

α -GLUCOSIDASE FROM *MUCOR JAVANICUS*

III. Certain Properties of the Crystalline Enzyme*

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In the preceding papers (1, 2), the authors reported on the purification, crystallization, and some properties of α -glucosidase from the mycelia of *Mucor javanicus*. The present paper deals with the molecular weight, and the compositions of amino acids and carbohydrates of this enzyme.

MATERIALS AND METHODS

Enzyme preparation. The crystalline α -glucosidase from the mycelia of *Mucor javanicus* IFO 4570 was prepared as described in the previous paper (1). This preparation was found to be homogeneous on disc gel electrophoresis, gel electrofocusing and ultracentrifugation. Prior to analysis for chemical composition, the enzyme preparation was dialyzed against deionized water for 2 weeks at 4 °C. Aliquots of the dialyzed enzyme preparation were pipetted out and dried in a vacuum desiccator over concentrated sulfuric acid, and the moisture of the dried enzyme preparation was determined by drying to a constant weight at 105 °C. The nitrogen content was determined by the micro-Kjeldahl method.

Analytical ultracentrifugation. Ultracentrifugal studies were performed with a Hitachi model 282 analytical ultracentrifuge. The sedimentation constant of the protein was calculated by extrapolation of the plot of the sedimentation coefficient to infinite dilution. The diffusion constant was calculated by the height-area method.

Analysis for carbohydrates. Carbohydrate detection in each fraction from density-gradient column was performed by the method of Somogyi and Nelson (3, 4). The Surgenor *et al.* (5) modification of the Sørensen and Haugaard (6) method, known as the Tillman reaction, served for carbohydrate detection in the acid hydrolyzate of the effluent from Bio-Gel P-100 column. This method was also used for analysis of neutral sugars (hexose and pentose) in intact crystalline α -glucosidase. Glucose was estimated by the glucose oxidase method of Dahlqvist (7). Pentoses were determined according to the method of Mejbaum (8), known as the Bial reaction. The modified carbazole method of Bitter and Muir (9) was used for determination of uronic acids. Sialic acid was estimated by the periodate-thiobarbituric acid method of Warren (10). Amino sugars were detected colorimetrically by the method of Rimington (11), and determined with a Nihon Denshi model JLC-6AH amino acid analyzer. Neutral sugars were identified by paper chromatography. The identification and esti-

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mation of neutral sugars were also performed by gas-liquid chromatography of their trimethylsilyl (TMS) derivatives.

Analysis amino acids. The crystalline α -glucosidase preparation (about 2 mg) was lyophilized to dryness and hydrolyzed in 3 ml of 6 N HCl at 105 °C under argon gas phase in a sealed glass tube for 22, 48 and 72 hr. Each hydrolyzate was evaporated to dryness over pellets of NaOH *in vacuo* at 35 °C, and then the residue was analyzed by the method of Spackman *et al.* (12) with a Nihon Denshi model JLC-6AH amino acid analyzer. The amino acid values are given as averages of values obtained after hydrolysis for 22, 48 and 72 hr. Independent determinations of cystine and cysteine as cysteic acid were performed after oxidation of protein by performic acid for 24 hr, according to the method of Schram *et al.* (13). Tryptophan content was determined by measuring the ultraviolet absorption of the protein in 0.1 N NaOH, according to the method of Goodwin and Morton (14) and Beaven and Holiday (15).

RESULTS

1. Sedimentation and diffusion constant

The sedimentation constant, $s_{20,w}^0$, was calculated to be 6.08 S (Fig. 1) and the diffusion constant ($D_{20,w}$) was 4.82×10^{-7} cm²·sec⁻¹ (Fig. 2).

2. Molecular weight

The molecular weight of the crystalline α -glucosidase was calculated to be 122,400 from the sedimentation and diffusion constants determined independently using the Svedberg equation. Molecular weight was also determined to be 126,800 by the low speed sedimentation equilibrium method (10,000 rpm) with the Hitachi light absorption scanning recorder and a multichannel cell (Fig. 3).

3. Criteria of glycoprotein

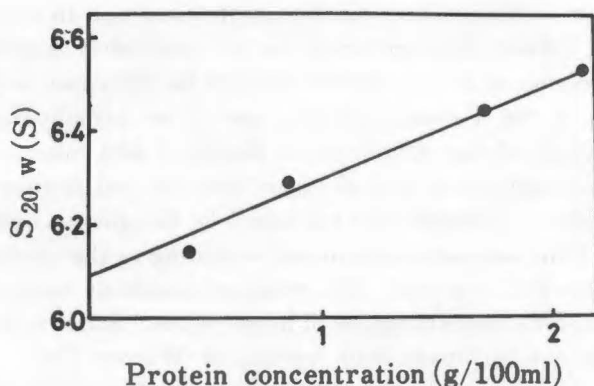


Fig. 1. Dependence of α -glucosidase sedimentation coefficient on protein concentration. Sedimentation velocity experiments were carried out at different concentrations of crystalline enzyme protein in 0.01 M acetate buffer, pH 5.3. Experimental conditions: temperature, 20 °C; speed, 58,000 rpm; cell used, a double sector cell.

