Purification and Properties of Cyclodextrin Glycosyltransferase of an Alkalophilic *Bacillus* sp.

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Received November 7, 1975

Extracellular cyclodextrin glycosyltransferase (α -1,4-glucan 4-glycosyltransferase, cyclizing, EC 3.2.1.19) of an alkalophilic *Bacillus* sp. (ATCC 21783) was purified about 74-fold and shown to be a single, homogeneous protein by disc polyacryl amide gel electrophoresis and ultracentrifugation. The molecular weight and isoelectric point were 88,000 and pH 5.4. The optimum pH for the enzyme action was 4.5~4.7. The apparent V_{max} and Km values for α -, β - and γ -cyclodextrin at the constant concentration of sucrose were 133.3, 23.4, 12.3 μ moles glucose/min per mg protein and 5.88, 0.39, 0.25 mM, respectively. The enzyme converted about 73% of starch, 65% of amylopectin, 45% of glycogen and 25% of amylopectin β -limit dextrin to cyclodextrins.

The Schardinger dextrins (cyclodextrins, CD) are homologous series of non-reducing, cyclic, D-glucose polymers ranging in size from six to twelve glycosyl ring structure and beyond.^{1,2)} The existence of exo- and endo-branched cyclic dextrins has been also reported.³⁾ These dextrins are synthesized from starch and related materials by cyclodextrin glycosyltransferase (CGTase) produced from *Bacillus* sp. Although the properties and action on starch of the enzyme of *B. macerans* (BMA) have been extensively studied,^{4~8)} the investigations of those from other strains have not been reported so much.^{9~12)}

We have isolated an alkalophilic soil bacterium which produce extracellular cyclodextrin glycosyltransferase by an alkalophilic medium containing 1% Na_2CO_3 . This paper deals with the purification procedures and some properties of the enzyme of this strain (alkalophilic *Bacillus* sp., ATCC 21783). The mode of action of the enzyme will be reported latter studies.

MATERIALS AND METHODS

Microorganism. An alkalophilic *Bacillus* sp. (ATCC 21783) was maintained on the II-medium agar slant¹³⁾ and stored at room temperature.

CGTase activity. The reaction mixture containing 5 mM α -cyclodextrin, 25 mM sucrose, 0.5 mg of glucoamylase in 0.35 ml of 0.2 M acetate buffer (pH 4.65) and 10 μ l of the enzyme suitably diluted with distilled water was incubated at 40°C for 10 min. The reaction was stopped by the addition of 1 ml of 3,5-dinitrosalicylic acid (DNS) solution and then heated in a boiling water bath for 5 min. Absorbance was determined at 510 nm with using glucose as a standard by DNS-method as described by Summer and Somers.¹⁴⁾ One unit of the enzyme is defined as the amount of enzyme to liberate 1 μ mole of glucose per minute under the described conditions.

Dextrinizing activity. Dextrinizing activity was determined by the measurement of dextrinizing power using amylose as a substrate according to Fuwa's method¹⁵⁾ with slight modification. In the standard assay, the reaction mixture containing 10 μ l of the enzyme suitably diluted with distilled water and 0.3 ml of 0.2% amylose in 0.2 M acetate buffer (pH 4.65) was incubated at 40°C for 10 min. The reaction was stopped with 1 ml of 0.5 M acetic acid and 0.5 ml of 0.02% $\text{I}_2/0.2\%$ KI solution. Distilled water was added to make a volume of 10 ml, and absorbance at 700 nm was measured. One unit of the enzyme is defined as the amount of enzyme which produces 10% reduction in the intensity of blue colour of amylose-iodine complex per minute under the described conditions.

Other analytical methods. The formation of cyclodextrins was monitored during the purification by trichloroethylene test. The reaction mixture con-

taining 2 ml of 3% potato starch in 0.1 M acetate buffer (pH 4.65), 0.5 ml of trichloroethylene and 0.5 ml of the enzyme was incubated for 30 min at 40°C. White precipitate was formed by vigorous stirring when cyclodextrins were formed. The yield of cyclodextrins was determined by the glucoamylase method described by Kobayashi *et al.*^{16,17)} Protein was estimated by the method of Warburg and Christian.¹⁸⁾ Disc polyacrylamide gel electrophoresis was carried out by the method of Davis.¹⁹⁾ Ampholine isoelectrofocusing was done by the method of Vesterberg and Svesson.²⁰⁾ Molecular weight was estimated by SDS-disc polyacrylamide gel electrophoresis described by Neville.²¹⁾ Total carbohydrate was determined by anthrone- H_2SO_4 method described by Morris.²²⁾

Materials. The amylase of *B. macerans* (IFO 3490) was prepared by the method of Kitahata *et al.*⁹⁾ Amylose and glucoamylase (Pure grade, 22.3 U/mg, EC 3.2.1.3) of *Rhizopus niveus* were purchased from Seikagaku Kogyo Co. Schardinger α -dextrin was obtained from Sigma Chemicals Co., U.S.A., and γ -dextrin was kindly donated by Dr. T. Kuge (Kyoto Pref. Univ.). Schardinger β -dextrin, maltotriose, corn starch and corn steep liquor were supplied by Nihon Shokuhin Kako Co. Amylopectin β - and α, β -limit dextrin were generous gifts of Dr. K. Watanabe of our laboratory. Ampholine carrier ampholite (pH 3.5~10) was from L.K.B.-Producter, Sweden. Ficoll was from Pharmacia Fine Chemicals Co., Sweden.

