Purification and Some Properties of Cyclodextrin Glycosyltransferase from a Strain of *Bacillus* Species

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An ultracentrifugally homogeneous preparation of the cyclodextrin glycosyltransferase was obtained from the culture broth of a strain of *Bacillus* species (No. 5 strain) by a purification methods including adsorption on corn starch, ammonium sulfate precipitation, chromatography on DEAE-cellulose column, and gel-filtration on Sephadex G-100. This enzyme preparation (No. 5 enzyme) was separated into two fractions in disc-electrophoresis and Ampholine-electrophoresis (Fr. 1: pI 6.07 and Fr. 2: pI 6.80) which had the ability to form cyclodextrin. These two fractions were similar to each other in the enzymic properties except the isoelectric point. These enzymes differ from the amylase from *Bacillus macerans* (BMA) in the enzymic properties. Furthermore, No. 5 enzymes differ from BMA in the action on starch. The authors consider that No. 5 enzymes mainly produce the cycloheptaamylose from starch and on the contrary, BMA mainly produces the cyclohexaamylose from starch.

Cyclodextrin glycosyltransferase-(α -1,4-glucan 4-glycosyltransferase, cyclizing) is an unique enzyme which has the ability to convert starch to cyclodextrin (homogeneous cyclic molecules of six or more α -D-glucopyranose units linked 1-4 as in amylose). This enzyme has also been reported to catalize coupling reactions whereby linear series of oligosaccharides are formed when α -dextrin (cyclohexaamylose) and a cosubstrate such as glucose, maltose or sucrose are incubated with a crude enzyme preparation.

Since in 1939 Tilden and Hudson announced the discovery of a cell free enzyme preparation from cultures of *Bacillus macerans* which had the ability to form the cyclodextrin from starch, this enzyme (BMA) has been investigated in detail and very actively for a long time.^{1~6)} However, so far no other source, microbial or otherwise, has been found for this unique enzyme.

Recently, we have obtained direct evidence for the presence of cyclodextrin glycosyltransferases in the culture filtrates from the strains of *Bacillus* species different from *B.*

macerans enzyme.⁷⁾ The present paper contains the purification and the general properties of the cyclodextrin glycosyltransferase (No. 5 enzyme) produced by the typical strain (No. 5 strain) selected from the several strains of *Bacteria* isolated from soil.

MATERIALS AND METHODS

Purified Bacillus macerans amylase. Stock culture of *Bacillus macerans* IFO 3490 was usually cultivated in a 500 ml of shaking flask containing 70 ml of the medium on a reciprocal shaker at 37°C for 70 hr. The medium used for the production of amylase was composed of corn steep liquor 1%, soluble starch 1%, ammonium sulfate 0.5% and calcium carbonate 0.5%. Solid ammonium sulfate was added to the supernatant (2500 ml) obtained by centrifugation of the culture to 30% saturation. The mixture was cooled to 5°C and then passed through a column containing a mixture of corn starch granules (150 g) and hyflo super-cel (60 g) under cooling. The adsorbed enzyme was eluted with 1200 ml of 0.033 M Na_2HPO_4 and the effluent was salted out with 0.65 saturation of ammonium sulfate. The resulting precipitate was separated by filtration, dissolved in 80 ml of the distilled water and dialyzed against the running water. The dialyzed enzyme solution (106 ml) was applied to a DEAE-Sephadex column (19 $\times$ 430 mm), equilibrated with 0.01 M tris-HCl buffer (pH 7.3). The column was washed with the same buffer and the enzyme was eluted with a linear gradient of sodium chloride concentration from 0 to 0.5 M

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FIG. 1. Ultracentrifugal Pattern of *Bacillus macerans* Amylase (BMA).

Protein concentration was 9 mg per ml.^{a)} The photographs were taken at intervals as indicated after reaching 60,000 rpm. Sedimentation was carried out at 20°C.

^{a)} Assuming that $E_{1\text{cm}}^{1\%}$ at 280 nm was 10.0.

FIG. 2. Disc-electrophoresis Pattern of Purified BMA.

The purified enzyme solution, about 100 μ l, which contained about 200 μ g protein, was subjected to a column of the electrophoresis at pH 9.4 as described in the text.

in the buffer. The active fractions were combined and concentrated to 5 ml using a collodion bag. The concentrated enzyme solution was finally purified by Sephadex G-75 gel column chromatography (17 $\times$ 1400 mm), equilibrated with 0.01 M phosphate buffer (pH 7.0). The active fractions were combined and used as the purified enzyme preparation. This preparation was purified about 200 fold and the specific activity was 260 (units/O.D. at 280 nm). This purified enzyme was homogeneous on the ultracentrifugation and also on the disc-electrophoresis as shown in Fig. 1 and Fig. 2.