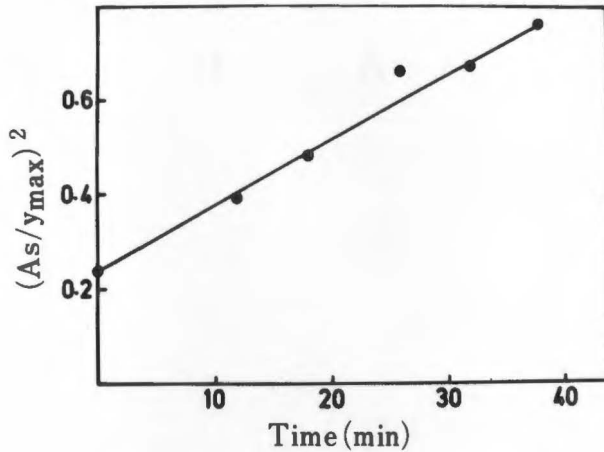


Fig. 2. Diffusion coefficient of α -glucosidase.

Ultracentrifugation was carried out at a concentration of 2.0 % crystalline α -glucosidase in 0.01 M acetate buffer, pH 5.3. Experimental conditions: temperature, 20 °C; speed, 15,000 rpm; cell used, a synthetic boundary cell. As, area; Ymax, the maximum height.

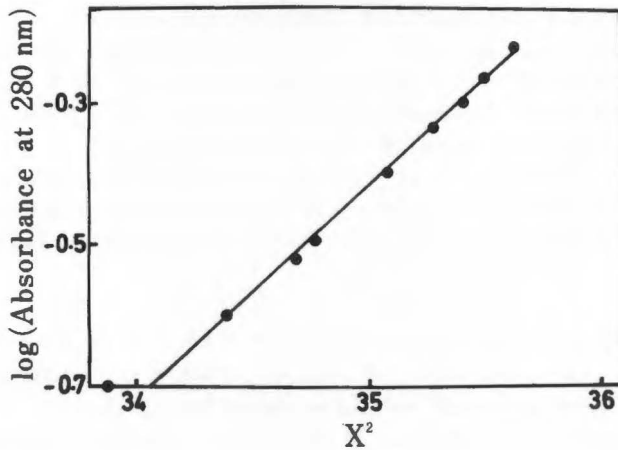


Fig. 3. Sedimentation equilibrium of α -glucosidase.

Experimental conditions were as follows: temperature, 28 °C; equilibrium time, 24 hr; cell used, a multichannel cell; concentration of the protein, about 0.03 % (in 0.01 M acetate buffer, pH 5.3). Abscissa, square of distance from center of rotation (x); ordinate, logarithm of absorbance at 280 nm.

(1) *Disc gel electrophoresis*. A duplicate gel (B) stained with periodate-fuchsin to identify carbohydrate showed that there was only one band of carbohydrate, and that the protein and carbohydrate materials in crystalline α -glucosidase migrated at identical rates on electrophoresis (Fig. 4).

(2) *Gel electrofocusing*. Gel electrofocusing was performed with 7.5 % polyacrylamide gels containing the carrier ampholyte (pH range 3 to 10) accord-

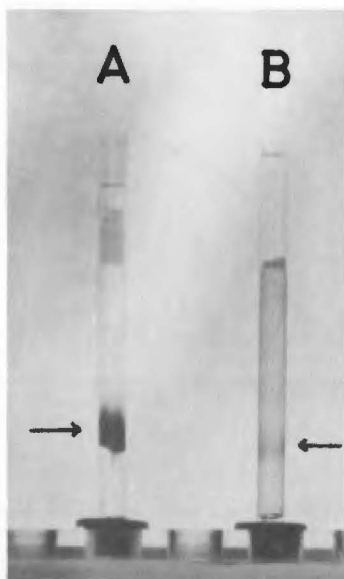


Fig. 4. Polyacrylamide disc gel electrophoresis of α -glucosidase.

