α-GLUCOSIDASE FROM *MUCOR JAVANICUS*

I. Purification and Crystallization

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Extensive studies have been made on α-glucosidases in animals, plants and microorganisms. α-Glucosidases from *Aspergillus niger* (1) and *Asp. oryzae* (2) have already been isolated in crystalline form from culture filtrates. No attempt, however, has yet been made to isolate α-glucosidase from *Mucor spp.*

The present paper describes the purification, crystallization and some properties of α-glucosidase from the mycelia of *M. javanicus*. It is also shown that this crystalline enzyme preparation has B_2-5′-α-gl-synthesizing activity, as well as α-glucosidase activity.

MATERIALS AND METHODS

Microorganism and culture. The culture of *M. javanicus* was obtained from the Institute for Fermentation, Osaka, Japan (4570). The culture medium contained 40 g of maltose (technical grade), 1 g of NH_4NO_3, 1 g of $NaNO_3$, 1 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7\,H_2O$, 0.5 g of KCl and 10 g of $CaCO_3$ in 1000 ml of deionized water. Maltose and $CaCO_3$ were sterilized separately before inoculation. Pieces of the mycelia of the mold were picked up from a Czapek-Dox agar slant and inoculated into a 500 ml shaking flask containing 100 ml of the above culture medium. Incubation was carried out at 30 ℃ for 2 days on a reciprocal shaker (68 strokes per minute, 9 cm amplitude), and 5 ml of the 2-day culture was used as the inoculum into a 500 ml shaking flask containing 100 ml of culture medium. After 2 days of incubation under shaking conditions at 30 ℃, mycelia from 300 flasks were harvested by filtration, washed with deionized water, and stored at −20 ℃. The mycelia yield was approximately 1 kg on wet weight basis.

Chemicals and reagents. Maltose (technical grade) and maltose (HHH, purity about 99.0 %) (Hayashibara Biochemical Laboratories, Inc., Okayama, Japan), riboflavin (E. Merck, Darmstadt, Germany), glucose oxidase and peroxidase from horseradish type II (Sigma Chemical Co., St. Louis, Missouri, U. S. A.), Sephadex G-200 and DEAE-cellulose (Pharmacia Fine Chemicals AB, Uppsala, Sweden), Ampholine (LKB Produkter AB, Broma, Sweden) and N, N, N′, N′-tetramethylenediamine, N, N′-methylenebisacrylamide and acrylamide (Tokyo Kasei Co., Tokyo, Japan) were used. All other chemical reagents used were of analytical grade and were obtained from Ishizu Pharmaceutical Co., Osaka, Japan.

Enzyme assays

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(*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 acetate buffer, pH 5.3, and enzyme solution in a final volume of 0.5 ml. After incubation for 15 min at 37 ℃, the reaction was stopped by heating in a boiling water bath for 5 min. The amount of glucose formed was determined according to the method of Papadopoulos and Hess (3), as modified by Dahlqvist (4). One unit of α-glucosidase activity is defined as the amount of enzyme which forms 1 μmole of glucose from maltose under the described conditions.

(*2*) *Assay for glucosyltransferase activity.* In standard assay, activity was determined by measuring the transference of the 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 acetate buffer, pH 5.3, enzyme solution, and deionized water in a final volume of 10 ml. After incubation for 30 min at 50 ℃, the reaction was stopped by heating for 5 min in a boiling water bath. Two milliliters of reaction mixture was applied as a band 11 cm wide to Toyo No. 2 filter paper. The paper chromatograms were developed twice by the ascending method with a solvent system of n-butyl alcohol-pyridine-water (6: 4: 3, v/v). After development, the paper was dried and examined by ultraviolet light. The band corresponding to B_2-5′-α-gl was cut out and eluted with 10 ml of deionized water for 60 min in the dark at 50 ℃. The eluate was cooled and filtered. The amount of B_2-5′-α-gl in the filtrate was determined by measuring the optical density at 450 nm. Recovery of standard crystalline B_2-5′-α-gl was 91 %.

Protein determination. The protein concentration was determined by measuring the absorbances at 280 nm and 260 nm (5). The protein concentration of each fraction of column was followed by measuring absorbance at 280 nm.

