Biosci. Biotechnol. Biochem., **67** (2), 334–340, 2003

Isolation of *Paenibacillus illinoisensis* That Produces Cyclodextrin Glucanotransferase Resistant to Organic Solvents

Noriyuki DOUKYU,† Hirokazu KUWAHARA, and Rikizo AONO

Department of Biological Information, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Nagatsuta 4259, Midori-ku, Yokohama 226-8501, Japan

Received August 13, 2002; Accepted September 26, 2002

A bacterium that secreted cyclodextrin glucanotransferase (CGTase) in a medium overlaid with *n*-hexane was isolated and identified as *Paenibacillus illinoisensis* strain ST-12 K. The CGTase of the strain was purified from the culture supernatant. The molecular mass was 70 kDa. The enzyme was stable at pH 6 to 10 and active at pH 5.0 to 8.0. The optimum temperature at pH 7.0 was 65°C in the presence of 5 mM $CaCl_2$. The enzyme produced mainly β-cyclodextrin. The total yield of α-, β-, and γ- cyclodextrins was increased 1.4-fold by the addition of ethanol. In particular, the yield of β-cyclodextrins in the presence of 10% (vol/vol) ethanol was 1.6-fold that without ethanol. The CGTase was stable and active in the presence of large amounts of various organic solvents.

Key words: cyclodextrin glucanotransferase; cyclodextrin; organic solvent; organic-solvent-tolerant; *Paenibacillus illinoisensis*

Cyclodextrin glucanotransferase (EC 2.4.1.19; CGTase) produces cyclodextrins from starch via intramolecular transglycosylation. Cyclodextrins are cyclic ring structures consisting of six, seven, or eight glucose residues joined by α-1-4 linkages (α-, β-, and γ-cyclodextrin, respectively). Cyclodextrins have been widely used in the fields of food, pharmaceuticals, agricultural chemistry, cosmetics, and perfumes because of their ability to form inclusion complexes with a wide variety of chemicals by partially encapsulating them into their cavities.[1] Several attempts have been made to improve the product selectivity of CGTase by the addition of organic solvents.[2,3] CGTase from *Bacillus circulans* strain 251 produces α-, β-, and γ-cyclodextrins in the ratio of 9:82:9 with the addition of *t*-butanol, although the product ratio is 15:65:20 without the solvent.[3] The addition of organic solvents can increase the overall yield of cyclodextrin production.[3,4] A CGTase from *Bacillus* sp. strain BE101 produced twice as much cyclodextrin after the addition of ethanol.[4] In addition, CGTase is used for the transglycosylation of water-insoluble flavonoids in the presence of organic solvent.[5] However, organic solvents often affect enzyme stability and activity.[6,7] Some times, highly polar organic solvents inactivate CGTases.[4,5] Enzymes with stability and activity in the presence of organic solvents would be useful for technological applications in which such solvents are used. Organic-solvent-resistant microorganisms are useful for the screening for extracellular enzymes in the presence of organic solvents. An organic solvent-stable lipase has been found in a cyclohexane-tolerant microorganism.[8] We have reported that cyclohexane-tolerant *Burkholderia cepacia* strain ST-200 produces cholesterol oxidase that resists organic solvents, detergents, and heat; the enzyme is secreted into the culture supernatant.[9,10] In this study, we isolated a microorganism that produced CGTase in a medium overlaid with *n*-hexane, and purified the CGTase to examine its stability and activity in the presence of various organic solvents.

Materials and Methods

Media. An organism that produced CGTase was screened for with a screening medium consisting of 1% potato starch (Wako Pure Chemical Industries, Osaka, Japan), 1% Bacto Tryptone (Difco Laboratories, Detroit, MI), 0.5% Bacto Yeast Extract (Difco), 1% NaCl, and 10 mM $MgSO_4$. When necessary, the medium was solidified with 1.5% (wt/vol) agar. LBMg medium, consisting of 1% Bacto Tryptone (Difco), 0.5% Bacto Yeast Extract, 1% NaCl, and 10 mM $MgSO_4$, was used at times to culture isolates.

