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PURIFICATION AND PHYSICOCHEMICAL PROPERTIES OF AN EXTRACELLULAR CYCLOAMYLOSE (CYCLODEXTRIN) GLUCANOTRANSFERASE FROM *Bacillus macerans*

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ABSTRACT

An extracellular cycloamylose (cyclodextrin) glucanotransferase (EC 2.4.1.19) from *Bacillus macerans* was purified to homogeneity by adsorption on starch, ammonium sulfate fractionation, column chromatography on DEAE-cellulose, and gel filtration on Sephadex G-100. The enzyme had a molecular weight of 67,000 and consisted of one polypeptide chain. The isoelectric point was pH 5.4. Temperature and pH optima were 60° and 5.4–5.8, respectively. The purified enzyme was quite stable at 50° (pH 6.0), but lost ~80% of its activity at 60° for 30 min (pH 6.0). Prolonged digestion by trypsin did not affect the catalytic properties of the enzyme. The K_m for starch was 5.7 mg/ml.

INTRODUCTION

Schardinger was the first to describe the crystalline cycloamyloses (cyclodextrins or Schardinger dextrins) found in starch-containing media after growth of *Bacillus macerans*[1]. However, Freudenberg and Meyer-Delius were the first to show that the cycloamyloses were closed rings of (1→4)-linked α-D-glucosyl residues[2]. Later, Tilden and Hudson demonstrated that the cycloamyloses are not excretory products of the organism, but are formed from starch by the action of an extracellular enzyme[3]. The most common products of this enzyme are cyclohexa-amylose and cyclohepta-amylose containing six and seven D-glucosyl residues, respectively, but five different products have been described[4]. The enzyme, now named cyclodextrin glycosyltransferase or cycloamylose glucanotransferase (EC 2.4.1.19), has often been referred to as *Bacillus macerans* amylase (BMA) in the literature. This enzyme is produced by *B. circulans*, *B. stearothermophilus*, and *B. megaterium*, in addition to *B. macerans*[5], and has been purified from *B. macerans*[6–8], *B. megaterium*[9], and an alkalophilic *Bacillus* sp.[10]. Recently, the enzyme from *B. macerans* has been crystallized by Kobayashi *et al.*[11]. We now report on the purification and the physico-

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chemical properties of an extracellular cycloamylose glucanotransferase from *B. macerans* isolated from dry onion powder.

MATERIALS AND METHODS

Bacterial strain. — The bacterium was isolated from dry onion powder and was identified as *B. macerans*[12]. Cultures of the organism were maintained at 4° on a 1% agar medium containing 0.5% of soluble starch (Difco Laboratories), 0.5% of yeast extract (Difco), 0.2% of $(NH_4)_2SO_4$, 0.1% of KH_2PO_4, 0.05% of $MgSO_4 \cdot 7H_2O$, and 0.03% of $CaCl_2 \cdot 2H_2O$. The final pH was adjusted to 6.2 with NaOH.

Enzyme production. — The cycloamylose glucanotransferase (CGTase) was produced by using a medium containing the same constituents as listed above. The production was performed in a Microferment Fermentor (New Brunswick) in a 10-liter batch at 37°, with stirring at 200 r.p.m. and with a constant air-flow at 2000 ml/min, for ~5 days.

Enzyme assay and detection of cycloamyloses. — The enzyme activity was determined by the method of Hale and Rawlins[6]. One CGTase unit was defined as the amount of enzyme which hydrolyzed 10 mg of starch per min. The cycloamyloses were stained and observed under a microscope as described by Tilden and Hudson[13].