Ultracentrifugal analysis. Ultracentrifugal studies were performed with a Hitachi analytical ultracentrifuge model UCA-1.

Disc gel electrophoresis. Enzyme electrophoresis was carried out in polyacrylamide gel, pH 4.0, as described by Reisfeld *et al* (6). Gel was stained for protein with 1 % amido black 10 B in 7 % acetic acid and destained with 7 % acetic acid.

Determination of isoelectric point. Isoelectric focusing experiments were performed at 4 ℃ in a 110 ml capacity column of LKB 8100 Ampholine Electrofocusing Equipment. The carrier ampholyte covering a pH range between 3 and 10 was used at an average final concentration of 1 %. After electrophoresis was completed (48 hr), 2 ml fractions were collected from the bottom of the column by means of a peristaltic pump. The pH of each fraction was determined at 4 ℃ on a Hitachi Horiba pH meter model M-5. Absorbance was measured at 280 nm on a Hitachi Perkin Elmer spectrophotometer model 139.

Gel electrofocusing. Gel electrofocusing was performed with 7.5 % poly-

acrylamide gels containing carrier ampholyte (pH range 3 to 10) according to the method of Wrigley (7). Gel was stained for protein with 0.2 % bromphenol blue in a mixed solution of ethyl alcohol-water-acetic acid (50: 45: 5, v/v) and destained in another mixed solution of ethyl alcohol-water-acetic acid (30: 65: 5, v/v).

RESULTS

1. Isolation of enzyme

An outline of the purification procedure and the results are shown in Table 1.

Step 1: Preparation of crude extract. Mycelia (1 kg) was suspended in 5.1 liters of 1 M acetate buffer, pH 5.3, containing 4 M urea at 30 ℃ for 48 hr, and the pH of the suspension was maintained at 5.3 by adjusting with 4 M acetic acid. After incubation, the mycelial debris was removed by filtration, and the filtrate was chilled to near 0 ℃. Unless otherwise indicated, all of subsequent operations were carried out at between 0 ℃ and 4 ℃.

Step 2: Fractionation with acetone. To the crude extract of mycelia acetone previously chilled to −20 ℃ was added dropwise, under stirring to give a 50 per cent concentration. The precipitate was collected by centrifugation and dissolved in 0.05 M acetate buffer, pH 5.3. A small amount of insoluble material was removed by centrifugation. To the resulting supernatant solution was added 1.45 ml of 1 M $CaCl_2$.

Step 3: Fractionation with polyethylene glycol. To enzyme solution containing 5×10^{-3} M $CaCl_2$ was added polyethylene glycol 6000 (P.E.G.) to give a concentration of 20 per cent (v/w). After the mixture was allowed to stand for 1 hr, the resulting precipitate was collected by centrifugation and

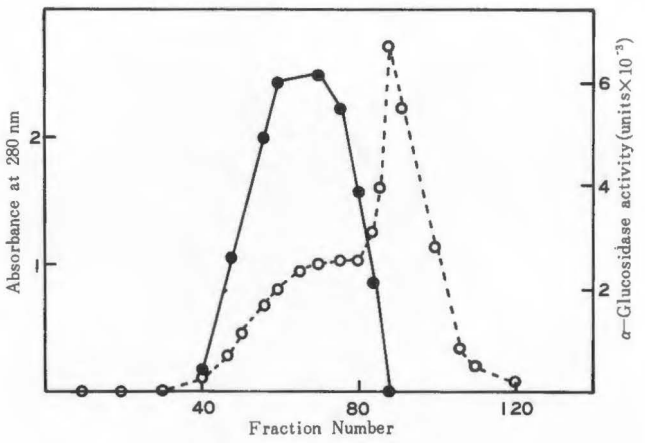

Fig. 1. Sephadex G-200 column chromatography.

Protein applied: 675 absorbancy units at 280 nm. Flow rate: about 20 ml per hr. Each fraction: 10 ml. o------o, Absorbance at 280 nm; •——•, α-glucosidase activity per 0.01 ml of each fraction.

dissolved in 0.05 M acetate buffer, pH 5.3.