Disc gel electrophoresis was carried out on 7.5 % polyacrylamide gel columns with a β -alanine-acetic acid buffer, pH 4.5, according to the method of Reisfeld *et al.* (22). Crystalline α -glucosidase (210 μ g) was applied, and run at a constant current of 5 mA per column (0.5 ϕ x 8 cm) for 155 min at 4 $^{\circ}$ C. Migration was toward the cathode. After electrophoresis, the gel (A) was stained for protein with 1 % amido black 10 B in 7 % acetic acid, and destained electrophoretically with 7 % acetic acid. Carbohydrate was detected on the duplicate gel (B) with the periodate-fuchsin stain according to the method of Zacharius *et al.* (23). The arrows refer to the protein and carbohydrate bands.

ing to the method of Wrigley (16). Crystalline α -glucosidase gave a single band of carbohydrate. The position of the band of carbohydrate on the gel was very similar to that of protein stained with bromphenol blue (Fig. 5).

(3) *Gel filtration on Bio-Gel P-100.* The crystalline α -glucosidase (5.2 mg) was applied to a column (1.8 ϕ x 70 cm) of Bio-Gel P-100 equilibrated previously with 0.02 M acetate buffer, pH 5.3. The column was eluted with the same buffer at a flow rate of about 5 ml per hour, and the effluent was collected in 5 ml fractions. The elution pattern is shown in Fig. 6. The distribution of Tillman's reaction-positive material was found to coincide with the absorbance peak at 280 nm.

(4) *Density-gradient centrifugation.* The density-gradient centrifugation technique (17) was employed for obtaining sedimentation patterns of protein and carbohydrate materials in crystalline α -glucosidase. A linear glycerol gradient of 5 to 40 % was prepared in 0.02 M acetate buffer, pH 5.3, and then equilibrated for 4 hr at 4 $^{\circ}$ C. After the enzyme preparation (about 1.2 mg of protein dissolved in 0.1 ml of the buffer) was placed on top of the gradient,

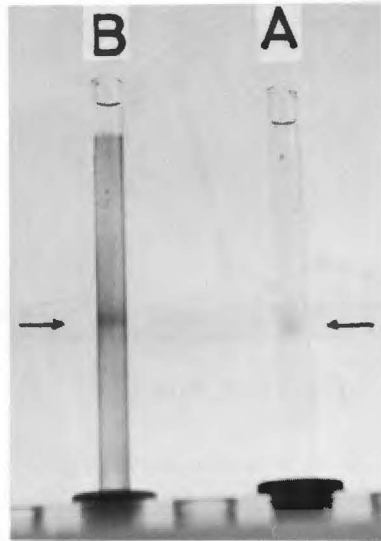


Fig. 5. Gel electrofocusing of α -glucosidase.

Gel electrofocusing was performed with 7.5 % polyacrylamide gel containing the carrier ampholyte (pH range 3 to 10), according to the method of Wrigley (16). Sample solution containing 60 μ g of crystalline α -glucosidase was photopolymerized on the top of the separation gel, and electrofocusing was run at a constant voltage of 80 V for 19 hours at 4 $^{\circ}$ C using a disc electrophoretic apparatus with 0.5 ϕ x 8 cm. Migration was toward the cathode. After electrofocusing, the gel (A) was stained for one hour in order to visualize the protein band with 0.2 % bromphenol blue in a mixed solution of ethyl alcohol-water-acetic acid (50: 45: 5, v/v), and destained with a mixed solution of ethyl alcohol-water-acetic acid (30: 65: 5, v/v). The duplicate gel (B) was stained for carbohydrate with periodate-fuchsin. The arrows refer to the protein and carbohydrate bands.

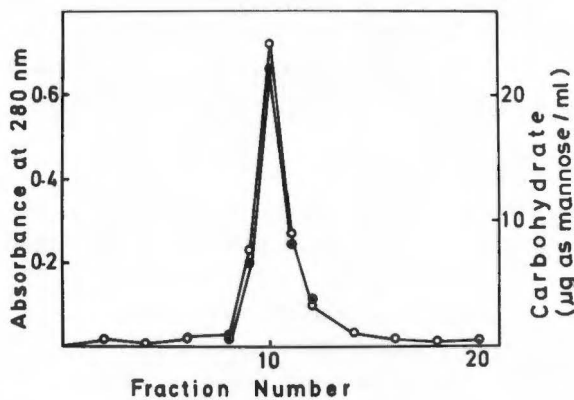


Fig. 6. Bio-Gel P-100 column chromatography of α -glucosidase.

The experimental details are described in the text. Protein was determined by measuring absorbance at 280 nm, and carbohydrate was determined by the colorimetric method known as Tillman's reaction with mannose as the standard. \circ ----- \circ , Absorbance at 280 nm; \bullet — \bullet , carbohydrate (μ g as mannose per ml).