Isolation of an organism that produced CGTase on a medium overlaid with n*-hexane.* Soil samples were gathered from several locations in the Kanto area in Japan. A small amount of soil sample was suspended in sterile 0.8% NaCl, and a portion of the suspension was spread on a screening-medium agar plate. The surface of the agar was overlaid with *n*-hexane to a

† To whom correspondence should be addressed. Fax: +81-45-924-5819; E-mail: ndoukyu@bio.titech.ac.jp

Abbreviations: CGTase, cyclodextrin glucanotransferase; DNS, 3,5-dinitrosalicylic acid; LB medium, Luria-Bertani medium

thickness of 3 to 5 mm. The plates were warmed at 30°C for 4 days in a tightly closed vessel. After incubation, the *n*-hexane was removed and 0.02% I_2 in 0.2% KI solution (about 10 ml) was poured onto the agar surface. Microorganisms that formed halos around their colonies were isolated by repeated single-colony isolation on the screening-medium agar. The microorganisms were grown at 30°C for 48 h in the screening medium, and then the culture was centrifuged (6,000 × *g*, 15 min, 4°C). The starch-hydrolyzing activity in the supernatants was measured by the two methods described below. Microorganisms that hydrolyzed starch but did not generate reducing sugars were selected as CGTase producers. Cyclodextrin production by the culture supernatants of the CGTase producers were examined by TLC as described below. Microorganisms that produced cyclodextrin from starch were selected. The enzyme stability in the presence of an organic solvent was examined to select an organic-solvent-tolerant enzyme. Seventy-five microliters of chloroform or ethanol was added to 300 μl of CGTase solution (30 μl of the culture supernatant and 300 μl of 100 mM sodium phosphate, pH 7.0). The mixture was shaken at 30°C for 12 h. The solution was diluted 4-fold with the sodium phosphate buffer and the activity was measured by the iodine method described below. Thermal stability of the enzyme also was examined. The residual activity at pH 7.0 was measured after incubation for 1 h at 40°C or 50°C by the iodine method.

Assays of starch-degrading activity. In one test, the starch-hydrolyzing activity was assayed with soluble starch as the substrate by measurement of changes in iodine staining. A 20-μl portion of enzyme solution was added to 300 μl of a reaction mixture consisting of 0.5% soluble starch (Wako Pure Chemicals) and 100 mM sodium phosphate buffer (pH 7.0). This mixture was incubated at 30°C for 10 min. The reaction was stopped by the addition of 0.5 ml of 5 M acetic acid. Then, 0.5 ml of 0.02% I_2 in 0.2% KI solution was added to this mixture, and the absorbance of the final mixture was measured at 660 nm. One unit of enzyme activity was defined as the amount of enzyme that reduced the intensity of A_{660} of amylose-iodine complexes by 10% per minute under the conditions described.

In the other assay of starch-hydrolyzing activity, changes in the concentration of reducing sugars during incubation with starch was measured by the 3,5-dinitrosalicylic acid (DNS) method.[11] The reaction mixture used was the same as above. This mixture was incubated at 30°C for 15 min. One unit of enzyme activity was defined as the amount of enzyme forming reducing sugars corresponding to 1 μmol of glucose per minute in the 3,5-dinitrosalicylic acid reaction.

Purification of CGTase. Strain ST-12 K was grown at 30°C for 14 h in LBMg medium containing 0.2% soluble starch, and the culture was centrifuged (6,000 × *g*, 15 min, 4°C). Proteins in the supernatant were precipitated with $(NH_4)_2SO_4$ (70% saturation) at 4°C overnight. The precipitate was recovered by centrifugation (15,000 × *g*, 30 min, 4°C) and dissolved in 10 mM Tris-HCl (pH 8.0). This solution was dialyzed against the same buffer at 4°C and put on a column (2.5 by 10 cm) of DEAE-cellulose DE53 (Whatman, Maidstone, England) equilibrated with the Tris-HCl buffer. The column was washed with 100 ml of the Tris buffer at the flow rate of 0.5 ml/min and then eluted with a linear gradient of NaCl concentrations of 0 to 50 mM in 300 ml of the Tris buffer at the flow rate of 0.5 ml/min. The fractions with starch-hydrolyzing activity were pooled. After the addition of $(NH_4)_2SO_4$ to 30% saturation, the solution was put on a column (2.5 by 20 cm) of butyl-Toyopearl 650M (Tosoh, Tokyo, Japan) equilibrated with 30% saturated $(NH_4)_2SO_4$-10 mM Tris-HCl (pH 8.0). The column was washed with 150 ml of the Tris buffer containing $(NH_4)_2SO_4$ (30% saturation) and then eluted with a decreasing linear gradient of $(NH_4)_2SO_4$ concentrations of 30% saturation to 0% in 300 ml of the Tris buffer at the flow rate of 0.5 ml/min. The enzyme solution was dialyzed twice against the 10 mM Tris-HCl buffer (pH 8.0) at 4°C and kept at 4°C until being used.

