Purification and Properties of α -Glucosidase from *Bacillus cereus*

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α -Glucosidase has been isolated from *Bacillus cereus* in ultracentrifugally and electrophoretically homogeneous form, and its properties have been investigated. The enzyme has a sedimentation constant of 1.4 S and a molecular weight of 12,000. The highly purified enzyme splits α -D-(1 $\rightarrow$ 4)-glucosidic linkages in maltose, maltotriose, and phenyl α -maltoside, but shows little or no activity toward polysaccharides, such as amylose, amylopectin, glycogen and soluble starch. The enzyme has α -glucosyltransferase activity, the main transfer product from maltose being maltotriose. The enzyme can also catalyze the transfer of α -glucosyl residue from maltose to riboflavin. On the basis of inhibition studies with diazonium-1-H-tetrazole, rose bengal and *p*-chloromercuribenzoate, it is assumed that the enzyme contains both histidine and cysteine residues in the active center.

In the previous papers,^{1,2)} we reported the purification, crystallization and properties of an α -glucosidase from the mycelia of *Mucor javanicus*. There have been far fewer studies on α -glucosidase from bacteria than on α -glucosidases from molds and yeast. Crude or partially purified preparations have so far been used in the studies on the bacterial α -glucosidase. In the course of our investigations on the nature of α -glucosidases from microorganisms, an α -glucosidase was isolated in a highly purified form from a bacterium which was isolated from soil and identified to be *Bacillus cereus*. It was found that the values of the sedimentation constant and the molecular weight of the α -glucosidase were considerably lower than those which were reported for other glycosidases.³⁾ This α -glucosidase was different in the substrate specificity and the transglucosylation action from other α -glucosidases. The enzyme catalyzed also the synthesis of riboflavin α -glucoside from maltose and riboflavin. The present paper deals with the purification and the properties of this α -glucosidase.

MATERIALS AND METHODS

Microorganism

About 30 strains of bacteria were isolated from soil in our laboratory by repeating a plate culture with

a medium containing 3% maltose (technical grade), 0.5% peptone, 0.2% K_2HPO_4 , 0.03% $MgSO_4 \cdot 7H_2O$, 0.01% $CaCl_2 \cdot 2H_2O$ and 2% agar in tap water, and being adjusted to pH 7.0. These strains were grown aerobically at 30°C for 16 hr in 100 ml of the following medium: 1 g of glycerol, 0.5 g of maltose (technical grade), 0.5 g of yeast extract, 0.5 g of peptone, 0.2 g of K_2HPO_4 , 0.03 g of $MgSO_4 \cdot 7H_2O$ and 0.01 g of $CaCl_2 \cdot 2H_2O$ were dissolved in 100 ml of tap water and adjusted to pH 7.0. After incubation, the cells were harvested by centrifugation, washed, and suspended in deionized water to give a cell concentration of 8% (wet weight). The procedure used for the estimation of the α -glucosidase activity of the strains was the same as that given under enzyme assays 1) and 2) in analytical techniques except for the replacement of enzyme with 0.1~1 ml of the cell suspension. One strain was selected from the isolated strains, on the basis of the activities of both maltose decomposition and riboflavin α -glucoside formation. This strain was identified as *Bacillus cereus* in the following ways:

(a) Morphological characters: Vegetative cell, rods, 1.0 to 1.2 by 3.0 to 5.0 μ , with rounded ends. Occurring singly or in short chains on bouillon agar, in long chains on soy bean agar. Gram-positive. Motile. Spore, ellipsoidal to short cylindrical, 1.0 to 1.2 by 1.2 to 2.0 μ . Para-central to subterminal. Many spores were formed in 24 hr. Sporangia, not appreciably swollen.

