), Blue dextran (MW 2,000,000); Elution volume (Ve), Sample elution volume. In (B) the standards are phosphorylase (Phs), MW 97,000; Fructose-6-phosphate (Fru), MW 84,000; Bovine serum albumin (BSA), MW 66,000; Glutamic dehydrogenase (Glu), MW 55,000; Ovalbumin (Ova), MW 45,000; Glyceraldehyde-3-phosphate dehydrogenase (GPD), MW 36,000.

was strongly inhibited by metal ions such as Mn^{2+}, Hg^{2+}, Ag^{+}, and Cu^{2+}, but was relatively stable to K^{+} and Cd^{2+}. Unlike CGTase of *Paenibacillus* sp. JB-13 and *B. autolyticus* 11149 [34], *Bacillus* sp. AL-6 [7] was relatively stable to Hg^{2+}.

The enzyme solution was mixed with 100 mM potassium phosphate buffer (pH 7.0) containing inhibitors and preincubated at 37°C for 30 min, and the substrates (CD and sucrose) were then added. As shown in Table 4, the enzyme activity was strongly inhibited by SDS and potassium permanganate, but it was only moderately inhibited by ammonium persulfate, L-cysteine hydrochloride, and NaN_3.

Molecular Weight and Isoelectric Point

The molecular weight of the CGTase was estimated to be 66,000 by Sephacryl S-200HR column chromatography (Fig. 10A). The molecular weight was also determined to be 66,000 by SDS-PAGE (Fig. 10B). These results indicate that the CGTase is a monomeric enzyme. The reported CGTases had similar molecular weights as those from *B. macerans* [37], *B. megaterium* [9], *B. coagulans* [2], and *B. stearothermophilus* [10].

The isoelectric point of the enzyme preparation was determined by the isoelectric focusing method and found to be pH 5.3 (data not shown), which was different from the optimum pH of 4.5 as reported by Kitahata [9, 10]. However, Nakamura [26] found an isoelectric point of pH 5.4 for the CGTase in their alkalophilic *Bacillus* sp.

K_m

The Michaelis constants for various substrates were determined by the Lineweaver-Burk plot using the reciprocals of the reaction velocity and substrate concentrations. Table 5 summarizes the Michaelis constants and maximum velocities for the reactions. The K_m and V_{max} values with soluble starch as a substrate were 55.9 g/l and 0.27 mg/ml/min, respectively. In the coupling activity, the K_m value of this enzyme for α-CD was similar to that for β-CD, but was about 13 times smaller than that for γ-CD. In the ring opening, the K_m value of CGTase for γ-CD was also larger than those for α-CD and β-CD.

Table 5. Kinetic parameters of the CGTase from *Paenibacillus* sp. JB-13 on various substrates.

Reaction		K_m	V_{max}
CD synthesis	Soluble starch	55.9 g/l	0.27 mg/ml/min
Coupling	α-CD	2.16 mM	196.1 unit/ml/min
	β-CD	2.05 mM	89.3 unit/ml/min
	γ-CD	28.95 mM	526.3 unit/ml/min
CD opening	α-CD	13.1 mM	434.8 unit/ml/min
	β-CD	11.1 mM	227.3 unit/ml/min
	γ-CD	23.57 mM	1,428.6 unit/ml/min

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