Assay of enzyme activity. The culture liquor did not contain any significant quantity of the α -amylase, and so the enzyme activity was measured as follows. The reaction mixture containing 0.5 ml of enzyme solution and 4.5 ml of 0.55% soluble starch in 0.05 M acetate buffer (pH 5.5) was incubated at 40°C for 10 min. After that, 0.5 ml of the reaction mixture is removed and added to 4 ml of 0.01 M iodine in 0.25 M potassium iodide. This mixture was diluted to 20 ml with the distilled water, and then the light transmission of the solution was measured at 660 nm in a Hitachi 181 spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme which gave a linear increase of 1% transmission per minute at 40°C.

In each step of the purification, trichloroethylene test was performed in order to check the formation of cyclodextrin.⁷⁾

Analytical methods. The total sugars were measured by phenol-H₂SO₄ method.⁸⁾ Iodine test is followed by the method of Tilden and Hudson microscopic test.²⁾ Protein concentration was determined spectrophotometrically by measuring the absorbance at 280 nm in a Hitachi 181 spectrophotometer.

Analysis of homogeneity. Disc-electrophoresis of the enzyme preparation was carried out by the method of Davis and Nagai^{9,10)} at pH 9.4 with an apparatus of M. S. Instruments Co. For the determination of the isoelectric point, isoelectric focusing was performed by the method of Matsuo¹¹⁾ in a 110 ml column using carrier Ampholites (pH 3~10, LKB-Producter AB, Sweden) for 64 hr at 300 volt, 4°C. Ultracentrifugation was carried out at 60,000 rpm at 20°C with a Hitachi UCA 1 analytical ultracentrifuge.

RESULTS

Purification of the No. 5 enzyme

A *Bacillus* sp., strain No. 5, isolated from soil was used throughout this investigation. A variety of media was tested for the production of much more cyclodextrin glycosyltransferase activity. So far tested, the suitable medium was that consisted of wheat bran 1%, polypepton 1%, corn steep liquor 1%, soluble starch 4% and dry yeast 0.5%. The strain No. 5 was cultivated in 500 ml of shaking flask containing 60 ml of medium on a reciprocal shaker at 37°C for 70 hr. The organisms were removed from the culture liquor by centrifugation.

The supernatant was used as the crude enzyme solution. Solid ammonium sulfate was added to the crude enzyme solution (2000 ml) to 25% saturation, and then the precipitate was removed by filtration with Celite. The resulting clear solution was cooled to 5°C, and charged on corn starch layer (corn starch 150 g and Hyflo Super-Cel 60 g). The adsorbed enzyme was eluted with 960 ml of 0.033 M Na_2HPO_4 and the effluent was salted out with 0.65 saturation of ammonium sulfate. After standing overnight, the precipitate was collected by filtration and dissolved in distilled water. This enzyme solution was dialyzed overnight against the running water, and the insoluble material formed was removed by centrifugation. The dialyzed enzyme solution (180 ml) was placed on the DEAE-cellulose column (20×414 mm), previously equilibrated with 0.01 M Tris-HCl buffer (pH 8.0). Active fractions which passed through the column, were combined (300 ml) and concentrated to 10 ml by using a collodion bag. The concentrated enzyme solution was applied to gel-filtration with Sephadex G-75 column (28×1300 mm), equilibrated with 0.01 M phosphate buffer (pH 7.0). Filtration was performed with the same buffer. The elution pattern thus obtained is shown in Fig. 3. The frac-

FIG. 3. Gel-Filtration of Cyclodextrin Glycosyltransferase from the Strain of No. 5 on a Sephadex G-75.

The experimental conditions are described in the text. ●—●, activity of cyclodextrin glycosyltransferase; ○---○, absorbance at 280 nm.

tions from No. 47 to No. 56 were collected and used as the enzyme preparation to examine the homogeneity. The results of the purification are summarized in Table I. The enzyme preparation of the final stage was purified about 130-fold, and the specific activity was 258 (units/O.D. at 280 nm).

Homogeneity of the enzyme preparation

In order to check the homogeneity of the enzyme preparation, ultracentrifugation, disc-

TABLE I. PURIFICATION PROCEDURE OF CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM THE STRAIN OF No. 5

	Vol. (ml)	Total activity (units)	Specific activity (units/O.D. _{280nm})	Yield (%)
Culture liquor	2000	1.34×10^5	1.91	100
Added $(\text{NH}_4)_2\text{SO}_4$ (0.25 satn.) and filtrated by celite				
Supernatant	2400	1.51×10^5	2.00	113
Adsorbed on corn starch and eluted by 0.033 M Na_2HPO_4				
Effluent	960	1.00×10^5	14.0	75
Salted out with $(\text{NH}_4)_2\text{SO}_4$ (0~0.6 satn.) and dialyzed				
Dialyzed soln.	180	1.06×10^5	128	79
Charged on DEAE-cellulose and washed				
Effluent	300	8.05×10^4	200	60
Concentrated and gel-filtrated by Sephadex G-75				
Purified enzyme	105	6.75×10^4	258	50

FIG. 4. Ultracentrifugal Pattern of No. 5 Enzyme. The conditions were same as described in Fig. 1.