RESULTS

Purification of the enzyme

Unless stated otherwise, all operations were carried out at room temperature.

1. *Preparation of the crude CGTase.* The organism was grown aerobically for 2 days at 37°C in the medium consisting (g/liter) 10 g of soluble starch, 50 g of corn steep liquor, 1 g of K_2HPO_4 , 0.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and separately sterilized 10 g of Na_2CO_3 . The cells were removed by centrifugation at 0°C. To 1 volume of the supernatant, 3 volumes of cold acetone (-20°C) were added, and the mixture was stored in a cold room for overnight. The precipitate formed was collected by centrifugation and resolved with tap water. This solution was dialyzed against running tap water for 2 days.

2. *Starch adsorption.* Corn starch (3%) and ammonium sulfate (20%) were added to the dialyzate and stirred for 60 min at 0~

4°C. The corn starch which adsorbed the enzyme was collected by filtration and washed with 10 mM phosphate buffer (pH 7.0) containing 20% (w/v) of ammonium sulfate and 1 M NaCl. The enzyme was eluted from corn starch with 10 mM of the same buffer containing 3 M NaCl and 0.1 M maltose. The effluent was dialyzed for 2 days against running tap water and concentrated by polyethylene glycol (PEG) 6000.

3. *Biogel p-100 gel filtration.* The concentrate was passed through a Biogel p-100 column (2.6×100 cm) which was equilibrated with 10 mM glycine-NaOH-NaCl buffer (pH 8.5) containing 0.1 M NaCl. The active fractions were collected and concentrated by PEG 6000. The concentrate was dialyzed against 10 mM glycine-NaOH-NaCl buffer (pH 8.5).

4. *DEAE-cellulose chromatography.* The dialyzate was loaded onto a DEAE-cellulose column (1.5×45 cm) equilibrated with the same buffer described above. After the column was washed with the same buffer, the enzyme was eluted with a linear gradient of NaCl. A linear gradient of NaCl was obtained by using a Ultrograd (LKB-producter AB, Sweden). Active fractions were collected and concentrated by PEG 6000.

5. *Sephadex G-100 gel filtration.* The concentrate was passed through a Sephadex G-100 column (1.5×100 cm) which equilibrated with the same buffer containing 0.1 M NaCl. A typical elution pattern is shown in Fig. 1. Active fractions were collected and concentrated by Ficoll 400. This enzyme was used for the following experiments. The results are summarized in Table I.

Homogeneity of the enzyme

The purified enzyme gave a single band of protein by disc gel electrophoresis (Fig. 2) and showed a symmetrical peak by ultracentrifugation ($s_{20,w}$; 4.6) and isoelectrofocusing (pI; 5.4). In 10 mM phosphate buffer (pH 8.0), the enzyme showed a typical absorption spectrum of protein. The ratio of absorption at 280 nm to 260 nm was about 1.9. This value indicated the absence of nucleic acid.

TABLE I. PURIFICATION SUMMARY OF THE CGTASE OF ALKALOPHILIC *Bacillus* sp.

Steps	Volume (ml)	Activity (Units)	Protein (mg)	Sp. activity (Units/mg)	Recovery (%)
Culture fluid	300	10,440	9550	1.09	100
Starch adsorption	540	10,750	250	43.0	103
Biogel p-100	26.5	9,290	140	66.4	89
DEAE-cellulose	190	4,810	64	75.2	46
Sephadex G-100	30	3,800	47	81.1	35

FIG. 1. Gel Filtration of Cyclodextrin Glycosyltransferase of Alkalophilic *Bacillus* sp. on a Sephadex G-100 Column.

The experimental details are described in the text. Symbols express; the enzyme activity (●—●), absorbance at 280 nm (○—○).

FIG. 2. Disc Gel Electrophoresis Pattern of the Purified Enzyme.

The purified CGTase containing about 20 μ g of protein was subjected to a column of the electrophoresis at pH 9.4.

The molecular weight was calculated as 88,000 by SDS-disc polyacrylamide gel electrophoresis.

Effect of pH on activity

Because of inability to determine at all of pH ranges tested by the standard assay, the

FIG. 3. Effect of pH on Activity.

