Purification and Characterization of α -Mannosidase and β -*N*-Acetylhexosaminidase from Watermelon Fruit

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Received March 11, 1988

Watermelon fruit was found to be rich in α -mannosidase (EC 3.2.1.24) and β -*N*-acetylhexosaminidase (EC 3.2.1.52). A simple procedure has been devised for the simultaneous isolation of α -mannosidase and β -*N*-acetylhexosaminidase from watermelon fruit. The purified enzymes were electrophoretically homogeneous and sufficiently free from other glycosidases. The molecular weights of α -mannosidase and β -*N*-acetylhexosaminidase were estimated to be 270,000 and 150,000 by Sephadex G-200 gel filtration. The enzymes had their maximum activities at pHs 4.0~4.5 and 5.0~5.5, and their *K_m* values for the corresponding *p*-nitrophenyl glycoside were 2.3 and 0.65 mM, respectively. α -Mannosidase required Zn^{2+} to retain its activity and liberated 60% of the mannose from ovalbumin glycopeptide.

Glycosidases are very useful for the structural and functional studies of glycoconjugates such as glycoproteins and glycolipids.^{1~2)} To date, many glycosidases have been isolated from a number of enzyme sources. In the utilization of glycosidases for structural and functional studies, the enzyme must be sufficiently free from contamination by other glycosidases. In spite of remarkable development of methods for enzyme purification, it is still quite difficult to isolate a homogeneous glycosidase entirely free from other glycosidases.

We found that watermelon fruit contained large amounts of α -mannosidase (EC 3.2.1.24), β -*N*-acetylhexosaminidase (EC 3.2.1.52), and α -galactosidase (EC 3.2.1.22). In our previous study,³⁾ α -galactosidase was purified from watermelon fruit and characterized. In this paper, we describe the purification of α -mannosidase and β -*N*-acetylhexosaminidase from watermelon fruit and the properties of the purified enzymes.

MATERIALS AND METHODS

Materials. *p*-Nitrophenyl β -D-*N*-acetylglucosaminide and *p*-nitrophenyl α -D-*N*-acetylgalactosaminide were purchased from Koch-Light Laboratories, Ltd. The other *p*-nitrophenyl glycosides were purchased from Nakarai Chemicals, Ltd. DE-53 (DEAE-cellulose) and CM-52 (CM-cellulose) were purchased from Whatman, Ltd. Diaflo PM-10 and YM-5 membranes were purchased from Amicon, Ltd. Ovalbumin glycopeptide (mannose 45.0%, glucosamine 23.8% by gas chromatography⁴⁾) was prepared from ovalbumin by the method of Yamashina and Makino.⁵⁾

Enzyme assays. Activities of glycosidases were assayed at 37°C using the corresponding *p*-nitrophenyl glycoside as the substrate. The enzyme solution (10~50 μ l) was added to 0.3 ml of 2 mM *p*-nitrophenyl glycoside dissolved in 0.05 M sodium citrate buffer, pH 4.6. After incubation for a preset time, 3.0 ml of 0.2 M sodium borate buffer, pH 9.8, was added to stop the reaction and the absorbance of the resultant solution was measured at 400 nm. One unit of enzyme was defined as the amount of enzyme which hydrolyzes 1 μ mol of *p*-nitrophenyl glycoside per min under the conditions described above.

Analytical methods. Protein was measured by the meth-

od of Lowry *et al.*⁶⁾ with bovine serum albumin as the standard. Disc polyacrylamide gel electrophoresis was done at pH 9.5 in 7.5% gel by the procedure of Davis.⁷⁾ Gel was stained for protein with 0.25% Coomassie Brilliant Blue R-250 in ethanol-acetic acid-water (9:2:9, v/v/v) and destained with ethanol-acetic acid-water (25:8:65, v/v/v). Mannose liberated from ovalbumin glycopeptide was determined by gas chromatography in the following procedure. The incubation mixture to which 100 μ g of glucitol was added as the internal standard was passed through a Dowex 50-X8 column (H^+ , 200~400 mesh, 0.5 ml), and the effluent was evaporated to dryness. The residue was further dried at 50°C in a stream of nitrogen and then trimethylsilylated in 0.2 ml of a mixture of pyridine-hexamethyldisilazane-trimethylchlorosilane (10:2:1, v/v/v).⁸⁾ A sample (2~3 μ l) of the reaction mixture was injected into a gas chromatograph with a hydrogen flame ionization detector. The conditions used were as follows: column, glass column (0.2 $\times$ 200 cm) packed with 5% SE-30 on Shimalite W (80~100 mesh); carrier gas, N_2 30 ml/min; column temperature, 190°C.