FIG. 5. Disc-electrophoretic Pattern of No. 5 Enzyme.

The conditions were same as described in Fig. 2.

FIG. 6. Isoelectric Focusing of No. 5 Enzyme.

The experiment was performed in a 110 ml column containing carrier ampholites pH 3~10 (1%, v/v) for 64 hr at 300 volt, 4°C.

●—●, activity of cyclodextrin glycosyltransferase;
○---○, absorbancy at 280 nm; ----, pH.

electrophoresis with polyacrylamide gel and isoelectric focusing were performed. The enzyme preparation showed a single symmetrical peak in the ultracentrifuge analysis as shown in Fig. 4. However, by the disc-electrophoresis at pH 9.4, this enzyme pre-

paration gave two bands as shown in Fig. 5. Also in the isoelectric focusing, the preparation was separated into two fractions, Fr. 1 (pI: 6.07) and Fr. 2 (pI: 6.80), both of which had the cyclodextrin glycosyltransferase activity. An electrophoretic profile is shown in Fig. 6. Therefore, in the following experiments, each fraction (Fr. 1, Fr. 2) was used as the purified enzyme preparation.

Enzymatic properties

1) *Effect of pH on activities and stabilities of three enzymes.* The pH-activity curves of three purified enzymes, Fr. 1, Fr. 2 and BMA are presented in Fig. 7. These three enzymes were similarly most active in the pH

FIG. 7. pH-Activity Curves of Three Purified Enzymes of Fr. 1, Fr. 2 and BMA.

The reaction mixtures consisted of 0.5 ml of the enzyme solution, 2.5 ml of 1% soluble starch and 2 ml of 0.1 M Mcllvaine buffer, were incubated at 40°C for 10 min.

●—●, Fr. 1; △—△, Fr. 2; ○---○, BMA.

range from 5 to 5.7, when checked by the standard assay method. However, in alkaline side the pH-activity curves of Fr. 1 and Fr. 2 were much broader than that of BMA.

FIG. 8. pH-Stability Profiles of Three Enzymes of Fr. 1, Fr. 2 and BMA.

Aliquots of the enzyme solution (1 ml) were incubated in 0.05 M McIlvaine buffer (pH 5.0~10.0), NaCl-NaOH-glycine buffer (pH 10.0~12.0) at 40°C for 2 hr. After that this solution were diluted with 0.1 M acetate buffer (pH 5.5) 4 ml and assayed for remaining activity.

●—●, Fr. 1; △—△, Fr. 2; ○---○, BMA.

FIG. 9. Temperature-activity Relationships of Three Enzymes of Fr. 1, Fr. 2 and BMA.

The reaction mixtures consisted of 0.5 ml of the enzyme solution, 2.5 ml of 1% soluble starch and 2 ml of 0.1 M acetate buffer (pH 5.5) were incubated at the indicated temperature for 10 min.

●—●, Fr. 1; △—△, Fr. 2; ○---○, BMA.

To examine the pH-stability relationships of three purified enzymes, the enzymes were incubated for 2 hr at 30°C in buffer solution of various pH values, and the residual activities were determined at pH 5.5. As shown in Fig. 8, two enzymes (Fr. 1 and Fr. 2) were stable over the range of pH 7.0 to 10.0, and BMA was stable over the range of pH 8.0 to 10.0.

2) *Effect of temperature on activities and stabilities of three enzymes.* The enzyme reaction was carried out at pH 5.5 for 10 min at various temperatures. The temperature optimums for the enzyme reaction of three enzymes (Fr. 1, Fr. 2 and BMA) were about 55°C as shown in Fig. 9.

To check the thermal stability, these enzymes were incubated at pH 7.0 and various temperatures, and after 10 min, the remaining activities were assayed by the standard method. The result is shown in Fig. 10. Two enzymes of No. 5 were stable at temperatures below 55°C, and BMA was stable at temperatures below 60°C.

Comparison of the cyclodextrins formed by the

FIG. 10. Thermal Stabilities of Three Enzymes of Fr. 1, Fr. 2 and BMA.

Aliquots of the enzyme solution in 0.05 M phosphate buffer (pH 7.0) were incubated at the indicated temperature for 10 min, cooled immediately at 0°C for 30 min and assayed for the remaining activities.

●—●, Fr. 1; △—△, Fr. 2; ○---○, BMA.

FIG. 11. Comparison of the Cyclodextrins Formed by the Action of Three Enzymes of Fr. 1, Fr. 2 and BMA on Soluble Starch.