Enzyme purification. — After ~5 days of growth, the culture medium was centrifuged at 4° for 30 min at 10,000*g*, to give a clear supernatant solution that was brought to 10% saturation by addition of solid ammonium sulfate with stirring at 4°. The solution was then passed through a layer of 100 g of corn starch mixed with Celite (1 : 1) on a Büchner funnel to adsorb the enzyme. The starch layer was washed with water at 4° until the enzyme started to emerge. The enzyme was then eluted with 0.02M phosphate buffer (pH 8.0) preheated to 60°. The CGTase-containing eluate was rapidly cooled on ice. The enzyme-containing, protein fraction that precipitated between 40 and 80% saturation with ammonium sulfate at 4° was dissolved in 60 ml of 0.02M phosphate buffer (pH 7.0) and dialyzed against 2 litres of 5mM sodium phosphate buffer (pH 7.0). The enzyme was then subjected to chromatography on a column (2 × 25 cm) of DEAE-cellulose with the same starting buffer, followed by stepwise changes to 0.1M, 0.175M, and 0.2M phosphate buffers with 0.5M NaCl. The CGTase was eluted in the 0.175M buffer, in fractions of 5 ml at a flow rate of 30 ml/h. The fractions having the highest specific activity were combined, and concentrated to 5 ml with an Amicon ultrafiltration unit, using a PM 10 membrane filter under an atmosphere of nitrogen at a pressure of 3 kPa at 4°. The concentrated enzyme-extract was applied to a column (2.5 × 40 cm) of Sephadex G-100 equilibrated at room temperature with 0.02M phosphate buffer (pH 6.0). Fractions of 5 ml were collected at a flow rate of 30 ml/h.

Electrophoretic studies. — Gel electrophoresis was performed in 7% polyacrylamide gels with 25mM Tris–glycine (pH 8.5) as the running buffer. The gels were loaded with 20–200 μg of protein and stained with Coomassie Brilliant Blue R-250[14]. Isoelectric focusing was performed in 7% polyacrylamide gels by using BioLyte 3/10

(Bio-Rad Laboratories) with 0.2% phosphoric acid as the anode solution and 0.4% ethanolamine as the cathode solution. Each gel was loaded with 50 μg of protein of purified CGTase. A current of 2 mA/gel was applied until the voltage reached 400 V, and then the voltage was kept constant for the duration of the experiment (3 h at 8°). The gels were fixed in 12% trichloroacetic acid for 60 min, and the ampholytes were washed from the gels with 6 changes of 5% trichloroacetic acid at 2-h intervals. The gels were stained as described above. The pH gradient in the gels was determined by slicing blank gels (subjected to isoelectric focusing simultaneously with the sample gels) into 0.5-cm sections immediately after isoelectric focusing. Each section was eluted overnight at room temperature with 1 ml of freshly boiled, distilled water. The pH was measured with a Beckman Expandomatic SS-2 pH meter.

Determinations of molecular weight. — Molecular weight was determined by dodecyl sulfate-gel electrophoresis in 9% polyacrylamide gels, in the presence of 2-mercaptoethanol[15]. The following standard proteins (Sigma Chemical Company) were used: bovine serum albumin (mol. wt. 68,000), *B. subtilis* alpha-amylase (50,000), DNase (31,000), and lysozyme (14,400). Molecular weight was also determined by sedimentation equilibrium experiments, performed at 22° with a Beckman Analytical Ultracentrifuge, Model E, using a double-beam photoelectric scanner at 280 nm. The molecular weight was determined in 0.1M sodium phosphate buffer (pH 6.0) at a rotor speed of 12,000 r.p.m. Calculations were made according to Yphantis[16]. The partial specific volume calculated from the amino acid composition was 0.722 ml/g.

Amino acid analysis. — The amino acid composition of the CGTase was determined with a Biocal BC 200 automatic, amino acid analyzer. Hydrolysis under vacuum was performed in 6M HCl at 108° for 24 h. Separation was performed by the method of Moore and Stein[17], modified as described in the Biocal 200 manual. Tryptophan was determined spectrophotometrically by the method of Beaven and Holiday[18].

Protein determination. — Protein was determined by the method of Lowry *et al.*[19]. Continuous reading of absorbance at 280 nm was used for protein estimation in column chromatography.

Stability tests. — For testing of temperature stability, samples (0.5 ml) of purified CGTase (50 μg of protein/ml) in 0.02M phosphate buffer (pH 6.0) were heat-treated for 1–30 min at 40°, 50°, and 60°. The samples were rapidly cooled in ice–water and the activities determined. The temperature stability was also tested at 60° at different pH values (pH 5, 6 and 8) under the same conditions.

Digestion of the CGTase (150 μg of protein/ml) was performed at 37° in 0.02M phosphate buffer (pH 7.6), using trypsin (60 μg/ml; Sigma Chemical Company). The CGTase activity was followed at pH 6.0 for 24 h.