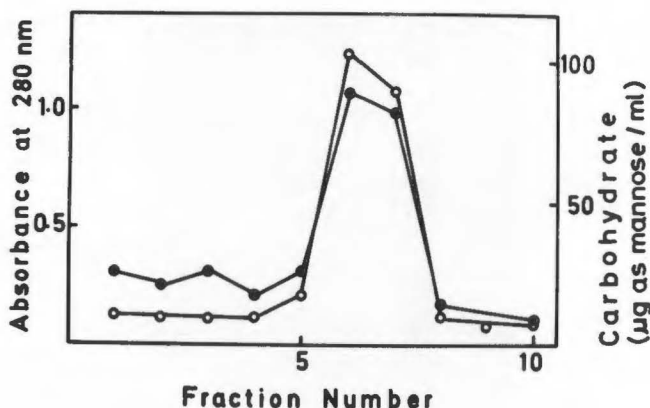


Fig. 7. Sedimentation pattern of α -glucosidase on glycerol density-gradient centrifugation.

The experimental details are described in the text. \circ — \circ , Absorbance at 280 nm; \bullet — \bullet , carbohydrate (μg as mannose per ml).

centrifugation was carried out at 4 °C in a RPS-40 A swinging rotor of a Hitachi model 65 P ultracentrifuge for 22 hr at 35,000 rpm. After centrifugation, the tube content was fractionated into 0.39 ml by punching a hole in the bottom of the tube with a needle. Each fraction was assayed first for protein content by measuring absorbance at 280 nm, and subsequently for carbohydrate content by the following procedure. Each fraction was hydrolyzed with 2 N H_2SO_4 at 100 °C for 4 hr in a tightly stoppered tube, and the hydrolyzate was cooled and neutralized with 4 N NaOH. After neutralization, the carbohydrate content in each hydrolyzate was determined by the method of Somogyi (3) and Nelson (4). Protein and carbohydrate materials in crystalline α -glucosidase were present in the same zone of the density-gradient tube (Fig. 7). From the results described in this section, the carbohydrate material was a constituent of crystalline α -glucosidase.

4. Composition of carbohydrate

(1) *Tillman's reaction.* The absorption spectrum of the product formed from crystalline α -glucosidase (210 μg of protein) in the Tillman reaction was similar to those from hexoses (Fig. 8).

(2) *Bial's reaction.* Reaction mixtures containing crystalline α -glucosidase (1268 μg of protein) and mannose gave a pale green color in the Bial reaction. Their absorbance at 660 nm per unit weight was very low, and marked turbidity appeared. In the case of pentoses or hexuronic acids, the reaction mixture gave a greenish blue color having a characteristic absorption spectrum. Fig. 9 shows the absorption spectrum of the color produced from α -glucosidase by the Bial reaction together with those from the reference sugars. These results suggested the absence of pentose in α -glucosidase, and the Tillman

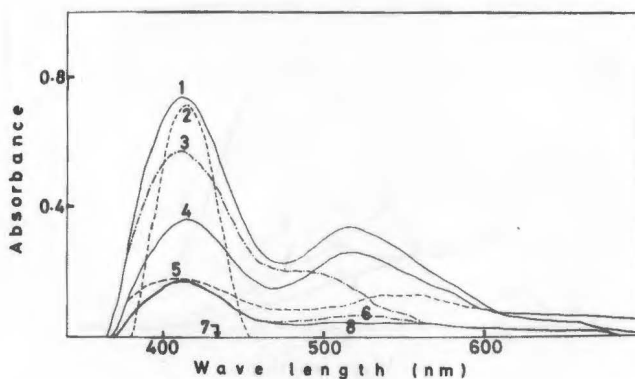


Fig. 8. The Tillman reaction of α -glucosidase.

The sample solution (1 ml) and 5 ml of 0.1 % orcinol in 67 % H_2SO_4 was mixed in a glass tube, and the resulting mixture was heated for 10 min at 80 °C in the dark. After cooling the tube with running tap water, the absorption spectrum of the reaction mixture was recorded on a Hitachi model EPS-3 T recording spectrophotometer. 1, Mannose (40 μg); 2, xylose (40 μg); 3, fucose (40 μg); 4, galactose (40 μg); 5, glucuronic acid (40 μg); 6, glucose (40 μg); 7, glucosamine (46 μg); 8, crystalline α -glucosidase (210 μg of protein).