(b) Cultural characters: Gelatin stab: Liquefaction small crateriform at first, then becoming stratiform. Gelatin agar streak plate: Wide zone of hydrolysis. Agar slants: Growth thin to moderate, rough, opaque, somewhat whitish, non-adherent, spreading, irregular edged with arborescent outgrowth. Glucose agar slants: Growth abundant, heavier and softer than on

agar. Glucose nitrate agar slants: Scant growth. Tyrosine agar slants: Growth moderate with arborescent outgrowth. Soy bean agar slants: Growth much abundant, whitish, very widely spreading by means of long, sometimes few paralleled, curled chains of cells, turning to the left or right. Milk agar streak plate: Wide zone of hydrolysis of casein. Potato: Growth abundant, spreading, somewhat raised, soft, creamy white. Glucose asparagine agar slants: Growth abundant, whitish, rough, irregular edged with few arborescent outgrowth. Broth: Heavy, uniform turbidity with soft, easily dispersed sediment and with soft ring pellicle. Milk: Peptonized after slight coagulation.

(c) Physiological characters: Acid but no gas (with ammonium salts as source of nitrogen) from glucose and sucrose. No acid from mannitol, arabinose or xylose. All of starch hydrolysis, acetylmethylcarbinol production, citrate utilization as sole source of carbon, nitrate reduction, and methylene blue reduction were positive. Indole production, negative. Oxygen demand: Aerobic, facultatively anaerobic.

(d) Pathogenicity: Non-pathogenic.

The above-mentioned properties of the selected strain were found to be identical with those of *B. cereus*, described in Bergey's Manual.

Culture

The medium used for maintaining and culturing *B. cereus* had the following composition (figures in g per liter): glycerol, 10; maltose (technical grade), 5; peptone, 10; yeast extract, 2; K_2HPO_4 , 2; $MgSO_4 \cdot 7H_2O$, 0.3; and $CaCl_2 \cdot 2H_2O$, 0.1. *B. cereus* was picked up from the agar slant of the medium, and inoculated into 500 ml-shaking flasks containing 80 ml of the medium. The incubation was carried out at 30°C for 24 hr on a reciprocating shaker (68 strokes per min, 9 cm amplitude), and 160 ml of a 24 hr culture was used as an inoculum into a 30 liter jar fermenter (rotation of impeller at 300 rpm and aeration at 15 liters per minute) containing 16 liters of the medium. After incubation at 30°C for 2 days, the bacterial cells from four jar fermenters were harvested by centrifugation. The yield of cells was 1860 g, on wet weight basis.

Chemicals and reagents

The markers for the determination of the molecular weight were obtained from Schwarz Mann Inc., (Orangeburg, New York, U.S.A.). Other chemicals and reagents used were the same as described in the previous papers.^{1,2)}

Analytical techniques

Enzyme assays

1) Assay for α -glucosidase activity. When maltose

was used as substrate, the standard reaction mixture contained 0.1 ml of 1% maltose aqueous solution, 0.25 ml of 0.1 M phosphate buffer, pH 6.9, enzyme solution, and deionized water in a final volume of 0.5 ml. After incubation for 15 min at 37°C, the reaction was stopped by heating the mixture in a boiling water bath for 5 min. The amount of glucose formed was determined according to the method of Papadopoulos *et al.*,⁴⁾ as modified by Dahlqvist.⁵⁾ One unit of α -glucosidase activity was defined as the amount of enzyme which formed 1 μ mole of glucose from maltose under the conditions described above. Phenol was determined by the method of Folin and Ciocalteu.⁶⁾

2) Assay for α -glucosyltransferase activity. In the standard assay, the activity was determined by measuring the transfer of α -glucosyl residue from maltose to riboflavin. The reaction mixture contained 2 ml of 5% maltose aqueous solution, 5 ml of 0.06% riboflavin in 0.1 M phosphate buffer, pH 6.9, enzyme solution, and deionized water in a final volume of 10 ml. After incubation at 37°C for 30 min, the reaction was stopped by heating the mixture for 5 min in a boiling water bath. The amount of riboflavin α -glucoside formed in the reaction mixture was estimated by paper chromatography, as described in the previous paper.¹⁾

Protein determination

The protein concentration was determined by measuring absorbances at 260 nm and 280 nm.⁷⁾ The protein concentration of each fraction from a column was followed by measuring the absorbance at 280 nm with a Hitachi Perkin-Elmer model 139 spectrophotometer.

Ultracentrifugal analysis

Ultracentrifugal studies were performed with a Hitachi model 282 analytical ultracentrifuge.