Step 4: Sephadex G-200 column chromatography. The enzyme solution obtained from Step 3 was applied to a Sephadex G-200 column (5.8ϕ x 60 cm) equilibrated with 0.01 M acetate buffer, pH 5.3. The column was eluted with the same buffer at a flow rate of about 20 ml per hour. The protein was eluted with 0.01 M acetate buffer, pH 5.3, and the elution pattern is shown in Fig. 1. Fractions (Nos. 40 to 84) showing enzyme activity were collected and concentrated by addition of P. E. G. to a concentration of 20 per cent (v/w). The precipitate obtained by centrifugation was dissolved in 0.05 M Tris-HCl buffer, pH 7.0.

Step 5: DEAE-cellulose column chromatography. The above enzyme solution was applied to a DEAE-cellulose column (2.8ϕ x 20 cm) equilibrated previously with 0.05 M Tris-HCl buffer, pH 7.0. Elution was carried out with the same buffer. The active fractions (Nos. 14 to 24) were pooled, and concentrated to 9.5 ml at 25 – 30 ℃ under reduced pressure, and dialyzed overnight against 1.5 liter of 0.1 M acetate buffer, pH 5.3.

Step 6: Crystallization. After dialysis, 40 ml of deionized water was added to the enzyme solution, and 2 % Ampholine, pH 7 – 9, was added dropwise until the solution became slightly turbid, and the solution was kept at 4 ℃. Needle-shaped crystals appeared after 5 hr (Fig. 2). A summary of the purification procedure is given in Table 1.

2. Criteria of purity

(1) Ultracentrifugation. The sedimentation patterns of the crystalline enzyme are shown in Fig. 3. The boundary of the enzyme moved as a single symmetrical peak. The $S_{20,w}$ value was calculated to be 0.56×10^{-12} cm.sec^{-1}.

(2) Disc gel electrophoresis. When the crystalline enzyme was subjected to electrophoresis in polyacrylamide gel at pH 4.0, a single band of protein was

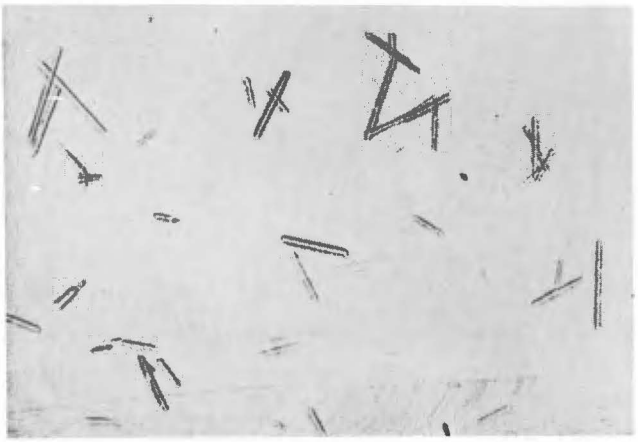

Fig. 2. Photomicrograph of the crystalline α-glucosidase isolated from *M. javanicus* (x 100).

observed migrating toward the cathode. No contaminants were detectable (Fig. 4).

(*3*) *Gel electrofocusing.* The crystalline enzyme gave a single band of protein on polyacrylamide gel electrofocusing (Fig. 5).

TABLE 1

Purification of α-glucosidase from *M. javanicus*

Step	Protein		α-Glucosidase (glucosyltransferase)		
	A_{280} [a]	A_{280}/A_{260}	Total units (A_{450}) [a]	Specific activity	Recovery
				units/A_{280}	%
1. Urea extraction	120,000	0.49	440,000 (30,000)	3.7 (0.3)	100 (100)
2. Acetone fractionation	2,900	0.42	328,000 (20,250)	113 (7.0)	75 (68)
3. P. E. G. fractionation	675	0.45	244,000 (16,000)	361 (23.7)	55 (53)
4. Sephadex G-200 column chromatography	328	0.68	180,000 (16,000)	549 (48.8)	41 (53)
5. DEAE-cellulose column chromatography	114	1.80	140,000 (12,900)	1228 (113.2)	32 (43)
6. Crystallization	59	1.48	48,300 (3,600)	819 (61.0)	11 (12)

a) Absorbance at 280 nm (or 450 nm).

