PURIFICATION AND PROPERTIES OF CYCLODEXTRIN GLYCOSYLTRANSFERASE (CGTASE) FROM AN ALKALINE-TOLERANT *BACILLUS* SP. PS304

PRASERT SUNTINANALERT[a], JINTANA PETMANEECHOTE[a], NISARAT DAMNIAN[a] AND NONGPORN HUTADILOK-TOWATANA[b]

[a] *Department of Microbiology,* [b]*Department of Biochemistry, Faculty of Science, Prince of Songkla University, Hatyai Campus, Songkhla 90112, Thailand.*

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ABSTRACT

A cyclodextrin glycosyltransferase (CGTase) produced from Bacillus *sp. PS304 was purified up to 29 folds by ammonium sulfate precipitation and DEAE-cellulose column chromatography. The purified enzyme was stable at a pH range from 4 to 12 and was most active at pH 5 and 8.5. The temperature optimum for its activity was 55°C. The enzyme was stable up to 45 °C for 1 h. The isolectric point (pI) of the enzyme was 6.5. The apparent molecular weight as determined by SDS-PAGE was 76 kDa. The enzyme activity was found partially suppressed by 10 mM copper ion (Cu^{2+}) and was completely inhibited in the presence of 3,4-dichloroisocoumarin at 1 mM.*

INTRODUCTION

Cyclodextrin glycosyltransferase (1,4-α-D-glucan 4-α-D-glucanotransferase, cyclising, EC 2.4.1.19, CGTase) hydrolyzes starch and related carbohydrates as amylose, amylopectin, glycogen and maltooligosaccharides into short chains of glucose and then links the two ends of each resulting fragment to form cyclodextrin, a cyclic polymer of glucose[1]. With increasing industrial demands on cyclodextrin nowadays, considerable interest has been focused on the production of this compound by using microorganism-derived CGTases. So far, various *Bacillus* spp. such as *Bacillus* sp. No. 5[2], *Bacillus circulans*[3,4] and alkalophilic *Bacillus* spp.[5,6] have been discovered producing CGTases, and the purification and characterization of these enzymes have also been studied[2-6]. Recently, from a soil sample collected from the southern part of Thailand, we succeeded in isolating a strain of an alkaline-tolerant *Bacillus* sp. PS304 which was capable of producing CGTase in a wide pH range between 5 and 9.5[7]. This bacterial enzyme, however, seems to be novel since it was demonstrated to produce three forms of cyclodextrin in the ratio differing from those reported earlier[3-5].

In this study, we therefore describe the purification and some properties of the CGTase produced from *Bacillus* sp. PS304.

MATERIALS AND METHODS

Microorganism

An alkaline-tolerant *Bacillus* sp. PS304 was isolated from soil sample as described earlier[7]. The bacterial isolate was maintained in agar slant supplemented with 1 % corn starch and then stored at -70 °C in nutrient broth pH 8.0 containing 15% glycerol for further use.

Enzyme assay

The dextrinizing activity of the CGTase was determined by the iodine method in accordance with Suntinanalert *et al*[7]. One unit of the enzyme activity was defined as the

amount of enzyme that hydrolyzes 10 mg of soluble starch in 30 min under the specified conditions. The production of cyclodextrins was determined by high performance liquid chromatography (HPLC) (Thermo Separation Products, U.S.A.) using Shodex PS Pak DC613 oligosaccharide column (6 x 150 mm), running at a flow rate of 1.5 ml/min at 60°C. The α, β and γ-cyclodextrin were identified by integrator comparing with standard markers.