RESULTS

Purification of α -Mannosidase and β -N-Acetylhexosaminidase from Watermelon Fruit

Step 1. Extraction. After seeds and peel had been removed, the fruit (3.5 kg) of a

watermelon (6 kg) was cut into small pieces and homogenized with a blender. The homogenate was filtered through cheese cloth and the filtrate was centrifuged at 5000 $\times g$ for 20 min. Table I shows glycosidase activities in the total supernatant fluid. Solid ammonium sulfate was added to the supernatant to 70%

TABLE I. GLYCOSIDASE ACTIVITIES IN THE WHOLE FRUIT AND SEEDS FROM A WATERMELON

Glycosidase	Juice	500 seeds
α -Mannosidase	550 units	0.3 units
β -N-Acetylhexosaminidase	530	0.3
α -Galactosidase	480	0.3
β -Glucosidase	100	
β -Galactosidase	60	
α -Glucosidase	60	
β -Mannosidase	20	
α -N-Acetylgalactosaminidase	10	
α -Fucosidase	10	
β -Glucuronidase	10	

The watermelon, with a weight of 6 kg, supplied 3.5 l of fruit juice and about 500 seeds. All the seeds were crushed in a steel mortar and extracted with 0.05 M sodium citrate buffer, pH 4.6. The extract was used for measurement of glycosidase activities.

FIG. 1. DE-53 Chromatography of the Enzyme Solution Obtained at Step 1.

The enzyme solution (80 ml) containing 0.6 g of protein was applied to a DE-53 column (3.0 $\times$ 42 cm) which had been equilibrated with 0.05 M sodium phosphate buffer, pH 7.3. The column was eluted with the same buffer and then eluted with a linear NaCl gradient in the same buffer at a flow rate of 50 ml/hr. Fractions (10 ml) were collected. —, absorbance at 280 nm; ○, α -mannosidase activity; ●, α -galactosidase activity; △, β -N-acetylhexosaminidase activity.

saturation and the mixture was left overnight. The precipitate was collected by centrifugation, dissolved in a minimal volume of 0.025 M sodium phosphate buffer, pH 7.3, and dialyzed exhaustively against the same buffer. The dialyzate was centrifuged at $15,000 \times g$ for 30 min to remove insoluble matter.

Step 2. DE-53 Chromatography. The enzyme solution from Step 1 was chromatographed on DE-53. The elution profile is shown

in Fig. 1. α -Mannosidase and α -galactosidase were separately eluted with the buffer used for equilibration of the column. After the α -galactosidase peak had completely emerged, the buffer was changed to a sodium chloride gradient to elute β -N-acetylhexosaminidase. The fractions containing the individual enzyme activity as shown by horizontal bars were pooled and the enzymes were precipitated by reverse dialysis against saturated ammonium sulfate

FIG. 2. Sephacryl S-200 Gel Filtration of the α -Mannosidase Preparation Obtained at Step 2.

The enzyme solution (10 ml) was applied to a Sephacryl S-200 column (4.0×90 cm) previously equilibrated with 0.05 M sodium phosphate buffer, pH 7.0. The column was eluted with the same buffer at a flow rate of 30 ml/hr and 10-ml fractions were collected. ----, absorbance at 280 nm; $\circ$, α -mannosidase activity.

FIG. 3. Sephacryl S-200 Gel Filtration of the β -N-Acetylhexosaminidase Preparation Obtained at Step 2.

The enzyme solution (10 ml) was applied to the Sephacryl S-200 column used for the α -mannosidase preparation. The conditions were the same as described in Fig. 2. ----, absorbance at 280 nm; $\triangle$, β -N-acetylhexosaminidase activity.

solution. The precipitates collected by centrifugation were dissolved in minimal volumes of 0.05 M sodium phosphate buffer, pH 7.3, and dialyzed against the same buffer.

Step 3 (for α -mannosidase). *Sephacryl S-200 gel filtration of the α -mannosidase preparation.* The α -mannosidase solution from Step 2 was filtered on Sephacryl S-200 gel. The elution profile is shown in Fig. 2. α -Mannosidase was eluted immediately after the void volume of the column. The fractions containing α -mannosidase were pooled and concentrated by ultrafiltration with a Diaflo PM-10 membrane.