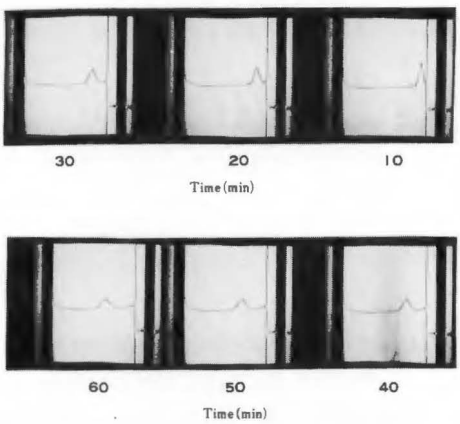

Fig. 3. Sedimentation patterns of crystalline α-glucosidase.

Ultracentrifugation was carried out with 0.25 % α-glucosidase in 0.01 M acetate buffer, pH 5.3, on a Hitachi analytical ultracentrifuge model UCA-1. Experimental conditions were as follows: temperature, 20 ℃; speed, 55,430 rpm; bar angle, 70°; cell used, a standard 12 mm single sector cell. The photographs were taken at indicated times after reaching 55,430 rpm.

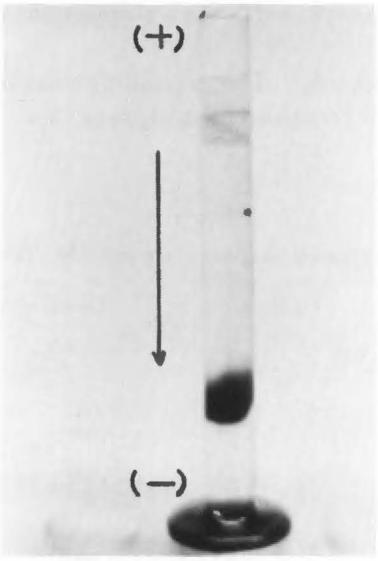

Fig. 4. Disc electrophoretic pattern of α-glucosidase.
β-Alanine-glacial acetic acid buffer, pH 4.5, was used. Crystalline enzyme (240 μg) was applied to the gel. Electrophoresis was carried out at pH 4.0, 5 mA per tube for 150 min at 4 ℃.

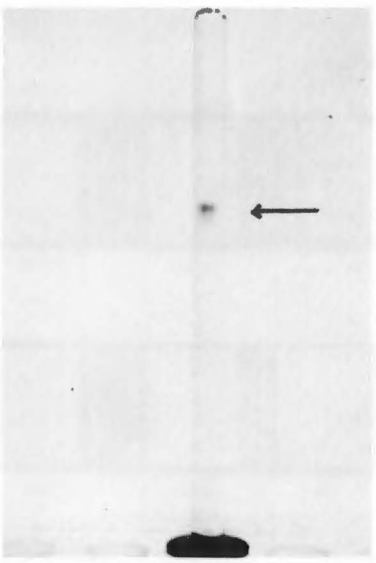

Fig. 5. Gel electrofocusing of α-glucosidase.
A sample solution containing 150 μg of crystalline α-glucosidase was photomerized on top of the separation gel, and electrofocusing was run at a constant voltage of 80 V for 48 hr at 4 ℃, using a disc electrophoretic apparatus with 0.5ϕ x 8 cm. Migration was toward the cathode.

3. Properties of enzyme

(*1*) ***Absorption spectrum.*** The absorption spectrum of crystalline α-glucosidase was taken with a Hitachi recording spectrophotometer model EPS-3 T (Fig. 6). In the wave length range investigated, the enzyme exhibited maximum absorption at 280 nm.