Purification of the enzyme

The bacteria were cultivated aerobically with 5% inoculum in 1 liter of starch medium pH 8.0 at 35°C for 24 h under continuous shaking. The cells were subsequently removed by centrifugation at 7,000 g, 4°C for 15 min and the supernatant was added with solid ammonium sulfate to give 80% saturation at 4°C. The precipitates formed were then collected by centrifugation and dissolved in 50 mM phosphate buffer pH 7.0. After being exhaustively dialyzed against the same buffer at 4°C, the retentate was loaded on to a DEAE-cellulose (Whatman DE-52) column (1.6 x 6.5 cm) that had been previously equilibrated with 50 mM phosphate buffer pH 7.0. The column was washed with the same buffer at a flow rate of 10 ml/h and fractions of 2 ml were collected. The bound protein was eluted from the column upon applying a linear gradient concentration of NaCl upto 1 M. The fractions containing CGTase activity were pooled, concentrated and then stored at -20°C for further study.

Protein measurement

Protein concentrations were determined by the method of Lowry *et al.*[8] using bovine serum albumin as a standard. For the chromatographic profile, the protein content in each fraction was measured by light absorption at 280 nm.

Gel electrophoresis

Polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulphate (SDS-PAGE) was carried out according to the method of Laemmli[9] using Pharmacia's standard low molecular weight kit to estimate the molecular weight of the enzyme.

Isoelectric focusing

Isoelectric focusing was performed in mini-slab polyacrylamide gel containing 7% polyacrylamide and 2% ampholytes pH 3-10 (Pharmacia)

Amino acid analysis

Amino acid composition of the enzyme was determined following acid hydrolysis with 6 M HCl vapour in a nitrogen atmosphere at 110°C for 20 h using a Picotag Work Station (Waters Co. Ltd., USA). After hydrolysis, the sample was dried and dissolved in Beckman's Na-S Sample Dilution Buffer. The amino acid content in this solution was then separated and analyzed by an automatic Beckman System 6300 Amino Acid Analyzer (Beckman Co. Ltd., USA). For tryptophan analysis, the sample was hydrolyzed in 0.8 M methanesulphonic acid under nitrogen gas at 110°C for 20 h prior to be determined by the analyzer. Cysteine was determined as cysteic acid after being oxidized in performic acid.

RESULTS

Purification of CGTase

Through two successive steps of purification, the CGTase from *Bacillus* sp. PS403 was purified approximately 29 folds with a 58% yield, as summarized in Table 1. In the first step,

when the supernatant of the *Bacillus* culture was concentrated by 80% saturation of ammonium sulfate, about 10% of the proteins in the culture medium including the CGTase became precipitated, as indicated by the appearance of the enzyme activity in that fraction. In the next step, by employing the DEAE column chromatography, the enzyme was successfully isolated in an unbound fraction. Under the conditions used, the enzyme was washed through immediately on loading and subsequent washing with the equilibrating buffer. This result was shown as a single protein peak (D1) with CGTase activity in the chromatographic profile in Figure 1, whereas the other proteins were still bound to the gel. This contaminant, however, could be eluted from the column as a protein peak (D2) with the buffer containing 0.5 M NaCl (Figure 1). As shown in Figure 2, the purified CGTase (peak D1) migrated as a single band on SDS-PAGE. Its molecular weight was then estimated to be 76 kDa. From amino acid analysis (Table 2), the purified enzyme contained high amounts of acidic amino acids as aspartic and glutamic acid, relatively low amounts of cysteine and histidine, and was devoid of glycine, methionine and tryptophan. Surprisingly, proline was also present as a major amino acid content of this enzyme.

Properties of the enzyme

By performing the starch-dextrinizing assay at different pH ranging from 4 to 12, the purified enzyme was demonstrated to have the highest activity at pH 5 and 8.5 with the optimum temperature at 55°C (Table 3). When the enzyme solution at pH 8.0 was incubated at various temperatures for 1 h prior to determining the remaining activity, the results showed that the CGTase was thermally stable between 30 and 45°C (Table 3). Upon increasing the temperature above 45°C, the enzyme was inactivated gradually. However, its activity was completely lost by heating at 60°C (data not shown). In addition, the enzyme had an isoelectric point (pI) of 6.5. When the CGTase activity was assayed in the presence of a variety of ions and chemicals, it was observed that most of the compounds tested did not have any significant effect on this enzyme except $CuSO_4$ at 10 mM that showed some inhibition and 3,4-dichloroisocoumarin at 1mM that inhibited the enzyme activity completely (Table 4).