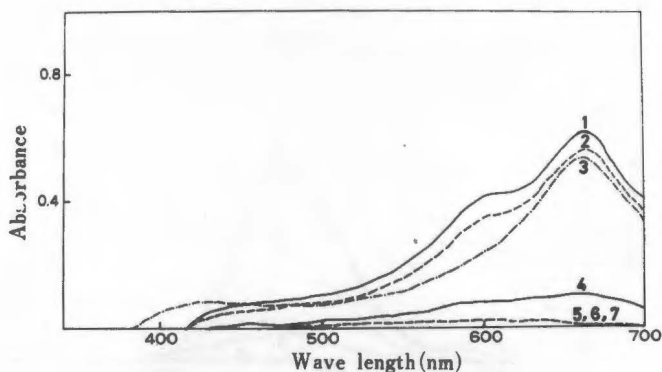


Fig. 9. The Bial reaction of α -glucosidase.

The sample solution (2 ml) was mixed with 2 ml of $FeCl_3$ -HCl reagent and 2 ml of orcinol reagent, and the mixture was heated for 20 min in a boiling water bath. After cooling it with ice-cold water, the absorption spectrum was measured. 1, Xylose (100 μg); 2, arabinose (100 μg); 3, ribose (100 μg); 4, glucuronic acid (100 μg); 5, mannose (100 μg); 6, fucose (100 μg); 7, crystalline α -glucosidase containing 70.7 μg of sugars calculated as mannose.

reaction result for α -glucosidase showed the absence of neutral sugars other than hexose. The neutral sugar content in intact α -glucosidase was calculated to be 5.59 g as mannose per 100 g of protein by applying the Tillman reaction.

(3) *Bitter and Muir's reaction*. The absorption spectrum of the color pro-

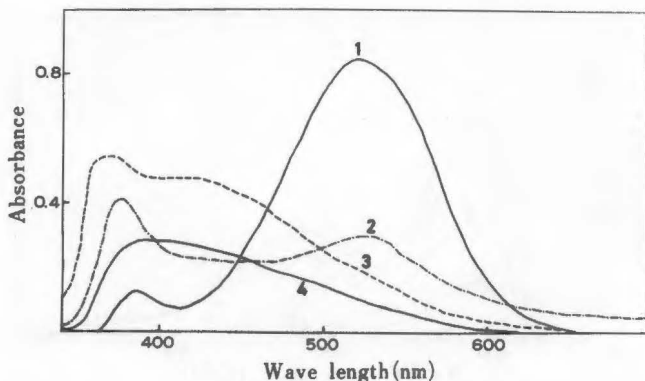


Fig. 10. The Bitter and Muir reaction of α -glucosidase.

To the sample solution (1 ml), 5 ml of conc. H_2SO_4 was added dropwise under cooling, and the mixture was heated for 10 min in a boiling water bath. After cooling with ice-cold water, 0.2 ml of 0.125 % carbazole solution in ethyl alcohol was added to the resulting mixture, and then the mixture was heated for 15 min in a boiling water bath. After cooling with ice-cold water, the absorption spectrum of the mixture was measured. 1, Glucuronic acid (30 μg); 2, galactose (200 μg); 3, mannose (353.6 μg); 4, crystalline α -glucosidase containing 106.1 μg of sugars calculated as mannose.

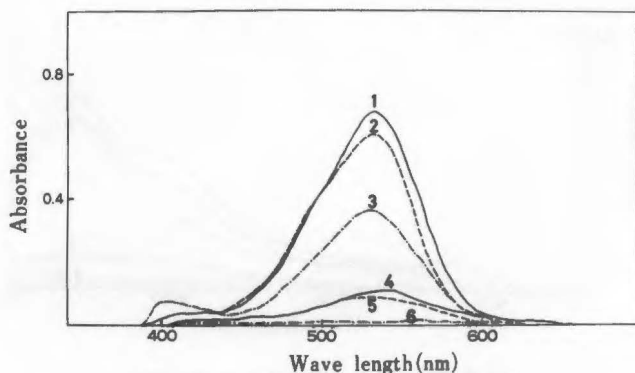


Fig. 11. The Elson and Morgan reaction of α -glucosidase.