(*2*) ***Isoelectric point.*** When the enzyme was subjected to isoelectric focus-

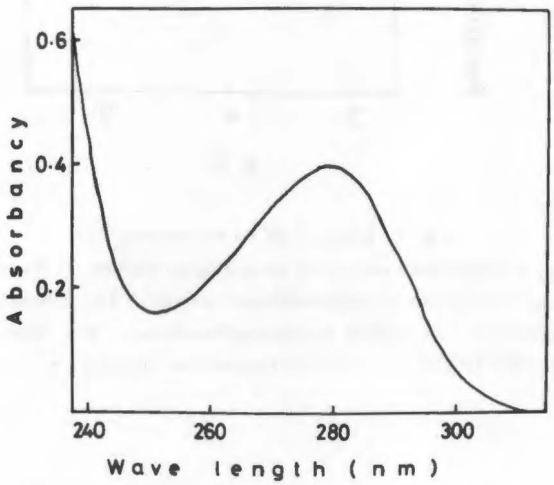

Fig. 6. Absorption spectrum of α-glucosidase.
The protein (2.2 mg) was dissolved in 4.2 ml of 0.05 M acetate buffer, pH 5.3, and the absorption spectrum was recorded on a Hitachi recording spectrophotometer model EPS-3T.

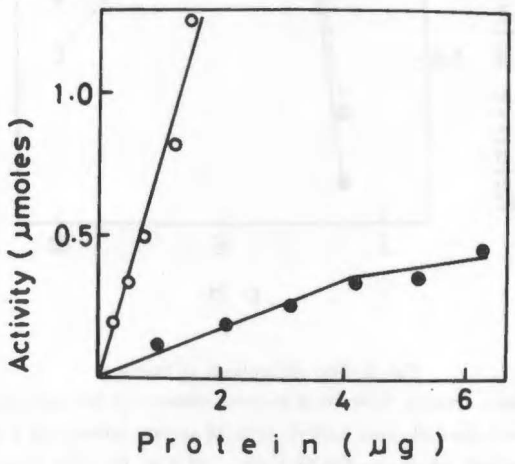

Fig. 7. Relationship between enzyme protein and activity.
α-Glucosidase and glucosyltransferase were estimated using various amounts of enzyme under the standard assay conditions. o——o, α-Glucosidase activity; •——•, glucosyltransferase activity in a final volume of 5 ml.

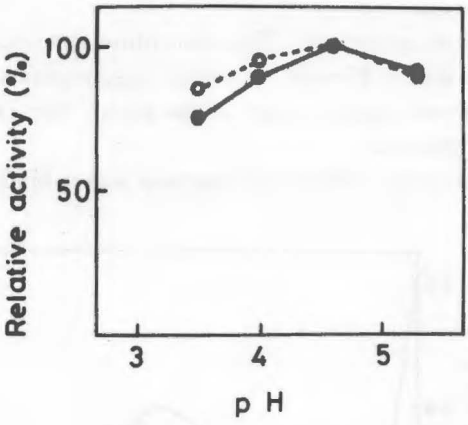

Fig. 8. Effect of pH on enzyme activity.

α-Glucosidase activity was estimated using 0.02 ml of enzyme solution (0.78 units) at various pH and 0.4 ml of the enzyme solution on glucosyltransferase activity. The following buffers were used: McIlvaine for α-glucosidase and acetate for glucosyltransferase. For other assay conditions see MATERIALS AND METHODS. ∘------∘, α-Glucosidase activity; •——•, glucosyltransferase activity.

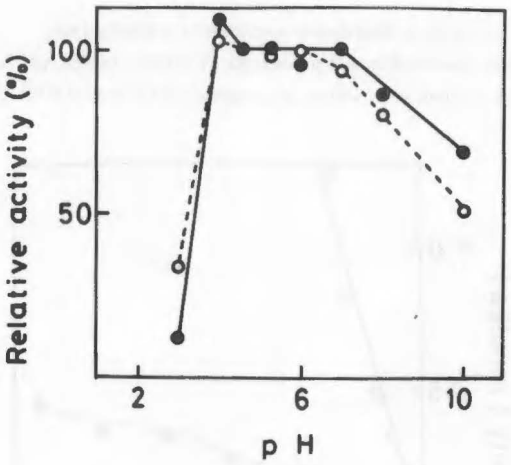

Fig. 9. The pH stability of enzyme.