DISCUSSION

In this study, the enzyme CGTase from an alkaline-tolerant *Bacillus* sp. PS304 was purified by using only two steps of purification. The appearance of high protein amount in the culture supernatant compared to that in 80% ammomium sulfate precipitates may arise from the detection by Lowry's method of short peptides and some free amino acids in the culture medium which could not be precipitated by ammonium sulfate. In addition, the yellow color of culture filtrate could interfere the optical density of this protein assay. The finding that the CGTase did not bind to the anion-exchange resin under the conditions used suggests the structure of the enzyme molecule to be either positively charged or uncharged at neutral pH. This has been supported by its migration to a pH of 6.5 in isoelectric focusing. These findings, however, seems to be contradict to the results of amino acid analysis that the total amount of the acidic amino acids (aspartic acid and glutamic acid) accounts for about 28% while the basic ones (histidine, lysine, and arginine) accounts for only 9%. In this regard, it is noteworthy that acid hydrolysis will result in the loss of amnonia from the side-chain amide groups of asparagine and glutamine giving rise to aspartic and glutamic acids, respectively[10]. Thus, aspartic acid and asparagine were combined and estimated as aspartic acid by the analytical method used, similar to glutamine and glutamic acid. And this is a plausible explanation of such high amounts of acidic amino acids compared to the others. Further determination on

Table 1 Purification of CGTase from *Bacillus* sp. PS304

Fraction	Activity (units)	Protein (mg)	Sp. activity (units/mg)	Recovery (%)	Purification (fold)
Culture supernatant	3,042	3,420	0.9	100	1
Ammonium sulfate precipitate (80%)	2,176	352	6	72	7
DEAE-cellulose (unbound)	1,753	69	25	58	29

Table 2 Amino acid composition of the CGTase. The purified enzyme was hydrolyzed and analyzed as already described in Materials and Methods.

Amino acid	% Amino acid
Alanine	12.23
Arginine	2.43
Aspartic acid	15.39
Cysteine	0.18
Glutamic acid	12.34
Glycine	0.0
Histidine	0.88
Isoleucine	2.18
Leucine	3.30
Lysine	5.43
Methionine	0.0
Phenylalanine	1.43
Proline	21.05
Serine	3.61
Threonine	3.08
Tryptophan	0.0
Tyrosine	1.04
Valine	4.23

Table 3 Some properties of the CGTase from *Bacillus* sp. PS304

Characteristics	Results
Molecular weight	76 kDa
pH stability[1]	4-12
pH optimum	5 and 8.5
Temperature stability[2]	30-45°C
Temperature optimum	55°C
Isoelectric point (pI)	6.5

1. The enzyme solution was incubated in the buffer at pH 4 to 12 for 1 h at 4 °C using 50 mM buffer of citrate pH 4.0-6.0, phosphate pH 6.0-8.0, tris-HCl pH 8.0-9.0, sodium carbonate and sodium hydrogen carbonate pH 9.0-11.0, and sodium carbonate and sodium hydroxide pH 11.0-13.0, before residual activity was measured.
2. The enzyme solution was incubated in 50 mM phosphate buffer at pH 8.0 for 1 h at 30 to 60°C, then residual activity was measured.

Table 4 Effect of various substances on the CGTase activity*

Substances	Concentrations (mM)	Relative activity (%)
Control	none	100
2-Mercaptoethanol	1	115
3,4-Dichloroisocoumarin	1	0
Iodoacetic acid	1	112
EDTA	10	101
$CaCl_2$	10	103
$CuSO_4$	10	31
$FeSO_4$	10	91
KCl	10	96
$MgCl_2$	10	98
$MnCl_2$	10	96
NaCl	10	85
$ZnSO_4$	10	103

* The reaction mixture contained one ml of diluted enzyme solution, 3 ml of 1% soluble starch, 2 ml of 50 mM phosphate buffer pH 8.0 and one of the components tested at the indicated concentration, and the enzyme activity was assayed as described previously in Materials and Methods.