RESULTS

Purification of the CGTase. — The strain of *B. macerans* used in this study

TABLE I

PURIFICATION OF CGTASE FROM *B. macerans*

Fraction	*Volume (ml)*	*Protein (mg/ml)*	*Total activity (U)*	*Specific activity*[a]	*Yield (%)*
Culture medium	8500	0.35	7140	2.4	100
Starch extract	1500	0.15	4500	20.0	63
Ammonium sulfate fraction (40–80%)	70	0.68	2730	57.4	38
Active fraction from DEAE-cellulose column	35	0.55	2220	115	31
Active fraction from Sephadex G-100 column	25	0.38	1210	127	17

[a]Specific activity = Total activity/[protein (mg/ml) × volume].

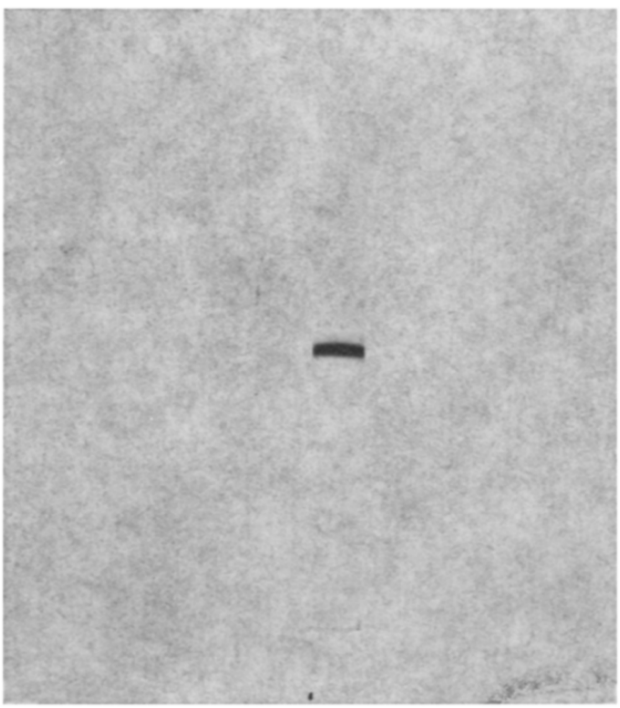

Fig. 1. Polyacrylamide disc-gel electrophoresis of the purified CGTase (50 μg of protein) on 7.0% polyacrylamide gel.

produced an extracellular CGTase, and no activity was found in the cells. The purification procedure is summarized in Table I. A 53-fold purification gave a yield of 17%, and led to a homogeneous protein as judged by polyacrylamide electrophoresis, isoelectric focusing, and ultracentrifugation.

Properties of the CGTase. — The molecular weight of the enzyme was calculated

TABLE II

AMINO ACID COMPOSITION OF CGTASE FROM *B. macerans*

	Amino acid residues (%)	
	CGTase (this work)	*CGTase (Ref. 8)*
Aspartic acid	14.8 (92)[a]	14.2
Threonine	9.43 (59)	9.54
Serine	6.14 (39)	6.92
Glutamic acid	6.48 (40)	6.34
Proline	4.11 (26)	3.63
Glycine	11.5 (72)	11.2
Alanine	8.72 (54)	9.10
Valine	6.73 (42)	7.21
Methionine	1.86 (12)	1.80
Isoleucine	4.78 (30)	5.15
Leucine	5.69 (36)	5.74
Tyrosine	4.20 (26)	4.39
Phenylalanine	5.13 (32)	4.80
Lysine	4.13 (26)	4.11
Histidine	1.75 (11)	1.57
Arginine	2.82 (18)	2.68
Tryptophan[b]	1.61 (10)	1.72
1/2 Cystine	0.00 (0)	0.00

[a]Number of amino acids to nearest integer based on molecular weight. [b]Determined spectrophotometrically.