Disc gel electrophoresis

The electrophoresis of the enzyme was carried out in a polyacrylamide gel, pH 9.0, by the method of Davis.⁸⁾ After the run, the gel was stained for proteins with 1% Amido Black 10 B in 7% acetic acid and destained with 7% acetic acid.

Determination of isoelectric point

Isoelectric focusing experiments were carried out at 4°C in a column of 110 ml capacity of an LKB 8100 ampholine electrofocusing equipment, as described previously.¹⁾

Determination of molecular weight

For the determination of the molecular weight, the enzyme was subjected to electrophoresis by the method

of Weber *et al.*¹⁰⁾ with the use of polyacrylamide gel containing sodium dodecyl sulfate at a concentration of 0.1 per cent. After electrophoresis, the distances (in cm) which the marker proteins moved were plotted against the logarithms of their molecular weights. The molecular weight of the purified enzyme was determined from its relative mobilities with the aid of this plot. The markers used were horse heart cytochrome *c* (mol. wt. 12,400), lysozyme (mol. wt. 14,307), sperm whale myoglobin (mol. wt. 17,800), beef pancreas chymotrypsinogen A (mol. wt. 25,000), ovalbumin (mol. wt. 45,000), and bovine serum albumin (mol. wt. 67,000).

RESULTS

1. Isolation of enzyme

Unless otherwise indicated, operations were carried out at 4°C. An outline of the purification procedure and the results obtained are given in Table I.

Step 1: Preparation of crude extract. The cells (1860 g) were suspended in 3.7 liters of 0.05 M phosphate buffer, pH 6.9, containing 1 mM mercaptoethanol. The suspension was crushed out with a Ōtake model 5615 french press (Tokyo, Japan). The intact cells and debris were removed by centrifugation at $14,310 \times g$ for 20 min.

Step 2: First ammonium sulfate fractionation. To the crude extract was added solid ammonium sulfate to 50% saturation. After 30 min, the resultant precipitate was removed by centrifugation and discarded. The su-

pernatant solution was then brought to 100% saturation by the further addition of solid ammonium sulfate. After standing overnight, the precipitate was collected by centrifugation at $14,310 \times g$ for 20 min, and dissolved in 0.05 M phosphate buffer, pH 6.9, containing 1 mM mercaptoethanol. The solution was dialyzed overnight against 0.02 M phosphate buffer, pH 6.9, containing 1 mM mercaptoethanol.

Step 3: DEAE-cellulose column chromatography. DEAE-cellulose was packed into two columns ($4\phi \times 22$ cm) and equilibrated with 0.05 M Tris-HCl buffer, pH 7.0, containing 1 mM mercaptoethanol. The dialyzed preparation was divided into two portions and each portion was applied to a separate column. Elution was carried out with a linear gradient of increasing concentration of sodium chloride (from 0 to 0.5 M) in the same buffer (Fig. 1). The activity appeared in Fractions 102 to 112. To the combined eluate was added solid ammonium sulfate to 100% saturation, and the solution was allowed to stand for 30 min. The precipitate formed was collected by centrifugation, and dissolved in 0.05 M phosphate buffer, pH 6.9, containing 1 mM mercaptoethanol. The solution was dialyzed overnight against 0.02 M phosphate buffer, pH 6.9, containing 1 mM mercaptoethanol.

Step 4: Second ammonium sulfate fractionation. To the dialyzed preparation from Step

TABLE I. PURIFICATION OF ENZYME FROM *B. cereus*

Step	Protein		α -Glucosidase		
	mg	280 nm/260 nm	Unit	S.A. ^{a)}	Recov. %
1. Crude extract	131,580	0.66	738,263	5.6	100
2. Ammonium sulfate fractionation, saturation 50~100%	31,200	0.61	573,813	18.4	77.7
3. DEAE-cellulose chromatography	1,544.7	1.21	180,800	117.0	24.5
4. Ammonium sulfate fractionation, saturation 60~70%	568.7	1.69	107,143	188.4	14.5
5. DEAE-Sephadex A-25 chromatography	142.3	1.59	28,139	197.7	3.8
6. Preparative disc gel electrophoresis	22.1	0.77	1,855	83.9	0.3

^{a)} Unit/protein (mg).