Crystalline α -glucosidase (7.172 mg of protein) was dissolved in 2 ml of 1 N HCl and hydrolyzed in a sealed glass tube for 4 hours at 102 $^{\circ}C$. After cooling, the hydrolyzate was neutralized with 1 N NaOH. To 2 ml of this solution, 1 ml of the Elson and Morgan reagent was added, and the mixture was heated for 20 min in a boiling water bath. After cooling, 2.5 ml of ethyl alcohol and 1 ml of the Ehrlich reagent were added successively to the mixture, and then the mixture was allowed to stand for 45 min to develop color. The mixture was made up to 7.5 ml with ethyl alcohol, and its absorption spectrum was measured. 1, Galactosamine (100 μg); 2, glucosamine (100 μg); 3, crystalline α -glucosidase (3.586 mg of protein); 4, N-acetylglucosamine (100 μg); 5, N-acetylgalactosamine (100 μg); 6, glucose (100 μg).

duced from the crystalline α -glucosidase by the Bitter and Muir reaction is shown in Fig. 10, together with those from the reference sugars. The reaction mixture containing hexuronic acid or galactose exhibited a characteristic pink color in the Bitter and Muir's reaction, while the mixture containing the α -glucosidase (1902 μ g of protein) or mannose gave a yellow color, and an absorption spectrum with a maximum at 530 nm was not produced. These results suggested that hexuronic acid was not present in α -glucosidase.

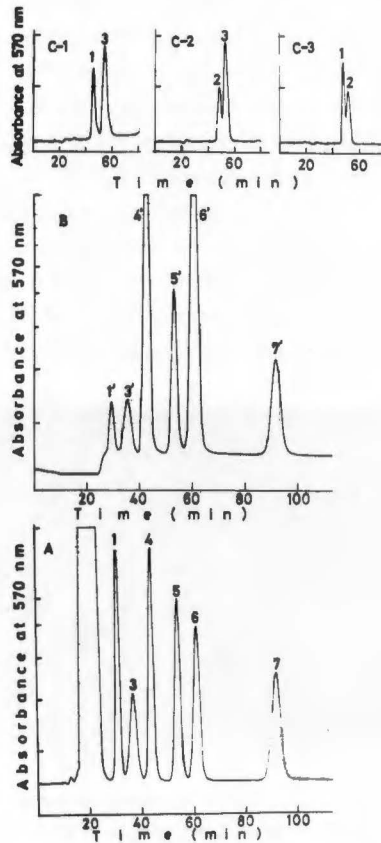


Fig. 12. Column chromatograms of hexosamine components.

A, Mixture of standard amino acids and glucosamine. B, 24-Hr hydrolyzate of α -glucosidase. C-1: Mixture of glucosamine and tryptophan. C-2, Mixture of galactosamine and tryptophan. C-3, Mixture of glucosamine, galactosamine, N-acetylglucosamine and N-acetylgalactosamine. Buffer: 0.35 N sodium citrate buffer, pH 5.20, containing 0.2 % ethyl alcohol. Column: $8\phi \times 150$ mm (C-1, C-2, C-3), $8\phi \times 100$ mm (A, B). Column resin: Nihon Denshi LCR-2. The column was equilibrated at 60°C . The flow rate of the buffer was 0.84 ml per minute in experiments C-1, C-2 and C-3, and 0.98 ml per minute in experiments A and B. The standard solution (A) contained $1/30$ μ mole of each amino acid and glucosamine. 1, Glucosamine; 2, galactosamine; 3, tryptophan; 4, lysine; 5, histidine; 6, ammonia; 7, arginine.

(4) *Reaction with periodate-thiobarbituric acid.* Crystalline α -glucosidase (3.536 mg of protein) was hydrolyzed with 1 ml of 0.1 N H_2SO_4 for 60 min at 80 °C. The detection of sialic acids in 0.2 ml of hydrolyzate was performed by the periodate-thiobarbituric acid method (10), and no absorbance was observed at 549 nm.

(5) *A modification of Elson and Morgan's reaction.* Crystalline α -glucosidase (7.172 mg of protein) was dissolved in 2 ml of 1 N HCl and heated in a sealed glass tube for 4 hr at 102 °C. After cooling, the hydrolyzate was neutralized with 1 N NaOH. The neutralized hydrolyzate gave a pinkish red color in a modified Elson and Morgan reaction; the characteristic absorption spectrum with a maximum at 530 nm was given by the color (Fig. 11).

(6) *Chromatography of the hydrolyzate with p-toluene sulfonic acid on ion-exchange resin.* Crystalline α -glucosidase (1.926 mg) was hydrolyzed with 1 ml of 3 N p-toluene sulfonic acid containing 0.2 % 3-(2-aminoethyl) indole for 24 and 48 hr at 105 °C in a sealed glass tube under argon gas phase. After cooling, 2 ml of 1 N NaOH was added to the hydrolyzate. This solution was filled up to 5 ml with deionized water, adjusted to pH 2.0 with 4 N NaOH, and filtered. The filtrate was diluted three times with distilled water, and the diluted solution was analyzed by using an amino acid analyzer. As shown in Fig. 12,