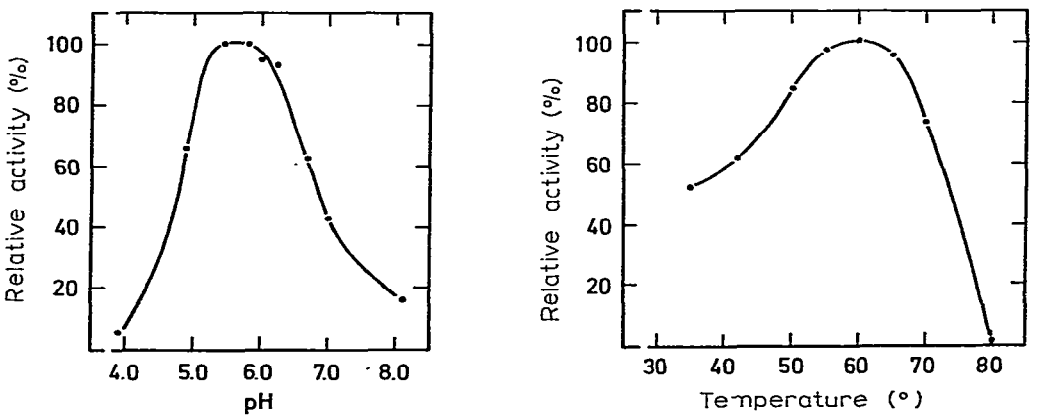

Fig. 2. Effect of pH on the CGTase activity. The assay was performed at 60° with acetate buffer (pH 3.9–5.4) and phosphate buffer (pH 5.8–8.1).

Fig. 3. Effect of temperature on the CGTase activity at pH 6.0.

to be 67,000 by ultracentrifugation and 75,000 by dodecyl sulfate-gel electrophoresis, indicating the protein to consist of one polypeptide chain only. The isoelectric point was pH 5.4, and the relative mobility on 7.0% polyacrylamide gel was 0.48 (Fig. 1).

The amino acid composition of the CGTase from *B. macerans* is given in Table II, in comparison with the data of DePinto and Campbell[8].

The effect of substrate concentration on the enzyme was determined at 60° and pH 6.0 by using soluble starch as the substrate. The K_m value calculated from the Lineweaver–Burk plot was 5.7 mg/ml. The CGTase reported in this work produced cyclohexa-amylose only, as judged by the method described by Tilden and Hudson[13]. Neither cyclohexa-amylose nor cyclohepta-amylose in concentrations up to 500mM changed the rate of hydrolysis of starch by the enzyme.

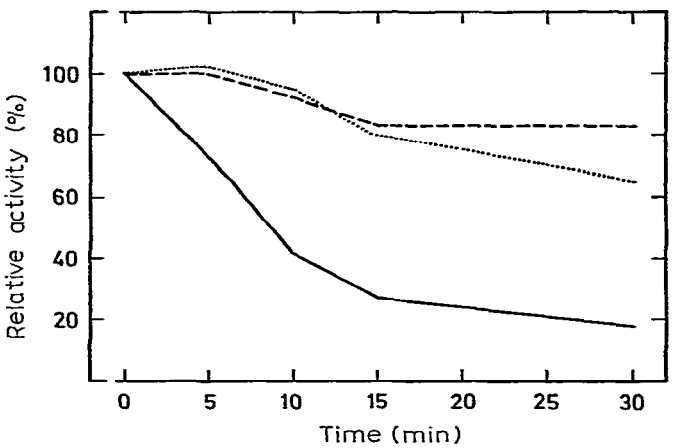

Fig. 4. Thermostability of the CGTase at 40° (– – –), 50° (....), and 60° (——). The enzyme was heated in 0.02M phosphate buffer (pH 6.0); at appropriate intervals, an aliquot of the solution was taken and the remaining activity determined.

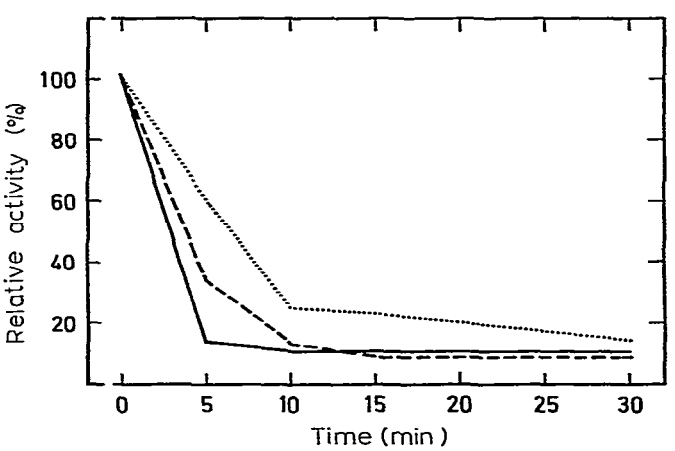

Fig. 5. Thermostability of the CGTase at pH 5 (——), pH 6 (....), and pH 8 (– – –). The enzyme was heated in 0.02M acetate buffer (pH 5.0) or phosphate buffer (pH 6.0 and 8.0); at appropriate intervals, an aliquot of the solution was taken and the remaining activity determined at pH 6.0.