FIG. 1. DEAE-cellulose Column Chromatography.

Applied protein: 15,600 mg.

Flow rate: about 45 ml per hour.

Fraction volume: 15 ml.

●—●, absorbance at 280 nm; ○---○, α -glucosidase activity per 0.01 ml of each fraction.

3 was added solid ammonium sulfate to 60% saturation. After 30 min, the precipitate was removed by centrifugation, and then more solid ammonium sulfate was added to 70% saturation. After standing overnight, the precipitate was collected by centrifugation and dissolved in 0.05 M phosphate buffer, pH 6.9, containing 1 mM mercaptoethanol. The solution was dialyzed overnight against 0.02 M phosphate buffer, pH 6.9, containing 1 mM mercaptoethanol.

Step 5: DEAE-Sephadex column chromatography. The dialyzed solution was then applied to a column of DEAE-Sephadex A-25 ($2.8\phi \times 19$ cm) which had been equilibrated with 0.05 M Tris-HCl buffer, pH 7.0, containing 1 mM mercaptoethanol. Elution was carried out with the same buffer to which was added NaCl. The concentration of NaCl was increased in a stepwise fashion (Fig. 2). The active fractions (No. 75 to 99) were collected, and precipitated by the addition of solid ammonium sulfate to 100% saturation. The precipitate was collected by centrifugation and dissolved in 0.05 M phosphate buffer, pH 6.9, containing 1 mM mercaptoethanol. The solution was dialyzed overnight against 0.02 M phosphate buffer, pH 6.9, containing 1 mM mercaptoethanol.

FIG. 2. DEAE-Sephadex A-25 Column Chromatography.

Applied protein: 568.7 mg. Flow rate: about 45 ml per hour. Fraction volume: 15 ml.

●—●, absorbance at 280 nm; ○---○, α -glucosidase activity per 0.01 ml of each fraction.

Step 6: Preparative disc gel electrophoresis. The dialyzed preparation was subjected to preparative disc gel electrophoresis. The equipment was made up of a running column ($6.8\phi \times 15$ cm) and two buffer reservoirs. The running column was composed of 90 ml of a spacer gel and 360 ml of a separating gel. The concentration of acrylamide was 2.5% in the spacer gel and 7.0% in the separating gel, respectively. Each of the upper and lower reservoirs contained 1000 ml of buffer. The gels and the electrode buffer were prepared by the same method as that of D. E. Williams *et al.*,⁹⁾ except that mercaptoethanol was added to both gels and buffer in concentration of 3 mM in order to preserve the enzyme activity. The dialyzed preparation was divided into 10 portions. To each portion was added sucrose to a final concentration of 20%, and the mixture was layered on the surface of the spacer gel. Electrophoresis was carried out with the anode in the lower reservoir by applying a constant current of 60 mA. The electrode buffer was replaced by the new buffer at 3-hr intervals. The run was carried out until the blue tracking

disc of the indicator dye (bromphenol blue) had reached the one-quarter of the length of the separating gel from the end. The electrophoretic mobility of the active protein band was slightly slower than that of the indicator dye. The gel was removed from the running column, and cut into 3 to 5 mm sections. Each of the gel sections was homogenized with about 20 ml of 0.2 M phosphate buffer, pH 6.9. After standing for 30 min, the enzymatically active homogenates were combined, and filtered to remove the gel particles. The filtrate was dialyzed overnight against 0.02 M phosphate buffer, pH 6.9, containing 1 mM mercaptoethanol, and concentrated by ultrafiltration using a collodion bag. This concentrate was used as the purified enzyme. The dye showing absorption at 260 and 280 nm was not removed from the purified enzyme solution by the procedures of dialysis and ammonium sulfate precipitation. There-

fore, the presence of the dye might be a cause for the result that the ratio of absorptions at 280 and 260 nm and the specific activity of the enzyme solution obtained from Step 6 in Table I were lower than those of Step 5.

II. Criteria of purity

1) *Ultracentrifugal pattern.* The sedimentation pattern of the purified enzyme is shown in Fig. 3. The boundary of the enzyme moved showing a single and symmetrical peak. The value of $s_{20,w}^0$ was calculated as being 1.4 S (Fig. 4).