Measurement of protein concentration. Protein concentration was measured by the method of Bradford[12] with bovine serum albumin as the standard.

Zymograms. SDS-PAGE was done on a 10% (wt/vol) polyacrylamide gel by the method of Laemmli[13] with slight modifications. Soluble starch was added to the polyacrylamide gel to a final concentration of 1% (wt/vol). After the electrophoresis, the gel was washed with the Tris-HCl buffer containing 25% (vol/vol) isopropanol. Then the gel was washed with 100 mM Tris-HCl buffer (pH 8.0) and incubated in the same buffer for 12 h at 30°C. A zone at which starch was hydrolyzed was stained by soaking of the gel in a solution of 0.2% I_2-2% (wt/vol) KI.

TLC. After the enzymatic reaction described above with 0.5% soluble starch, 1 μl of the reaction mixture was spotted onto a 0.2-mm-thick silica gel 60 plate (Merck, Darmstadt, Germany) and the sample was developed with a solvent mixture of acetic acid-ethyl acetate-water (2:2:1, by vol). Glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose were used as the standards for starch-hydrolyzed products. Cyclodextrins also were standards. The oligosaccharides were detected by spraying of 1% (wt/vol) orcinol and 50%

(vol/vol) sulfuric acid on the TLC plate, which was then heated at 120°C for 10 min. Cyclodextrins were detected by being sprayed with 1% I_2 in methanol.

Effects of pH on enzyme activity and stability. The enzyme activity was assayed at 30°C at various pHs with 100 mM citric acid-NaH_2PO_4 (pH 3.0–5.5), CH_3COOH–CH_3COONa (pH 4.0–5.5), KH_2PO_4–Na_2HPO_4 (pH 5.5–7.5), Tris-HCl (pH 7.5–9.0), Na_2CO_3–$NaHCO_3$ (pH 9.0–11.0), and NaCl–NaOH (pH 11.0–13.0). Enzyme stability was measured at 30°C after 1 h of incubation at the various pHs just listed.

Measurement of cyclodextrin. A sample was analyzed with an NH_2-1251-N (4.6 by 250 mm) column (Senshu Science Co., Tokyo) attached to an HPLC apparatus. The column was eluted with acetonitrile-water (6:4, vol/vol) at the flow rate of 0.5 ml/min. The elution was monitored by an refractive index detector (RID-300, Japan Spectroscopic Co., Ltd.).

Commercial CGTase preparations. CGTase from *Bacillus macerans* was the product of Amano Enzyme, Inc. (Nagoya, Japan).

Results

Isolation of a microorganism that produced CGTase tolerant of organic solvents

First, we screened for microorganisms that produced starch-hydrolyzing enzymes on the screening-medium agar overlaid with cyclohexane. Although we examined about 500 soil samples, CGTase-producing microorganisms were not found. Next, we screened for the microorganisms on a screening-medium plate overlaid with *n*-hexane, which is less toxic than cyclohexane. From about 400 soil samples, 452 strains formed halos around their colonies. Of the strains, 38 strains had strong starch-hydrolyzing activity in the culture supernatant by iodine method. Of the 38 strains, the culture supernatants of 12 strains did not contain a detectable starch-hydrolytic activity generating reducing sugars by the DNS method. These 12 strains were selected as CGTase producers. Cyclodextrins were detected as the major products yielded by the culture supernatants of the 12 strains. Of the 12 selected strains, strain ST-12 K produced the CGTase with the highest thermal stability. This enzyme was more stable in the presence of chloroform and ethanol than the enzymes of the other 11 strains. Consequently, strain ST-12 K was selected as a producer of a CGTase tolerant of organic solvents.