Temperature and pH optima. — The pH optimum for the purified CGTase was found to be between 5.4 and 5.8 (Fig. 2). At both sides of the pH optimum, a rapid decrease in activity was found, giving only 30% of maximum activity at pH 4.5 and 7.5. The temperature optimum was found to be 60° (Fig. 3), with a rapid decrease in activity at higher temperatures and with no activity at 80°. A much slower decrease in activity was observed by reducing the temperature; at 35°, the CGTase had ~50% of the activity observed at 60°.

Stability of the CGTase. — The temperature stability of the enzyme at 40, 50, and 60° (pH 6.0) is shown in Fig. 4. The CGTase was quite stable at 40 and 50°, but lost activity at 60°. However, 20% of the initial activity was still left after 30 min at 60°. Fig. 5 shows that the enzyme is more stable at pH 6.0 than at pH 5.0 and 8.0. No loss in activity was observed by incubating the enzyme with trypsin for 24 h at 37° and pH 7.6.

DISCUSSION

The growth medium used for CGTase production in this study contained only 0.5% of soluble starch and 0.5% of yeast extract in addition to inorganic components. This medium is less complex than that used in other studies of the same enzyme[6,8–11]. The CGTase activity was detectable after ~3 days of growth, and reached maximum activity after 5 days.

The purification procedure involved the most commonly used techniques for purification of this enzyme, giving a homogeneous protein with a yield of 17% (Table I).

The molecular weight of this CGTase is ~67,000, and very different from that found earlier. DePinto and Campbell[8] found a molecular weight of 139,000 for an intracellular CGTase from *B. macerans*, and Kobayashi *et al.*[11] found a molecular weight of 145,000 for an extracellular CGTase consisting of two subunits having a molecular weight of 74,000. By using dodecyl sulfate-gel electrophoresis, we found a molecular weight of 75,000, indicating no subunits. However, the amino acid composition of our product is quite similar to that reported by DePinto and Campbell[8], except for the content of proline, which is higher in our enzyme. Smaller differences were also found for serine, valine, isoleucine, and phenylalanine (Table II). It is quite surprising that two molecules with such different molecular weights have similar amino acid compositions. Our enzyme does, however, have a molecular weight quite close to that of the monomer of the CGTase reported by Kobayashi *et al.*[11], although that monomer had no enzyme activity. The same enzyme in an alkalophilic *Bacillus* sp. was found to have a molecular weight of 88,000 by use of dodecyl sulfate-gel electrophoresis[10].

The isoelectric point was found to be pH 5.4, which is different from that (4.5) reported by Schwimmer[20]. However, Nakamura and Horikoshi[10] found an isoelectric point of 5.4 for the CGTase in their alkalophilic *Bacillus* sp.

The pH and temperature optima for the CGTase were pH 5.4–5.8 and 60°,

respectively (Figs. 2 and 3); exactly the same values were found by Kobayashi *et al.*[11], whereas DePinto and Campbell[8] found a pH optimum of 6.1–6.2. The properties of the enzyme from the alkalophilic *Bacillus* sp. were very different, with pH and temperature optima of 4.5–4.7 and 45°, respectively[10]. The temperature stability of the CGTase was also very similar to that reported by Kobayashi *et al.*[11]. At pH 6.0, the enzyme lost very little activity before the temperature passed 50° (Fig. 4).

The K_m value for soluble starch was found to be 5.7 mg/ml at pH 6.0 and 60°. This value is somewhat higher than that (3.33 mg/ml) reported by DePinto and Campbell[8] at pH 6.2 and 40°. Hale and Rawlins reported that maltose and D-glucose stimulated the enzyme activity, whereas cyclohepta-amylose inhibited the enzyme activity[6]. However, we could not confirm these results and found that neither cyclohexa-amylose nor cyclohepta-amylose had any effect up to 500mM.

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