2) *Disc gel electrophoresis.* When the purified enzyme was subjected to electrophoresis in a polyacrylamide gel at pH 9.5, a single band of protein migrating toward the anode was observed. No contaminants were detected (Fig. 5).

FIG. 3. Sedimentation Pattern of α -Glucosidase.

Ultracentrifugation of α -glucosidase was carried out at a concentration of 0.5% in 0.02 M phosphate buffer, pH 6.9. Experimental conditions: temperature, 20.0°C; speed, 58,000 rpm; cell used, a standard 12 mm double sector cell. The photographs were taken at indicated times after the maximum speed had been reached.

FIG. 4. Concentration Dependence of Sedimentation Constant of α -Glucosidase.

FIG. 5. Disc Electrophoresis of α -Glucosidase.

The purified enzyme protein (200 μ g) was applied to polyacrylamide disc gel electrophoresis at pH 9.5. Electrophoresis was carried out at a constant current of 5 mA per tube for 90 min at 4°C. Tris-glycine buffer, pH 8.3, was used.

III. Properties of enzyme

1) *Isoelectric point.* When the enzyme was subjected to isoelectric focusing, there appeared a single absorbance peak. The isoelectric point of this enzyme was at pH 4.5.

2) *Molecular weight.* The molecular weight of the enzyme, which was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, was 12,000 (Fig. 6).

FIG. 6. Determination of the Molecular Weight of α -Glucosidase.

The marker proteins used were bovine albumin, ovalbumin, chymotrypsinogen A, myoglobin, lysozyme, and cytochrome c. All proteins were run on duplicate gels. The arrow indicates the mobility of α -glucosidase. The extrapolated value for α -glucosidase is 12,000.

3) *Linearity of the enzyme activity.* A linear relationship between the protein concentration and both enzyme activities was observed (Fig. 7).

FIG. 7. Relationship between Enzyme Protein and Activities.

α -Glucosidase and α -glucosyltransferase activities were estimated using various amounts of enzyme under the standard assay conditions.

○—○, α -glucosidase activity in a final volume of 0.5 ml; ●—●, α -glucosyltransferase activity in a final volume of 2.5 ml.

4) *pH Optimum.* As shown in Fig. 8, the pH optimum for the α -glucosidase activity

laid at 6.8 to 7.3.

5) *pH stability*. No loss in the α -glucosidase activity was observed at the pH range of 6.8 to 7.3 (Fig. 9).

FIG. 8. Effect of pH on the Enzyme Activity.

α -Glucosidase activity was estimated using 0.02 ml of enzyme solution (0.38 units) at various pH. Phosphate buffer was used. For other assay conditions see MATERIALS AND METHODS.

FIG. 9. pH Stability of the Enzyme.

The enzyme solution (0.04 ml, 0.76 units) was preincubated for 20 hr at 30°C with 0.2 ml of different buffer: 0.05 M acetate buffer, pH 4.0 to 6.0; 0.05 M phosphate buffer, pH 6.0 to 8.0; 0.05 M veronal buffer, pH 7.0 to 9.0; 0.05 M glycine-NaOH buffer, pH 9.0 to 10.0.

After adjustment of pH with 0.2 M phosphate buffer, pH 6.9, the residual α -glucosidase activity was measured under the standard assay conditions.

6) *Temperature optimum*. The temperature optimum for the α -glucosidase activity was observed to be at 40°C (Fig. 10).

FIG. 10. Effect of Temperature on the Enzyme Activity.

α -Glucosidase activity was estimated using 0.02 ml of enzyme solution (0.38 units) at various temperature. For other assay conditions see MATERIALS AND METHODS.

7) *Heat stability*. As shown in Fig. 11, the enzyme was stable up to 35°C, whereas above this temperature its activity was markedly decreased.

FIG. 11. Heat Stability of the Enzyme.

The reaction mixture containing 0.04 ml of enzyme solution (0.76 units) and 0.36 ml of 0.05 M phosphate buffer, pH 6.9, was preincubated for 15 min at various temperatures.

After preincubation, the residual α -glucosidase activity was measured under the standard assay conditions.