Characterization and identification of strain ST-12 K

We analyzed a partial sequence of the 16S rDNA gene sequence of ST-12 K as described by Lane.[14] The 1480 bp sequences, corresponding to 96% of the complete sequence, was similar to the 16S rDNA sequences of *Paenibacillus* species as follows: 99%, *P. illinoisensis* (DDBJ/EMBL/GenBank accession no. D85397); 96%, *P. amylolyticus* (no. D85396); and 94% *P. borealis* (no. AJ011322). The microbiological and biochemical characteristics of strain ST-12 K were examined by methods reported for the classification of species of *Paenibacillus*.[15] Cells of strain ST-12 K were rods measuring 0.8 to 1.0 by 3.0 to 4.0 μm, they were Gram positive, motile, and spore-forming. The colonies were flat, smooth, circular, entire, and yellowish gray. No soluble pigment was produced on a nutrient agar. Strain ST-12 K had positive catalase test results but negative oxidase test results. Gelatin and starch were hydrolyzed and casein was weakly hydrolyzed. Acetate was used, but citrate, lactate, succinate, L-malate, and L-glutamate were not. Strain ST-12 K grew at 40°C, pH 5.7, or in the presence of 2% NaCl. Growth was inhibited at 4°C or in the presence of 5% NaCl. Acid was produced from L-arabinose, glycerol, D-mannose, and raffinose, but not from lactose or L-rhamnose. These microbiological characteristics were identical to those of *P. illinoisensis*.

Strain ST-12 K grew on LBMg medium overlaid with a 10% volume of dodecane (log P_{ow} 7.0), decane (log P_{ow} 6.0), nonane (log P_{ow} 5.5), *n*-octane (log P_{ow} 4.9), or diphenyl ether (log P_{ow} 4.2), but not in that overlaid with cyclohexane (log P_{ow} 3.4), or *p*-xylene (log P_{ow} 3.1). The growth was weak on the LBMg medium in the presence of *n*-hexane (log P_{ow} 3.9).

Purification of CGTase

The activity in the presence of dodecane, decane, and nonane was similar to that without any solvent. The activity in the presence of *n*-octane, diphenyl ether, or *n*-hexane was one tenth that without a solvent. Therefore, the CGTase was purified from the culture supernatant of ST-12 K grown on the medium without any solvent. Table 1 summarizes the purification steps used. The starch-hydrolyzing activity detected by the iodine method was eluted from the DE53 column with NaCl at 15 mM and from the column of butyl-Toyopearl 650M with $(NH_4)_2SO_4$ at 10% saturation. In the end, the CGTase was purified 4.5-fold from the culture supernatant. The purified CGTase had a specific activity of 1200 U/mg of protein. The purified preparation gave a single band by SDS-PAGE (Fig. 1). The molecular mass was estimated to be 70 kDa. A 70-kDa protein from the culture supernatant was seen also on the zymogram when ST-12 K grew on a medium with dodecane, decane, and nonane (not shown).

Effects of pH and temperature

The CGTase was active at pH 5.0 to 9.0 and most active at pH 5.0 to 8.0. The enzyme was stable at

Table 1. Purification of CGTase from Strain ST-12 K

Step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Culture supernatant	3,520	79.5	21,000	264	1	100
$(NH_4)_2SO_4$ precipitation	64	54.1	16,900	312	1.2	80
DEAE-cellulose chromatography	95	9.15	9,760	1,070	4.1	46
Butyl-Toyopearl chromatography	72	4.75	5,720	1,200	4.5	27

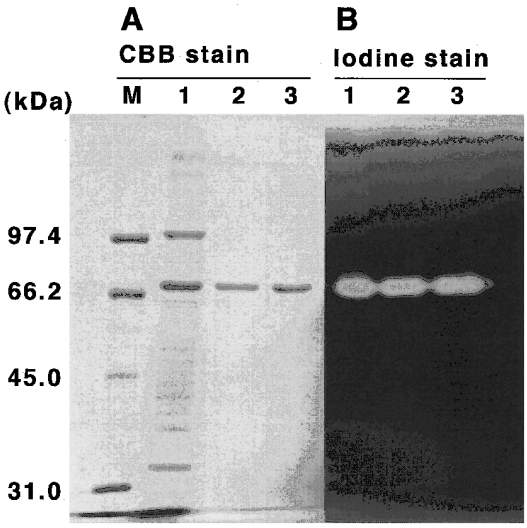

Fig. 1. SDS-PAGE of Fractions Obtained during Purification of the CGTase.