For assay of α-glucosidase activity, 0.04 ml of enzyme solution (1.56 units) was preincubated for 20 hr at 30 ℃ with 0.2 ml of the following buffers: 0.05 M acetate buffer, pH 3 to 6, 0.05 M phosphate buffer, pH 7 to 8; and 0.05 M glycne-NaOH buffer, pH 9 to 10. For assay of glucosyltransferase activity, 0.4 ml of enzyme solution (15.6 units of α-glucosidase activity) was preincubated for 20 hr at 30 ℃ with 2.6 ml of the above-mentioned buffers. After adjustment of pH to 5.3 with 1 M acetate buffer, pH 5.3, the residual enzyme activity was measured under the standard assay conditions. ∘------∘, α-Glucosidase activity; •——•, glucosyltransferase activity.

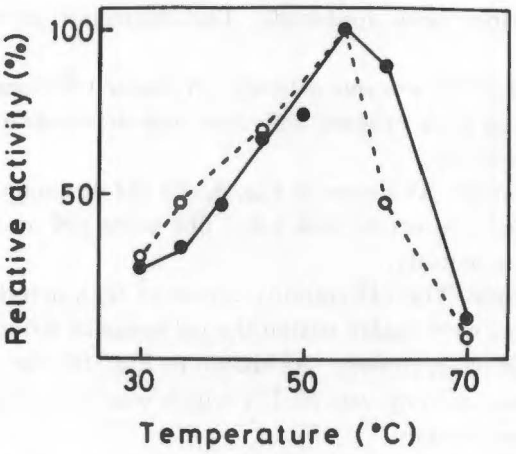

Fig. 10. Effect of temperature on enzyme activity.

α-Glucosidase activity was estimated using 0.02 ml of enzyme solution (0.78 units) at various temperatures and glucosyltransferase activity was estimated using 0.4 ml of enzyme solution. For other assay conditions see MATERIALS AND METHODS. o------o, α-Glucosidase activity; •——•, glucosyltransferase activity.

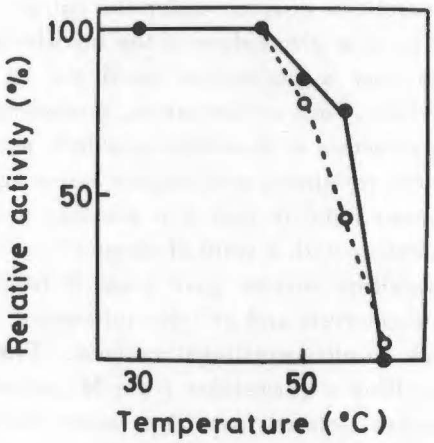

Fig. 11. Heat stability of enzyme.

For assay of α-glucosidase activity, the reaction mixture containing 0.04 ml of enzyme solution (1.56 units) and 0.36 ml of 0.05 M acetate buffer, pH 5.3, was preincubated for 15 min at various temperatures. For assay of glucosyltransferase activity, the reaction mixture containing 0.4 ml of enzyme solution (15.6 units of α-glucosidase activity) and 2.6 ml of 0.05 M acetate buffer, pH 5.3, was preincubated for 15 min at various temperatures. After preincubation, the residual enzyme activity was measured under the standard assay conditions. o------o, α-Glucosidase activity; •——•, glucosyltransferase activity.

ing, a single absorbance peak appeared. The isoelectric point of this enzyme was pH 8.6.

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

(*4*) *pH Optimum.* As shown in Fig. 8, the pH optimum for α-glucosidase activity with maltose as substrate was 4.6. The same pH maximum was found for glucosyltransferase activity.

(*5*) *pH Stability.* The pH stability curves of both activities are shown in Fig. 9. Both activities were stable within the pH range of 4.0 to 7.0.

(*6*) *Temperature optimum.* As shown in Fig. 10, the temperature optimum for α-glucosidase activity was 55 ℃, which was the temperature optimum for glucosyltransferase activity.

(*7*) *Heat stability.* As shown in Fig. 11, both enzyme activities were stable up to 45 ℃, whereas above 45 ℃ the activities were markedly reduced, and at 60 ℃, the activities were almost completely destroyed.