8) *Substrate specificity.* With various substrates, the α -glucosidase activity of the enzyme was examined, and the relative rates of hydrolysis were listed in Table II. Maltose, nigerose, phenyl α -malto-side, and maltotriose were readily served, but isomaltose, kojibiose, sucrose, soluble starch, glycogen, or amylose was very slightly susceptible or resistant to the enzyme. No enzyme activity was observed with maltitol, maltotriitol, cellobiose, melibiose, lactose, or raffinose.

TABLE II. SUBSTRATE SPECIFICITY OF THE ENZYME

The reaction mixture containing 0.04 ml of enzyme solution (0.76 units), 5.55 mM substrate and 50 mM phosphate buffer, pH 6.9, in a final volume of 0.5 ml was incubated at 37°C for 15 min. The reaction was stopped by heating for 5 min in a boiling water bath for the estimation of glucose liberated.

Substrate	Hydrolysis (relative rate %)
Methyl α -glucoside	0
Phenyl α -glucoside	trace
Isomaltose	trace
Kojibiose	0
Maltose	100
Nigerose	62.5
Sucrose	0
Trehalose	0
Turanose	0
Phenyl α -malto-side	46.6
Maltotriose	26.5
Panose	0
Soluble starch ^{a)}	0
Glycogen ^{a)}	0
Amylose ^{a)}	0
Amylopectin ^{a)}	0
Cellobiose	0
Lactose	0

^{a)} 0.1 ml of 1% substrate was used.

9) *Pattern of action on phenyl α -malto-side.* As shown in Fig. 12, the enzyme hydrolyzed phenyl α -malto-side to glucose and phenyl α -glucoside.

10) *Michaelis constant.* The value of K_m for maltose was calculated to be 5.55×10^{-3} M from the Lineweaver-Burk plot (Fig. 13).

FIG. 12. Pattern of Action on Phenyl α -Maltoside.

The reaction mixture (5 ml) containing 0.3 ml of enzyme solution (5.7 units), 0.5 ml of 2% substrate and 4.2 ml of 0.05 M phosphate buffer, pH 6.9, was incubated at 37°C. For estimation of glucose liberated, a portion of the reaction mixture was pipetted out at various time intervals and heated in a boiling water bath for 5 min. For estimation of phenol liberated, 0.5 ml of the reaction mixture was pipetted out at various time intervals, and the reaction was stopped by pouring into 2 ml of 5% Na_2CO_3 .

○---○, glucose liberated; ●—●, phenol liberated.

FIG. 13. Effect of Maltose Concentration on α -Glucosidase.

For the estimation of K_m value, the reaction mixture containing 0.03 ml of enzyme solution (0.57 units), 0.2 ml of 0.1 M phosphate buffer, pH 6.9, indicated amounts of maltose, and deionized water in a final volume of 0.5 ml was incubated at 37°C for 30 min. Velocity (V) was expressed as increased absorbance at 420 nm of the reaction mixture, and substrate concentration (S) as mg per 0.5 ml of the reaction mixture.

11) *Transfer reaction products.* When the enzyme was incubated with 5% maltose, two unknown substances (temporarily named

FIG. 14. A Paper Chromatogram of Transglucosylation Products from Maltose.

The reaction mixture (3 ml) containing 0.75 ml of enzyme solution (concentrated solution, 142.5 units), 1.5 ml of 10% maltose, and 0.75 ml of 0.2 M phosphate buffer, pH 6.9, was incubated at 37°C. At various time intervals, 0.1 ml of the reaction mixture was applied on a Toyo No. 2 filter paper, and developed three times by the ascending method with *n*-butanol-pyridine-water (6:4:3, v/v). After drying, the spots were made visible by the silver nitrate dip method.