Samples containing 1 U of CGTase were electrophoresed on an SDS-10% (wt/vol) polyacrylamide-1% soluble starch gel. The gel was stained with Coomassie brilliant blue R-250 (CBB) (A) or 0.2% I_2-2% (wt/vol) KI solution (B). Lane 1, proteins precipitated with 70% saturated $(NH_4)_2SO_4$ from the culture supernatant; lane 2, proteins in a solution gathered from fractions with activity from the DEAE-cellulose DE53 column; lane 3, proteins in a solution gathered from fractions with activity from the butyl-Toyopearl 650M column. M, Molecular size markers (kilodaltons).

from pH 6.0 to 10.0 after incubation for 1 h at 30°C. The optimum temperature at pH 7.0 was at 60°C (Fig. 2). The enzyme was stable at temperatures from 4 to 45°C. In the presence of 5 mM $CaCl_2$, the enzyme had optimal activity at pH 7.0 at 65°C and still had 85% and 40% of its activity after incubation for 30 min at 60°C and 70°C, respectively. However, the enzyme had lost almost all activity after 30 min at 80°C.

Formation of cyclodextrins from starch

Mostly, β-cyclodextrin was produced during the first hour (Fig. 3). Some α- and γ-cyclodextrins formed up to 3.26 mg/ml (33% of the starting amount of starch) was produced in 24 h, and the ratio of α-, β-, and γ-cyclodextrins was 24:69:7. By the addition of 10% (vol/vol) ethanol, up to 4.52 mg/ml cyclodextrins were produced (45% of the starting amount of starch) in 24 h, and the ratio of α-, β- and γ-cyclodextrins was 12:82:6. The effects of ethanol concentration on the yield and product selectivity of cyclodextrins are shown in Table 2. The highest conversion yield was 46%, reached when ethanol was added to 20%. This yield was close to that with 10% ethanol. The proportion of β-cyclodextrin produced was highest in the presence of 10% ethanol.

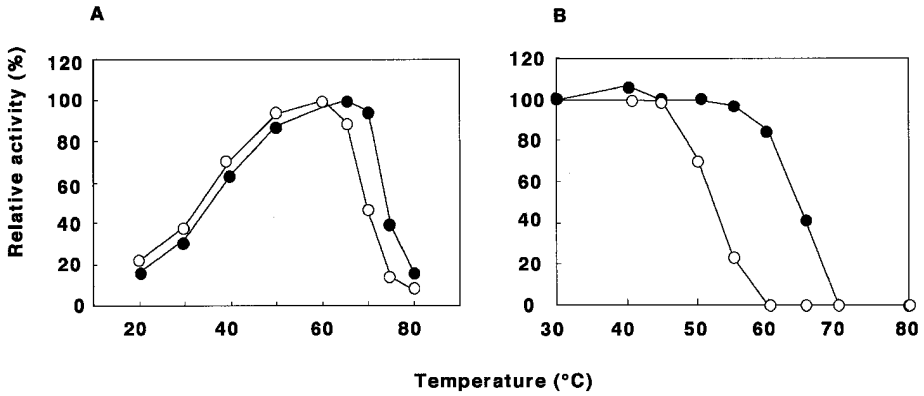

Fig. 2. Effects of Temperature on CGTase Activity and Stability.

(A) Enzyme activity was assayed by the iodine method at pH 7.0 at the temperatures indicated in the figure in the absence of $CaCl_2$ (○) or in the presence of 5 mM $CaCl_2$ (●). (B) Enzyme dissolved in 100 mM phosphate buffer (pH 7.0) was incubated for 30 min at the temperatures indicated in the absence of $CaCl_2$ (○) or in the presence of 5 mM $CaCl_2$ (●), and the relative activity was assayed by the iodine method at 30°C.

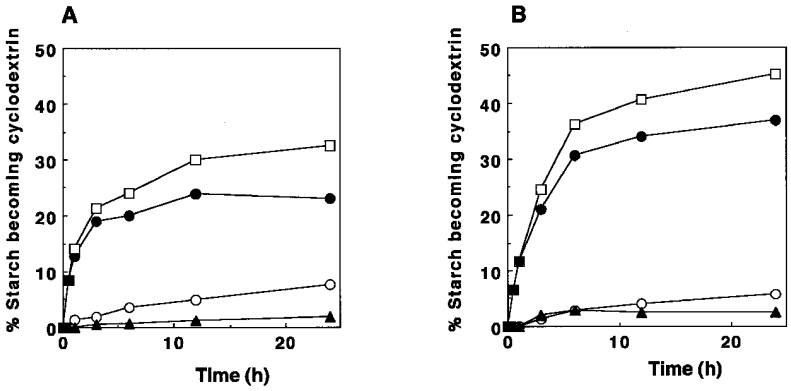

Fig. 3. Formation of α-, β-, and γ-Cyclodextrins from Starch.