The reaction mixtures of 3% soluble starch (12 ml) and each of three enzymes (3 unit per ml, 6 ml) were incubated at 40°C.

●—●, decrease in iodine colour (blue value); ■, amounts of cyclodextrin precipitated with trichloroethylene.

action of three enzymes (Fr. 1, Fr. 2 and BMA) from starch

Each of three enzymes (3 units/ml, 6 ml) was reacted with 3% soluble starch (12 ml) at 40°C. At certain intervals, 2 ml aliquots of the reaction mixture were removed to examine the decrease in iodine color (blue value), the amounts of cyclodextrin precipitated with trichloroethylene (TCE) and its iodine test. The results are shown in Fig. 11. The decrease in iodine color of three enzymes were similar to each other. Namely, three enzymes decompose the soluble starch in the same rate. Two of No. 5 (Fr. 1 and Fr. 2) among three enzymes produced the same amounts of cyclodextrin precipitated with TCE. However, in the case of BMA the amounts of cyclodextrin precipitated with TCE were much less than that of No. 5 enzymes. In the iodine test, the cyclodextrin formed by the action of BMA formed blue hexagonal plate prisms with I_2 -KI and that of two enzymes of No. 5 formed yellow-brown monoclinic prisms.

DISCUSSION

The cyclodextrin glycosyltransferase produced by No. 5 strain of *Bacillus* species was

purified by the following procedures: adsorption on corn starch, ammonium sulfate precipitation, chromatography on DEAE-cellulose column and gel-filtration on Sephadex G-100. The enzyme obtained was homogeneous in ultracentrifugation, but separated into two fractions in disc-electrophoresis, and ampholine electrophoresis (Fr. 1: pI 6.07, Fr. 2: pI 6.80), which had the ability to form cyclodextrin.

Two fractions of No. 5 enzymes were similar to each other in general properties such as specific activity, optimum pH and temperature and pH and temperature stability except the isoelectric point. Also in the action on starch, two fractions were similar to each other. Further detailed enzymic properties and the correlation between Fr. 1 and Fr. 2 will be described in later studies.

In order to compare No. 5 enzymes with the cyclodextrin glycosyltransferase produced by *Bacillus macerans* IFO 3490 (*Bacillus macerans* amylase, BMA), BMA was purified to an electrophoretically and ultracentrifugally homogeneous state by the same method.

BMA differs from No. 5 enzymes in enzymic properties such as in isoelectric point (BMA, pI: 4.62), optimum pH and temperature and

pH and temperature stability. Also in the action on starch, BMA is different from No. 5 enzymes.

As shown in Fig. 11, the amounts of the cyclodextrin precipitated with TCE in the reaction mixture of BMA on starch were much less than that of No. 5 enzymes. It is reported that the solubility of the cyclohexaamylose (cyclodextrin G6) in the presence of TCE is 0.24 g/100 ml and the cycloheptaamylose (cyclodextrin G7) is 0.03 g/100 ml.⁶⁾ Judging from these results, it is assumed that No. 5 enzymes mainly produce the cycloheptaamylose on the contrary, BMA mainly produces the cyclohexaamylose. Furthermore, the result in the iodine test supports to this assumption. Namely, the cyclodextrin formed by the action of BMA forms the complex with iodine-iodide, blue hexagonal plate prisms, which are characteristic of the cyclohexaamylose. On the other hand, that of No. 5 enzymes formed the complex with iodine-iodide, yellow-brown monoclinic prisms which are characteristic of the cycloheptaamylose. Further confirmation will be described in later studies.

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REFERENCES

- 1) E. B. Tilden and C. S. Hudson, *J. Amer. Chem. Soc.*, **63**, 2900 (1939).
- 2) *Idem*, *J. Bact.*, **43**, 527 (1942).
- 3) D. French, J. Pazur, M. L. Levine and E. Norberg, *J. Amer. Chem. Soc.*, **70**, 3145 (1948).
- 4) J. A. Depinto and L. L. Campbell, *Biochemistry*, **7**, 114 (1968).
- 5) *Idem*, *Arch. Biochem. Biophys.*, **125**, 253 (1968).
- 6) D. French, M. L. Levine, J. H. Pazur and E. Norberg, *J. Amer. Chem. Soc.*, **71**, 353 (1949).
- 7) S. Okada, N. Tsuyama and S. Kitahata, *Amylase symposium*, Japan, **7**, 61 (1972).
- 8) M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
- 9) B. J. Davis, *Ann. N.Y. Acad. Sci.*, **121**, Abst. 2,404 (1964).
- 10) Y. Nagai, *Tampakushitsu Kakusan Koso*, Bessatsu, **IX**, 3 (1967).
- 11) Y. Matsuo and T. Horio, *Tampakushitsu Kakusan Koso*, **12**, 737 (1967).