Step 3 (for β -N-acetylhexosaminidase). *Sephacryl S-200 gel filtration of the β -N-acetylhexosaminidase preparation.* The β -N-acetylhexosaminidase solution from Step 2 was filtered on Sephacryl S-200 gel. The elution profile is shown in Fig. 3. The fraction containing β -N-acetylhexosaminidase were pooled and concentrated by ultrafiltration with a Diaflo PM-10 membrane.

Step 4 (for α -mannosidase). *Sephadex G-200 gel filtration of the α -mannosidase preparation.* The α -mannosidase solution from Step 3 was filtered on Sephadex G-200 gel. The elution profile is shown in Fig. 4. The fractions containing α -mannosidase were pooled and

concentrated by ultrafiltration with a Diaflo PM-10 membrane. The concentrate was used for characterization as the final α -mannosidase preparation.

Step 4 (for β -N-acetylhexosaminidase). *CM-52 chromatography of the β -N-acetylhexosaminidase preparation.* The β -N-acetylhexosaminidase preparation from Step 3 was applied to a CM-52 column (1.8×12 cm) which had been equilibrated with 0.05 M sodium citrate buffer, pH 4.4. The column was eluted with the same buffer at a flow rate of 6 ml/hr, followed by elutions with 0.1 M sodium citrate buffer, pH 5.0, 0.1 M sodium citrate buffer, pH 5.6, and a NaCl gradient in the buffer. All the β -N-acetylhexosaminidase activity was eluted with the first buffer in the unadsorbed fraction and contaminating proteins which were retained on the column were eluted by subsequent elutions (data not shown). The fractions containing β -N-acetylhexosaminidase were pooled and concentrated by ultrafiltration with a Diaflo PM-10 membrane. The concentrate was used for characterization as the final β -N-acetylhexosaminidase preparation.

The overall purification process of α -mannosidase and β -N-acetylhexosaminidase is

FIG. 4. Sephadex G-200 Gel Filtration of the α -Mannosidase Preparation Obtained at Step 3.

The enzyme solution (1.5 ml) was applied to a Sephadex G-200 column (2.6×56 cm) previously equilibrated with 0.05 M sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl. The column was eluted with the same buffer at a flow rate of 10 ml/hr and 4-ml fractions were collected. ---, absorbance at 280 nm; O, α -mannosidase activity.

TABLE II. PURIFICATION OF α -MANNOSIDASE AND β -N-ACETYLHEXOSAMINIDASE FROM 3.5 LITERS OF WATERMELON FRUIT JUICE

Steps	α -Mannosidase				β -N-Acetylhexosaminidase			
	TU	TP	SA	Rec	TU	TP	SA	Rec
		(mg)	(units/mg)	(%)		(mg)	(units/mg)	(%)
Juice	550	4800	0.11	100	530	4800	0.11	100
(NH ₄) ₂ SO ₄ ppt	370	620	0.60	68	420	620	0.68	80
DE-53	320	140	2.3	58	320	140	2.3	60
Sephacryl S-200	210	8.3	25	39	270	6.0	45	51
Sephadex G-200 or CM-52	87	2.2	40	16	220	2.4	92	42

TU, total units; TP, total protein; SA, specific activity; Rec, recovery.

FIG. 5. Disc Polyacrylamide Gel Electrophoresis of Purified α -Mannosidase and β -N-Acetylhexosaminidase.

A, α -mannosidase: upper band, purified enzyme; lower band, bovine serum albumin which had been added as a stabilizer.

B, β -N-acetylhexosaminidase.

FIG. 6. Estimation of Molecular Weights of α -Mannosidase and β -N-Acetylhexosaminidase by Sephadex G-200 Gel Filtration.

Column, 2.6×56 cm; eluent, 0.05 M sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl. 1, ribonuclease A; 2, chymotrypsinogen A; 3, ovalbumin; 4, bovine serum albumin; 5, watermelon β -N-acetylhexosaminidase; 6, aldolase; 7, catalase; 8, watermelon α -mannosidase; 9, ferritin.

summarized in Table II.