The pH vs CGTase activity was measured as described in the text. The pH was adjusted with the following buffer systems; acetate buffer (pH 3~5), phosphate buffer (pH 5.5~8). Solid line expresses pH vs activity curve of BMA and dotted line expresses alkalophilic *Bacillus* sp. enzyme

CGTase activity was measured by following method. The reaction mixture containing 5 mM α -cyclodextrin, 25 mM sucrose in 0.3 ml of 50 mM various buffer solutions and 10 μ l of the enzyme was incubated at 40°C for 10 min. The reaction was terminated by heating in a boiling water bath for 10 min. The reaction mixture was incubated with 0.5 mg of glucoamylase in 0.5 ml of 0.5 M acetate buffer (pH 5.0) at 45°C for 2 hr. Glucose thus liberated was determined as described above. As a reference, BMA was tested under the same conditions. As shown in Fig. 3, the optimum pH for the enzyme action of the enzyme was 4.5~4.7.

Effect of pH on stability

The enzyme was dissolved in various buffer solutions and heated at 60°C for 30 min. After the enzyme was brought to pH 4.65,

the residual activity was measured under the standard assay method. The enzyme was stable at the range of pH 6~10 under the tested conditions (Fig. 4).

FIG. 4. Effect of pH on Stability.

About 50 μ g of the enzyme in 0.1 ml of 0.05 M various buffer solutions was incubated at 60°C. After 30 min, the enzyme solution was brought to pH 4.65 by the addition of 0.5 ml of 0.5 M acetate buffer (pH 4.65), and the residual CGTase activity was measured by the standard assay. The pH was adjusted with the following buffer systems; acetate (pH 4~5), phosphate (pH 6~8), glycine-NaOH-NaCl (pH 9~12).

FIG. 5. Effect of Temperature on Activity

The CGTase activity was measured at various temperatures as follows. The reaction mixture containing 5 mM α -cyclodextrin, 25 mM sucrose in 0.3 ml of 0.2 M acetate buffer (pH 4.65) and 10 μ l of the enzyme was incubated at various temperatures for 10 min. The reaction was stopped by heating in a boiling water bath for 10 min. Then, the reaction mixture was incubated with 0.5 mg (50 μ l) of glucoamylase solution at 45°C for 5 hr. Glucose thus formed was determined as described above.

Effects of temperature on activity

The enzyme activity was measured at pH 4.65 and various temperatures. As shown in Fig. 5, the optimum temperature for the enzyme action was about 45°C.

Thermal stability

The enzyme dissolved in 0.1 M glycine-NaOH-NaCl buffer (pH 9.0) was treated at various temperatures for 30 min. And the residual activity was measured at pH 4.65. The reaction was also done in the presence of 10 mM CaCl_2 or 1 mM EDTA. As a reference, the enzyme also treated by the same method in a 0.1 M acetate buffer (pH 4.65). As shown in Fig. 6, although the enzyme was very un-

FIG. 6. Thermal Stability.

About 50 μ g of the enzyme in 0.1 ml of 0.1 M glycine-NaOH-NaCl buffer (pH 9) was incubated at various temperatures for 30 min. The residual CGTase activity was measured by the standard assay. The reaction was also done in the presence of 10 mM CaCl_2 or 1 mM EDTA. As a reference, the enzyme also treated in 0.1 M acetate buffer (pH 4.65). Symbols express that (●—●) + CaCl_2 , (×—×) + EDTA, (○—○) control, and (△—△) treated at pH 4.65.

stable when the incubation was done at acidic condition, the enzyme stable up to 65°C and slightly stabilized by Ca^{2+} , and the addition of EDTA decreased its thermal stability.

Effect of co-substrates on activity

The effect of various cosubstrates on the dextrinizing activity was compared with that

of BMA. Each of carbohydrates which was added to 0.3 ml of 0.2% amylose in 0.2 M acetate buffer (pH 4.65) was incubated with 10 μ l of the enzyme at 40°C for 10 min. The decrease of blue colour of amylose-iodine complex was determined by the method described above.

TABLE II. EFFECT OF CO-SUBSTRATES ON DEXTRINIZING ACTIVITY

Compounds	Concn. (%)	Relative activity (%)	
		The CGTase	BMA
Control	—	100	100
Glucose	0.05	270	370
Maltose	"	330	550
Maltotriose	"	250	440
Sucrose	"	170	120
Fructose	"	140	97
Lactose	"	150	95
Sorbitol	"	140	95
Schardinger β -dextrin	0.3	100	65

As shown in Table II, the rate of the enzyme action was accelerated by the addition of various saccharides. Starch hydrolyzates such as glucose, maltose and maltotriose were less effective, but other carbohydrates were more effective than that observed by BMA. The enzyme was not inhibited by β -cyclodextrin at the tested concentration.