DISCUSSION

In the present investigation, a procedure for obtaining a crystalline preparation of α-glucosidase from *M. javanicus* was developed. Most of the α-glucosidase activity of *M. javanicus* was present in mycelia when the microorganism was grown on maltose medium; that is, *M. javanicus* did not secrete α-glucosidase as an extracellular enzyme under the culture conditions employed (8). Approximately 40 % of α-glucosidase of the mycelia was easily solubilized by extraction with urea, but α-glucosidase could not be extracted from the mycelia by ordinary methods, such as sonication, grounding of washed or dried mycelia with sea sand, extraction with acetone powders, autolysis with $(NH_4)_2HPO_4$ or toluene and treatment with surface active agents. The results reported in the present paper indicate that it is possible to obtain crystalline α-glucosidase from *M. javanicus* with a yield of about 12 % by rather simple purification steps. The crystalline enzyme gave a single band when examined by polyacrylamide gel electrophoresis and gel electrofocusing, and showed a single symmetrical moving peak on ultracentrifugal analysis. The optical temperature and pH stability of crystalline α-glucosidase from *M. javanicus* were similar to those of crystalline enzymes isolated from *Asp. niger* and *Asp. oryzae*. However, the optimal pH of the former was 4.6 which was different from the optimal pH 3.5 for the latter. α-Glucosidase from *M. javanicus* was more sensitive to heat than that from *Aspergillus*; almost all activity from the former was lost at 60 ℃ but the latter was stable in a range from 0° to 60 ℃. Throughout the purification and crystallization procedures of α-glucosidases, the α-glucosidase activity to hydrolyze maltose was always associated with glucosyltransferase activity to synthesize B_2-5′-α-gl from maltose and riboflavin. It was also observed that both activities of crystalline α-glucosidase varied in parallel

according to the following conditions: pH, temperature, and pretreatment at pH 3 to 10 and at 30 ℃ to 60 ℃. The results indicate that α-glucosidase from *M. javanicus* has intrinsic glucosyltransferase activity to synthesize B_2-5′-α-gl. Further investigations on the properties of crystalline α-glucosidase from *M. javanicus* will be reported in the next paper of the series.

SUMMARY

α-Glucosidase was purified and crystallized from the mycelia of *Mucor javanicus*, by a procedure involving extraction with urea, fractionations with acetone and polyethylene glycol 6000, successive separation on columns of Sephadex G-200 and DEAE-cellulose, and crystallization by addition of ampholine reagent. The crystalline enzyme was homogeneous on ultracentrifugal analysis, gel electrophoresis and gel electrofocusing. The purified enzyme showed a sedimentation constant of 5.6 S and an isoelectric point of pH 8.6. Certain properties of the purified enzyme were also investigated. It was demonstrated that the synthesis of riboflavin α-glucoside was catalyzed by the way of a transglucosylation activity of this α-glucosidase.

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LITERATURE CITED

1. Tsujisaka, Y. and Fukumoto, J. 1958. Maltase action of molds. Symp. Enz. Chem. Japan, 13: 84–92.
2. Sugawara, S., Nakamura, Y. and Shimomura, T. 1959. Fractionation of maltase and saccharogenic amylase in mold, and crystallization of maltase. Bull. Agric. Chem. Soc. Jpn., 23: 156–159.
3. Papadopoulos, N. M. and Hess, W. C. 1960. Determination of neuraminic (sialic) acid, glucose, and fructose in Spinal fluid. Arch. Biochem. Biophys., 88: 167–171.
4. Dahlqvist, A. 1961. Determination of maltase and isomaltase activities with a glucose-oxidase reagent. Biochem. J., 80: 547–551.
5. Warburg, 0. and Christian, W. 1942. Isolation and crystallization of enolase. Biochem. Z., 310: 384–421.
6. Reisfeld, R. A., Lewis, U. J. and Williams, D. E. 1962. Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. Nature, 195: 281–283.
7. Wrigley, C. W. 1968. Analytical fractionation of plant and animal proteins by gel electrofocusing. J. Chromatog., 36: 362–365; Gel electrofocusing-a technique for analyzing multiple protein samples by isoelectric focusing. Sci. Tools, 15: 17–23.
8. Suzuki, Y. and Uchida, K. 1971. Production of 5′-D-riboflavin-α-D-glucopyranoside in growing cultures by the mold *Mucor*. Agric Biol. Chem., 35: 805–812.