X_1 and X_2) which were chromatographically different from either glucose or maltose, were detected on a paper chromatogram (Fig. 14). The unknown substance X_1 was isolated and characterized by the following procedure. The reaction mixture containing 1 ml of 10% maltose, 0.5 ml of 0.2 M phosphate buffer, pH 6.9, 0.2 ml of the purified enzyme solution (95.44 units), and deionized water in a final volume of 2 ml was incubated at 37°C for 60 min. The reaction was stopped by heating the mixture in a boiling water bath for 5 min. All of the reaction mixture was applied on a sheet of filter paper (Toyo No. 2 filter paper, 30×60 cm) in a straight line, and developed three times with *n*-butanol-pyridine-water (6:4:3, v/v) by the ascending method. The sheet of paper was cut out in a band at the position corresponding to the spot of X_1 , which had been determined with a guide strip. X_1 was then extracted with deionized water from the band, and the extract was concentrated under reduced pressure. The concentrate showed a single reducing spot on the paper chromatogram. The position of X_1 on the chromatogram was similar to that of maltotriose, but not to that of kojibiose or nigerose. When 0.3 ml of the concentrate of X_1 or 0.045% solution of maltotriose was incubated with the crystalline α -glucosidase from *M. javanicus*^{1,2)} having no isomaltose- or panose-hydrolyzing activity, both X_1 and maltotriose

were completely hydrolyzed to glucose (Fig. 15). These results strongly suggest that X_1

FIG. 15. Hydrolysis of Maltotriose and Substance X_1 by Crystalline α -Glucosidase from *M. javanicus*.

The reaction mixture containing 15.16 μ g of crystalline α -glucosidase from *M. javanicus*, substrate (1305.6 μ g as glucose), and 1.7 ml of 0.1 M acetate buffer, pH 5.3, in a final volume of 5 ml was incubated at 37°C.

●—●, maltotriose; ○---○, substance X_1 .

is maltotriose. Further investigations on the chemical structure of X_1 are currently in progress in our laboratory. The enzyme catalyzed also the transfer of the α -glucosyl residue from maltose to riboflavin (Fig. 7).

12) *Inhibition studies.* Among the metal ions tested, Cu^{2+} , Zn^{2+} , Hg^{2+} , and Ni^{2+} were the potent inhibitors of the enzyme (Table III). No inhibition was observed with ethyl-

TABLE III. EFFECT OF METAL IONS

0.04 ml of enzyme solution (0.76 units), 0.02 ml of 0.1 M metal ion solution in deionized water, and 0.34 ml of 0.05 M phosphate buffer, pH 6.9, were pre-incubated at 37°C for 30 min, after which 0.1 ml of 1% maltose was added, and the resulting mixture was kept at 37°C for 15 min.

Metal ion	Hydrolysis (residual activity %)
None	100
AlCl ₃	124.1
BaCl ₂ ·2H ₂ O	66.1
CaCl ₂ ·2H ₂ O	86.8
CoCl ₂	96.1
Cu(CH ₃ COO) ₂	0
HgCl ₂	0
KCl	117.1
MgCl ₂ ·6H ₂ O	100.4
MnCl ₂ ·4H ₂ O	129.2
NiCl ₂ ·6H ₂ O	17.3
Pb(CH ₃ COO) ₂	130.7
ZnCl ₂	34.2

TABLE IV. EFFECTS OF INHIBITORS AND VARIOUS CARBOHYDRATES

The reaction mixture containing 0.04 ml of enzyme solution (0.76 units), 0.2 ml of 0.1 M phosphate buffer, pH 6.9, each reagent dissolved in deionized water, and deionized water in a final volume of 0.4 ml was preincubated at 30°C for 5 min, after which 0.1 ml of 1% maltose was added, and the resulting mixture was kept at 30°C for 15 min.

Inhibitor or carbohydrate	Concentration	Hydrolysis (residual activity %)
	mm	
None		100
EDTA ^{a)}	5	100
PCMB ^{a)}	0.05	0
DHT ^{a)}	0.04	0
	0.004	26.8
Rose bengal ^{b)}	0.1	12.0
	0.01	22.6
	0.001	60.2
Tris	50	7.5
	100	7.5
α -D-Glucose-1- phosphate	6.7	120.9
Maltitol	7.3	100
Sucrose	7.3	100
Turanose	7.3	64.4
	14.6	50.6

^{a)} Preincubation time: 30 min.

^{b)} Reaction mixture was preincubated for 30 min in the same procedure as described in the previous paper.²⁾

enediamine tetraacetate (EDTA). Tris and turanose inhibited α -glucosidase activity (Table IV).