Thirty microliters of the enzyme solution (20 U/ml) was added to the reaction mixture (300 ml) consisting of 1% soluble starch and 100 mM sodium phosphate buffer (pH 7.0) (A) or to a reaction mixture containing 10% (vol/vol) ethanol (B). The mixture was incubated at 30°C. The amount of cyclodextrins is shown as the percentage of soluble starch converted. (○), α-Cyclodextrin; (●), β-cyclodextrin; (▲), γ-cyclodextrin; (□), total cyclodextrin.

Table 2. Effects of Ethanol on Yield and Product Selectivity of Cyclodextrins[a)]

Ethanol (%)	Yield (%)[b)]	Product ratio (%)		
		α	β	γ
0	33	24	69	7
5	30	16	77	7
10	45	12	82	6
20	46	12	77	11
50	26	5	72	23

[a] Thirty microliters of the enzyme solution (20 U/ml) was added to the reaction mixture (300 μl) containing of 1% soluble starch and 100 mM sodium phosphate buffer (pH 7.0) in 0 to 50% (vol/vol) ethanol.

[b] Yields of cyclodextrins are shown as the percentage of converted soluble starch after 24 h of incubation at 30°C.

Table 3. Effects of Organic Solvents on Stability of CGTase

Solvent	log P_{ow}	Relative residual activity[a)] of CGTase from:	
		ST-12 K	*B. macerans*
None	—	100[b)]	100[b)]
Methanol	−0.76	102±9	98±1
Ethanol	−0.24	84±2	99±4
2-Propanol	0.28	82±6	96±6
Chloroform	1.9	47±2	75±2
Benzene	2.1	88±11	92±2
Toluene	2.6	102±6	100±3
p-Xylene	3.1	92±2	100±6
Cyclohexane	3.4	88±8	91±3
n-Hexane	3.9	84±5	90±1

[a)] An organic solvent (75 μl) was added to 300 μl of CGTase solution (0.2 U/ml in 100 mM sodium phosphate buffer, pH 7.0). The mixture was shaken at 30°C for 12 h. The solution was diluted 4-fold with the sodium phosphate buffer and the activity was measured by the iodine method.

[b)] The activity is relative to that in a control CGTase solution not exposed to any organic solvent.

Means and SD for three independent experiments are shown.

Table 4. Effects of Organic Solvents on Activity of CGTase

Solvent	log P_{ow}	Relative activity[a)] of CGTase from:	
		ST-12 K	*B. macerans*
None	—	100[b)]	100[b)]
Methanol	−0.76	78±4	18±3
Ethanol	−0.24	104±9	20±4
2-Propanol	0.28	80±8	15±5
Chloroform	1.9	91±9	65±3
Benzene	2.1	106±6	88±5
Toluene	2.6	104±6	88±8
p-Xylene	3.1	93±11	88±6
Cyclohexane	3.4	102±5	98±2
n-Hexane	3.9	94±9	94±4

[a)] An organic solvent (75 μl) was added to 300 μl of CGTase solution (0.2 U/ml in 100 mM sodium phosphate buffer, pH 7.0) containing 0.5% soluble starch. The mixture was shaken at 30°C for 2 to 10 min. The activity was measured by the iodine method.

[b)] The activity is relative to that in a control CGTase solution not exposed to any organic solvent.

Means and SD for three independent experiments are shown.

Effects of organic solvents on enzyme activity and stability

Table 3 shows the stability of the purified CGTase of ST-12 K in the presence of various solvents and also the stability of commercially available CGTase from *Bacillus macerans*. The enzyme from *B. macerans* has been extensively studied.[16] Highly polar hydrophobic solvents with log P_{ow} values (less than 2) inactivate most enzymes through structural denaturation.[17] Both enzymes were moderately stable in presence of methanol, ethanol, 2-propanol, benzene, toluene, *p*-xylene, cyclohexane, and *n*-hexane. The enzymes were partially inactivated by the addition of chloroform after incubation for 12 h at 30°C.