Determination of V_{max} and K_m values of glycosyl residues transfer action

The glycosyl residue transfer action to sucrose was examined by double reciprocal plot (Fig. 7). The apparent maximum velocity (V_{max}) and Michaelis constant (K_m) values for α -, β - and γ -cyclodextrin at a constant concentration of sucrose were 133.3, 23.4, 12.3 μ moles glucose/min per mg of protein and 5.88, 0.39, 0.25 mM, respectively.

Formation of cyclodextrins from starch and related materials

Formation of cyclodextrins from various substrates was investigated. As shown in Fig. 8, the enzyme converted 73% of potato starch, 65% of amylopectin, 45% of oyster

FIG. 7. Double Reciprocal Plot with α -, β - and γ -Cyclodextrin as Variable Substrate.

The reaction mixture containing 25 mM sucrose, 0.5 mg glucoamylase, 0~5 mM cyclodextrin in 0.35 ml of 0.2 M acetate buffer (pH 4.65) and 10 μ l of the enzyme was incubated at 40°C for 10 min. The reaction was stopped by the addition of 1 ml of 3,5-DNS solution, and the mixture was heated in a boiling water bath for 5 min. After the mixture was made up to 5 ml with distilled water, the absorbance was measured at 510 nm with using glucose as a standard. Symbols express that (●—●); α -cyclodextrin, (○—○); β -cyclodextrin, (×—×); γ -cyclodextrin.

glycogen and 25% of amylopectin β -limit dextrin at 1% (w/v) of substrates concentration.

DISCUSSION

The CGTase of an alkalophilic *Bacillus* sp. was purified about 74-fold by corn starch adsorption, Biogel p-100 gel filtration and DEAE-cellulose chromatography followed by Sephadex G-100 gel filtration.

The purified enzyme was homogeneous on ultracentrifugation, disc polyacrylamide gel electrophoresis and ampholine isoelectrofocusing. The enzyme thus obtained was very thermostable at alkaline region and having a optimum pH range for the enzyme action at 4.5~4.7. As a reference, BMA was compared with this enzyme. The enzyme was different from BMA in accordance with the isoelectric point (BMA, pI; 4.6), optimum pH for the

FIG. 8. Formation of Cyclodextrins from Starch and Related Materials.

The reaction mixture containing 1% (w/v) of various substrates in 2 ml of 0.2 M acetate buffer (pH 4.65) was incubated with 15 μ g (10 μ l) of the enzyme at 45°C for various periods. The aliquot (0.3 ml) was heated in a boiling water bath for 10 min to terminate the reaction and then re-incubated with 0.25 mg of glucoamylase at 40°C for 20 hr to hydrolyze saccharides which were not converted to cyclodextrins. The yield of cyclodextrins was calculated from the difference of total reducing sugar before and after the reaction of the CGTase. Symbols express that (○—○) potato starch, (●—●) potato amylopectin, (△—△) oyster glycogen and (×—×) potato amylopectin β -limit dextrin.

enzyme action and the effect of co-substrates on the dextrinizing activity. The rate of dextrinizing action of the purified enzyme was less accelerated by starch hydrolyzates such as glucose, maltose and maltotriose. In addition, the enzyme was not inhibited by β -cyclodextrin by which BMA was inhibited. The apparent V_{max} and K_m values determined for α -cyclodextrin were larger than those for β - or γ -cyclodextrin. This result shows that α -cyclodextrin is easily hydrolyzed and transferred the glycosyl residue to some acceptors, but the formation of the enzyme- α -cyclodextrin complex is slower than those of other cyclodextrins. The enzyme converted starch and related materials to cyclodextrins with somewhat high yields than those so far reported by BMA¹¹ or other enzymes.^{11,12} This fact suggests that the branching points of amylopectin will be bypassed or incorporated to cyclodextrins molecule.

In this work, although we failed to purify

another CGTase(s) of this bacterium, at least, two CGTases might be containing in the crude enzyme. The enzyme(s) having pH-optimum for the enzyme action in alkaline region was more unstable than the purified enzyme, especially, at the conditions of acidic and alkaline pH. The alkaline CGTase(s) in the crude enzyme was easily inactivated by the heat treatment (pH 10.5, 60°C, 5 min), though the CGTase having pH-optimum at 4.5~4.7 was remained almost all of its activity. Purification of these alkaline CGTase(s) is now studying.

As the results, we wish to conclude that the purified CGTase of alkalophilic *Bacillus* sp. (ATCC 21783) is different from other CGTases, especially in pH-optimum for the enzyme action, thermal stability and yields of cyclodextrins from various substrates.

Acknowledgements. The authors indebted to Drs. T. Kuge and K. Watanabe for the gifts of Schardinger γ -dextrin, amylopectin β and α , β -limit dextrin.

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