13) *Amino acid residue of active center.* On the basis of the results on the pH dependence of the enzymatic activity, the pK values were calculated to be approximately 6.0 and 8.3. The pK value 6.0 was considered to correspond to an imidazole group of the histidine residue and the other value 8.3 to a sulfhydryl group of the cysteine residue in proteins. Experiments were carried out with diazonium-1-H-tetrazole (DHT) and rose bengal, the specific modification reagents for the histidine residue, and *p*-chloromercuribenzoate (PCMB) for the cysteine residue. DHT was prepared in the same procedure as described in the previous paper.²⁾ The three reagents just described acted as inhibitors of the enzyme (Table IV). PCMB showed no inhibitory effect on the enzymatic activity, when the enzyme was preincubated in the presence of 5 mM of mercaptoethanol. By the addition of 5 mM of mercaptoethanol to the enzyme solution, 35% of the activity of the enzyme which was inhibited by the preincubation with PCMB before the introduction of maltose, was restored.

DISCUSSION

An α -glucosidase from the cells of *B. cereus* was prepared in a highly purified state by a procedure including crush with a french press, fractionation with ammonium sulfate, column chromatography on DEAE-cellulose and on DEAE-Sephadex A-25, and preparative polyacrylamide gel electrophoresis. The α -glucosidase gave a single protein band when examined by polyacrylamide disc gel electrophoresis, and showed a single boundary sedimentation pattern in the ultracentrifugal analysis. The enzyme was very different from α -glucosidases from other origins in values of $s_{20,w}^0$ and molecular weight, substrate specificity, and transglucosylation action. The α -glucosidase had a sedimentation constant of 1.4 S, corresponding to a molecular weight

of 12,000. Both physical values just described were much smaller than those of the glycosidases previously reported. The α -glucosidase of *B. cereus* hydrolyzed strongly α -(1 $\rightarrow$ 4)- and α -(1 $\rightarrow$ 3)-glucosidic linkages, and negligibly α -(1 $\rightarrow$ 6)- and α -(1 $\rightarrow$ 2)-glucosidic linkages. The α -glucosidases from plants¹¹⁻¹³) and from *M. javanicus*²¹) hydrolyzed soluble starch, and the α -glucosidases from animals¹⁴⁻¹⁷) hydrolyzed glycogen, whereas the α -glucosidase from *B. cereus* did not hydrolyze these polysaccharides. The α -glucosidase from *B. cereus* hydrolyzed phenyl α -maltoside to glucose and phenyl α -glucoside, while that from *Asp. niger*¹⁸) hydrolyzed phenyl α -maltoside to phenol and maltose, indicating that the former splits the α -(1 $\rightarrow$ 4)-glucosidic linkage between two glucosyl residues in phenyl α -maltoside, whereas the latter splits first the linkage between phenol and maltosyl residue. The α -glucosidases from brewer's yeast¹⁹⁻²⁴) hydrolyzed phenyl α -glucoside and sucrose faster than maltose, while the α -glucosidase from *B. cereus* hydrolyzed phenyl α -glucoside slower than maltose and showed no activity toward sucrose. The transglucosylation action of the α -glucosidase from *B. cereus* was examined by paper chromatography. Two unknown substances X₁ and X₂ were detected on chromatograms as the transglucosylation products from maltose at a concentration of 5 per cent. X₁ was presumed to be maltotriose from the similarities in the chromatographic mobility and in the rate of hydrolysis with the crystalline α -glucosidase from *M. javanicus* which was not capable of hydrolyzing isomaltose or panose. Studies on X₂ have not yet been undertaken because of its scanty amount isolated. Iso-maltose was detected as the main transglucosylation product from maltose digests with the α -glucosidases from *Asp. niger*,¹⁸) *Asp. oryzae*,²⁵) *Penicillium chrysogenum*²⁶) and *Schizosaccharomyces pombe*.²⁷) Like other glycosidases,^{1, 28, 31}) the α -glucosidase from *B. cereus* catalyzed the transfer of the α -glucosyl residue of maltose to riboflavin.

Finally, we examined the amino acid resi-

dues of the active center by the techniques of the specific modification. From the results, it is inferred that both histidine and cysteine residues in the active center are closely related to the activity of the α -glucosidase from *B. cereus*.

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