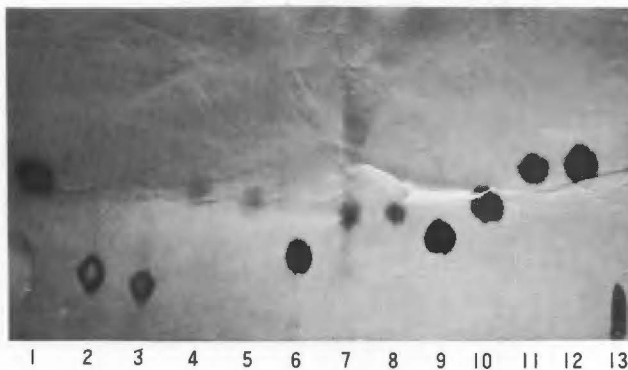


Fig. 13. A paper chromatogram of neutral sugar constituents of α -glucosidase.

Crystalline α -glucosidase (7.148 mg) was hydrolyzed with 3 ml of 2 N H_2SO_4 at 100 °C for 5 hr in a sealed glass tube. The hydrolyzate was cooled and neutralized with $BaCO_3$, and the resultant precipitate was removed by centrifugation. The supernatant solution was passed through the column of mixed ion-exchange resins of Dowex 50 W x 2 and Dowex 1 x 2 (130: 235). The effluent and washings with deionized water were evaporated to dryness *in vacuo*, and the residue was dissolved in 1 ml of deionized water. This solution (0.2 ml) was applied on a Toyo No. 50 filter paper and developed for 43 hr by the ascending method with a solvent system of n-butyl alcohol-ethyl alcohol-water (10: 1: 2, v/v). Aliquots of 0.05 ml of 0.2 % reference sugar solution were spotted on the same filter paper. After drying, sugars were detected by the silver nitrate dip method (24). 1, Xylose; 2, glucosamine; 3, galactosamine; 4, N-acetylglucosamine; 6, galactose; 7, neutral sugar constituent of α -glucosidase; 8, mannose; 9, glucose; 10, arabinose; 11, ribose; 12, fucose; 13, glucuronic acid.

hexosamines appeared prior to tryptophan in the chromatogram. Glucosamine was separated from galactosamine, but neither N-acetylglucosamine nor N-acetylgalactosamine was detected in the effluent from the column even after 80 min (C-3). One peak (peak 1') appeared prior to tryptophan (peak 3') in the chromatogram of the hydrolyzate of the α -glucosidase. The ratios of the retention time of glucosamine, galactosamine and the peak 1' of the hydrolyzate to that of tryptophan were 0.833, 0.906 and 0.840 (average values of 24 and 48 hr hydrolyzates), respectively. The value at the peak 1' was very similar to that of glucosamine. From the area of peak 1', the glucosamine content was calcu-

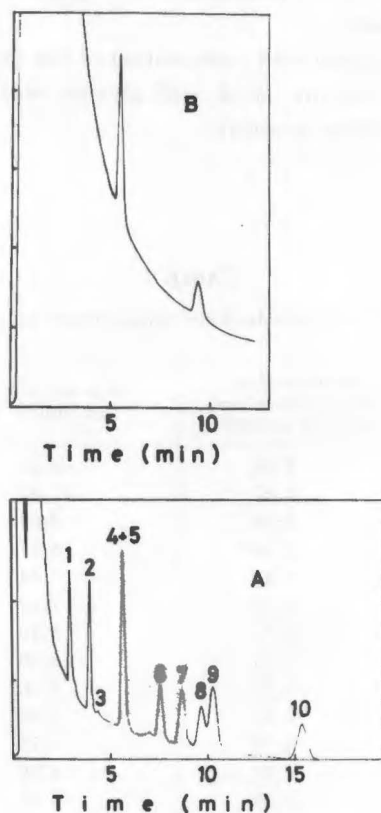


Fig. 14. Gas-liquid chromatograms of TMS derivatives of neutral sugar constituent of α -glucosidase and reference sugars.

A, Standard mixture of reference sugars. B, Neutral sugar of crystalline α -glucosidase. A Hitachi model 063 gas chromatograph equipped with a hydrogen flame ionization detector was used. Experimental conditions were as follows: column, a spiral column (3 ϕ x 2,000 mm) packed with 10 % Carbowax 20 M on Chromosorb W (60-80 mesh); carrier gas, helium at a flow rate of 40 ml per min. The column was run isothermally at 200 $^{\circ}$ C, with the injector at 250 $^{\circ}$ C. 1, α -Arabinose; 2, β -arabinose; 3, γ -arabinose; 4, α -galactose; 5, α -mannose; 6, β -galactose; 7, α -glucose; 8, β -mannose; 9, γ -galactose; 10, β -glucose.

lated to be 1.76 g per 100 g of protein.