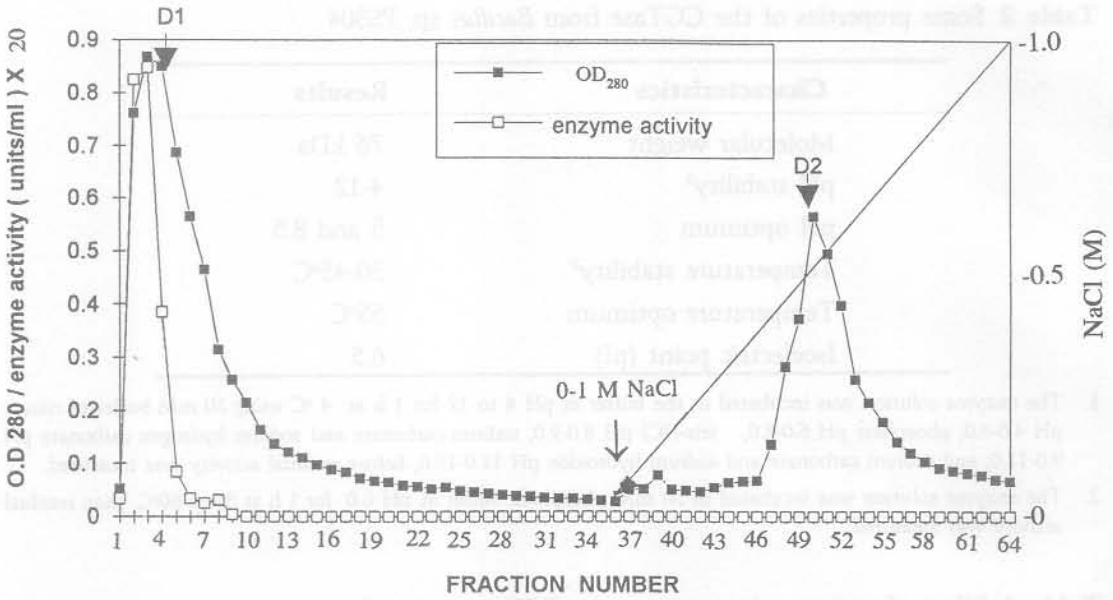

Fig. 1 Chromatographic profile of the CGTase on a DEAE-cellulose column. The arrow indicates the start of elution with a NaCl gradient in the equilibrating buffer.

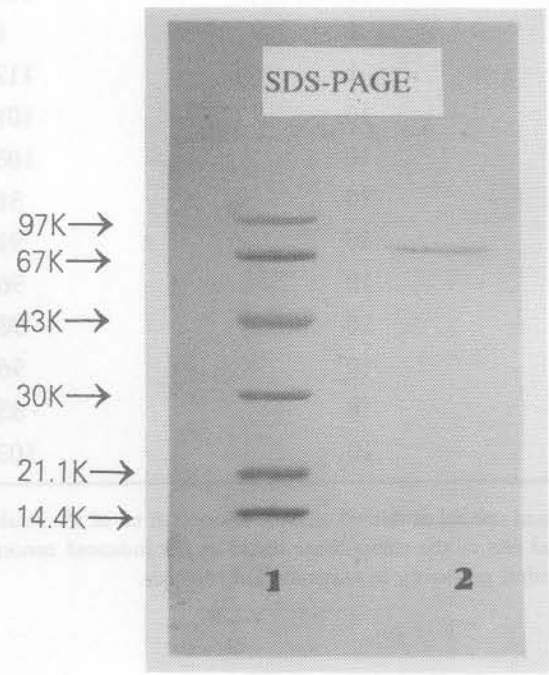

Fig. 2 SDS-PAGE of the purified CGTase from DEAE-cellulose fraction. Lane 1) Low molecular weight standard of phosphorylase (M.W. 97 kDa), albumin (M.W. 67 kDa), ovalbumin (M.W. 43 kDa), carbonic anhydrase (M.W. 30 kDa), trypsin inhibitor (M.W. 21.1 kDa), and lysozyme (M.W. 14.4 kDa). Lane 2) Purified enzyme from DEAE-cellulose (about 30 mg) (peak D1). A 12% gel containing 0.1% SDS was used.