Table 4 shows activity in the presence of organic solvents. The enzyme from *B. macerans* was inactivated by methanol, ethanol, 2-propanol, chloroform. The enzyme of ST-12 K was active in the

presence of all organic solvents tested in this assay with a 10-min reaction.

Discussion

Depending on the most abundant kind of cyclodextrin that CGTase produces, the enzyme is sometimes classified as α-, β-, or γ-CGTase. By this classification, the enzyme from strain ST-12 K is a β-CGTase. Of the three kinds of cyclodextrins, β-cyclodextrin is of the most practical use because its inclusion complexes are easily prepared and stable; the size of the apolar cavity is optimum for many molecules such as drugs and preservatives.[1] In addition, β-cyclodextrin is easily separated from reaction mixtures because of its low solubility in water. In view of these points, we wanted to isolate microorganisms producing enzymes that synthesize β-cyclodextrin. The CGTases from *Bacillus megaterium*, *Bacillus circulans*, a *Bacillus* sp., *Bacillus haropbilus*, *Thermoanaerobacterium thermosulfurigenes*, and *Bacillus ohbensis* produce mainly β-cyclodextrin.[16] On the basis of its 16S rDNA sequence, and its morphological and physiological characteristics, strain ST-12 K was classified as *P. illinoisensis*. No CGTase from *P. illinoisensis* was reported.

There have been several studies on improvement of product selectivity or increasing the yield of cyclodextrin production by CGTase in the presence of organic solvents.[2–4] However, there is no report about screening for a microbe that produces organic-solvent-tolerant CGTase. Cyclodextrins are mostly used in food, cosmetics, and pharmaceuticals, ethanol is a possible solvent to use in the production of cyclodextrins. Addition of ethanol also can improve the overall process economics by reducing bacterial contamination in enzymatic processes. CGTase from strain ST-12 K produced α-, β-, and γ-cyclodextrins at the ratio of 12:82:6 in the addition of 10% (vol/vol) ethanol, although the product ratio was 24:69:7 without the solvent. This product selectivity for β-cyclodextrin (82%) is the same as the highest selectivity for β-cyclodextrin (82%) obtained with *Bacillus circulans* strain 251 and the addition of *t*-butanol.[3] Moreover, the overall yield of cyclodextrin production with ST-12 K CGTase was up to 1.4-fold with the addition of ethanol. This improvement is greater than that of *Bacillus circulans* strain 251 with *t*-butanol (1.1-fold).[3] In particular, the yield of β-cyclodextrin in the presence of 10% (vol/vol) ethanol was 1.6-fold that in the absence of ethanol. With commercially available CGTase from *B. macerans*, the yield was lowered to 85% when ethanol was added (not shown). The production of cyclodextrins increases with the addition of toluene and decane.[4] In this case, the cyclodextrins formed an insoluble complex with organic solvents; the continuous removal of cyclodextrins by precipitation from the reaction system shifts the equilibrium in favor of cyclodextrin production. However, precipitation of cyclodextrins by the addition of ethanol was not observed in our study. The effects of solvent addition, such as increased yield and improved product selectivity, were related to reduced competition from the intermolecular transglycosylation reaction that causes degradation of cyclodextrin products.[3] Final concentrations of cyclodextrin products were higher when degradation of cyclodextrin by CGTase was inhibited by the addition of organic solvents. Inhibition of cyclodextrin degradation by CGTase was affected by the solvent added and the kind of cyclodextrin. Therefore, product selectivity seemed to depend on the solvent and the cyclodextrin.

ST-12 K CGTase was stable and active in the presence of various organic solvents. Although CGTase from *Bacillus macerans* was stable in the presence of methanol, ethanol, 2-propanol, or chloroform, the enzyme was not active in these solvents. The inactivation of *Bacillus macerans* CGTase by the addition of the organic solvents seemed to be reversible. The stability of CGTase from *Bacillus* sp. strain BE101 decreases in the presence of ethanol.[4] The residual activity of CGTase from *Bacillus* sp. strain BE101 is about 40% at 45°C after 12 h in the presence of 10% (vol/vol) ethanol. Under the same conditions, ST-12 K CGTase maintained 99% of its activity in the presence of ethanol. Our CGTase was stable and active in the presence of various organic solvents. The CGTase should be useful for technological applications with organic solvents. In particular, this enzyme can be used to improve the overall process economics of the enzymatic production of β-cyclodextrins in the presence of ethanol.

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