(7) *Paper chromatography and gas-liquid chromatography of neutral sugar constituents.* As shown in Fig. 13, the hydrolyzate treated with ion-exchange resin gave a single spot of neutral sugar corresponding to mannose on the paper chromatogram. In tests with glucose oxidase, glucose was shown to be absent in the hydrolyzate containing 400 μ g of sugars calculated as mannose per ml. The gas-liquid chromatography of the hydrolyzate also revealed that mannose was the sole constituent of neutral sugar in crystalline α -glucosidase (Fig. 14). The carbohydrate composition of the α -glucosidase is shown in Table 1. The hexosamine constituent is assumed to be glucosamine.

5. Amino acid composition

Table 1 shows the amino acid composition of the crystalline α -glucosidase. Aspartic acid, lysine, glutamic acid and glycine were abundant, while half cystine was present in minute quantity.

TABLE 1
Amino acid and carbohydrate compositions of α -glucosidase

	Amino acid or carbohydrate per 100 g of enzyme	N as per cent of total N	No. of residues per 122, 400 mol. wt.
Tryptophan	3.84	3.40	23
Lysine	9.67	11.95	81
Histidine	4.94	8.63	39
Ammonia	1.54 ^{a)}	8.17	111 ^{a)}
Arginine	4.84	10.04	34
Aspartic acid	11.09	7.52	102
Threonine	6.72	5.10	69
Serine	3.95	3.40	46
Glutamic acid	9.38	5.76	78
Proline	5.17	4.06	55
Glycine	4.78	5.75	78
Alanine	4.00	4.06	55
Half cystine	0.29	0.22	3
Valine	5.07	3.91	53
Methionine	2.07	1.25	17
Isoleucine	6.22	4.28	58
Leucine	6.54	4.50	61
Tyrosine	7.55	3.76	51
Phenylalanine	5.53	3.02	41
Glucosamine	1.76	0.89	12
Mannose	5.59		38
Total	109.01	99.66	994

a) These values are omitted from the total.

DISCUSSION

Crystalline α -glucosidase from the mycelia of *M. javanicus* was characterized as a glycoprotein, because the carbohydrate component was not split from the protein moiety under conditions of disc gel electrophoresis, gel electrofocusing, Bio-Gel column chromatography and density-gradient centrifugation. The amounts of carbohydrate residues were significant. The α -glucosidase was considered to contain hexose and hexosamine from the positive Tillman and Rimington reactions. Furthermore, results obtained by several chromatographic techniques led to the conclusion that mannose and glucosamine were the sole constituent of neutral sugar and hexosamine, respectively. Neither glucose, pentoses, hexuronic acid, nor sialic acid was detected by the glucose oxidase method, the method known as Bial's reaction, the modified carbazole method of Bitter and Muir, and the periodate-thiobarbituric acid method, respectively. The carbohydrate content of α -glucosidase shown in Table 1 was similar to that of glucosylase from *Rhizopus javanicus* (18), but differed from the carbohydrate contents of α -glucosidases from *M. rouxii* (19) and *Asp. fumigatus* (20) which contained glucose together with other carbohydrate residues. Table 1 shows that α -glucosidase consisted of 18 kinds of amino acid residues. The high content of aspartic acid, lysine and histidine that was closely related to the α -glucosidase activity of *M. javanicus* in the active center (2) was characteristic of the amino acid composition of α -glucosidase from *M. javanicus*, in comparison with those of α -glucosidases of cattle liver (21) and *Asp. fumigatus* (20).

SUMMARY

The crystalline α -glucosidase from *Mucor javanicus* has a sedimentation constant ($s_{20,w}^0$) of 6.1 S, a diffusion constant ($D_{20,w}$) of 4.8×10^{-7} cm²·sec⁻¹, and an average molecular weight of 124,600, as determined by two different methods. The α -glucosidase is a glycoprotein containing the following constituent: tryptophan₂₃, lysine₈₁, histidine₃₉, arginine₃₄, aspartic acid₁₀₂, threonine₆₉, serine₄₆, glutamic acid₇₈, proline₈₈, glycine₇₈, alanine₈₈, half cystine₃, valine₅₃, methionine₁₇, isoleucine₈₈, leucine₈₁, tyrosine₈₁, phenylalanine₄₁, glucosamine₁₂, and mannose₃₈.

The low content of half cystine, the high content of aspartic acid, lysine and histidine, and the presence of mannose as the sole constituent of neutral sugar are characteristic of this enzyme.

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