amino acid sequence of this enzyme may help elucidate this controversy. Purity of the enzyme from DEAE-cellulose chromatography was demonstrated by a single protein band on SDS-PAGE detected by Coomassie blue staining, indicating a single subunit of the molecule which was confirmed by its migration on normal PAGE (data not shown).

This CGTase seems to be alkaline tolerant with the optimum pH at 5 and 8.5. The enzyme is rather heat labile since it lost activity at 60°C. However, it displayed some properties similarly to those reported from other *Bacillus* CGTases. It resembles to that from *Bacillus circulans* E192 in term of the molecular size (M.W. 78 kDa[4]) but unlike the latter it showed different pH and temperature optimum (*B. circulans* E192 were at 5.5 and 60°C, respectively[4]). The kinetic data of this enzyme is not presented because the methods of kinetic determination described recently by Lee and Tao[11] using methyl orange is specific for only α-cyclodextrin but not for the β forms of cyclodextrin which was produced predominantly by this *Bacillus* sp. PS304[7]. Additonally, this CGTase is not metallo-enzyme since the enzyme was not inhibited by EDTA and no ions enhanced significantly the enzyme activity except for copper ion (Cu^{2+}) that showed some inhibition. The fact that this enzyme is unaffected by the sulfhydryl agents *i.e.* mercaptoethanol and iodoacetic acid implies the non-requirement of thiol group for the enzyme activity. Moreover, the CGTase was completely inhibited by 3,4-dichloroisocoumarin, the typical serine proteases inhibitor, suggesting that serine might be essential at the active site. Finally, the finding that this CGTase was rich in imino acid 'proline' may reflect the rigidity of the enzyme molecule[11]. The enzyme possesses high contents of acidic amino acids corresponding to the fact that alkalophilic proteins generally contain more acidic amino acid composition. Together with the sodium ion and hydrogen ion antiporter, they are believed to be involved in pH homeostasis which is a necessary function for life at high pH[12].

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บทคัดย่อ

การทำบริสุทธิ์เอนไซม์ cyclodextrin glycosyltransferase (CGTase) ที่ ผลิตจาก *Bacillus* sp. PS304 โดยการตกตะกอนโปรตีนด้วยแอมโมเนียมซัลเฟตและ DEAE-cellulose คอลัมน์โครมาโทกราฟี ตามลำดับ พบว่า สามารถทำให้เอนไซม์ CGTase มีความบริสุทธิ์เพิ่มขึ้น 29 เท่า เอนไซม์ที่เตรียมได้มีความเสถียรที่พีเอช 4 ถึง 12 แต่มีกิจกรรมสูงสุดที่พีเอช 5 และ 8.5 และมีความเสถียรที่อุณหภูมิสูงสุด 45°ซ ในขณะที่มีกิจกรรมสูงสุดที่อุณหภูมิ 55°ซ นอกจากนี้ เอนไซม์ยังมีค่า isoelectric point (pI) เท่ากับ 6.5 เอนไซม์ CGTase นี้มีน้ำหนักโมเลกุล 76 กิโลดาลตัน เมื่อตรวจโดย SDS-PAGE กิจกรรมของเอนไซม์ดังกล่าวถูกยับยั้งได้บางส่วนโดย 10 mM คอปเปอร์ไอออน (Cu^{2+}) และถูกยับยั้งได้ทั้งหมดโดย 3,4-dichloroisocoumarin ที่ความเข้มข้น 1 mM.