Properties of α -Mannosidase and β -N-Acetylhexosaminidase Purity

As the purity of the enzymes was judged by disc gel electrophoresis, the α -mannosidase and β -N-acetylhexosaminidase preparations were homogeneous (Fig. 5). To evaluate possible contamination by other glycosidases, the purified enzymes (0.4 unit) were incubated at 37°C for 2 hr with each of the following p-

nitrophenyl glycosides: α - and β -galactopyranosides, α - and β -mannopyranosides, α - and β -glucopyranosides, α -L-fucopyranoside, β -N-acetylglucosaminide, α -N-acetylgalactosaminide, and β -glucuronide. The purified α -mannosidase and β -N-acetylhexosaminidase contained no other glycosidase activities.

Molecular weight

The molecular weights of α -mannosidase and β -N-acetylhexosaminidase were estimated

to be 270,000 and 150,000, respectively, by gel filtration on a Sephadex G-200 column calibrated with standard proteins of known molecular weight (Fig. 6).

FIG. 7. Effect of pH on the Activities of α -Mannosidase and β -N-Acetylhexosaminidase.

○, α -mannosidase; △, β -N-acetylhexosaminidase. pH 3~6, sodium citrate buffer; pH 7~8, sodium phosphate buffer.

FIG. 8. Effect of pH on the Stability of α -Mannosidase and β -N-Acetylhexosaminidase.

○, α -mannosidase; △, β -N-acetylhexosaminidase. pH 3~6, sodium citrate buffer; pH 7~8, sodium phosphate buffer; pH 9~9.5, ammonium chloride buffer.

pH Optimum

The effect of pH on the activities of the enzymes was examined using the corresponding *p*-nitrophenyl glycosides as substrates in sodium citrate and sodium phosphate buffers (Fig. 7). α -Mannosidase and α -N-acetylhexosaminidase had their maximum activities at pHs 4.0~4.5 and 5.0~5.5, respectively.

pH Stability

The stability of the enzymes at various pHs was examined. The enzymes were incubated in buffers from pH 3.0 to 9.5 for 3 hr at 25°C and then the residual activities were assayed under the standard conditions (Fig. 8). The activities of α -mannosidase and β -N-acetyl-

FIG. 9. Effects of Zn^{2+} , Albumin, and EDTA on the Activity of α -Mannosidase.

α -Mannosidase (0.3 unit) was incubated at 30°C in 0.5 ml of 0.05 M sodium acetate buffer, pH 4.5, in the presence and absence of 0.2 mg of bovine serum albumin. After 2 hr an equal volume of 2 mM $ZnSO_4$ in the buffer, of 2 mM EDTA in the buffer, or of the buffer alone was added, and the incubation was continued. At intervals of two hours, portions of the reaction mixtures were assayed and the results were expressed as percentages of the original activity of the untreated enzyme sample. ●, enzyme + albumin + Zn^{2+} ; ▲, enzyme + Zn^{2+} ; ○, enzyme + albumin; △, enzyme alone; □, enzyme + albumin + EDTA.

hexosaminidase were fairly stable in the range of pH 7~9 and 6.5~8, respectively.

Effect of substrate concentration

The effect of substrate concentration on the reaction rate of α -mannosidase and β -N-acetylhexosaminidase was examined at their pH optima and 37°C. The initial velocities were measured by the method of Algranati.⁹⁾ The K_m values for the corresponding *p*-nitrophenyl glycoside were calculated from the Lineweaver-Burk plots to be 2.3 and 0.65 mM, respectively.

Effects of albumin, Zn^{2+} , and EDTA

Since α -mannosidases from some sources^{10~13)} are Zn^{2+} -containing enzymes, the effects of Zn^{2+} and EDTA on the activity of watermelon α -mannosidase were studied in the presence and absence of bovine serum albumin (Fig. 9). The α -mannosidase preparation gradually lost its activity during incubation at 30°C in 0.05 M sodium acetate buffer, pH 4.5. Bovine

serum albumin protected the α -mannosidase activity. Moreover, the addition of Zn^{2+} not only protected the enzyme activity against the progressive fall but also gradually restored the activity already lost. EDTA accelerated the inactivation of the enzyme.

Liberation of mannose from ovalbumin glycopeptide by α -mannosidase

Ovalbumin glycopeptide was exhaustively digested by watermelon α -mannosidase (Fig. 10). After 44 hr, 60% of the mannose in the glycopeptide was liberated and no significant further liberation was observed for the next 24 hr.

DISCUSSION

Watermelon fruit is quite useful as a source of α -mannosidase, α -galactosidase, and β -N-acetylhexosaminidase since it contains large amounts of these enzymes (Table I). We have devised in this study a simple procedure for the simultaneous isolation of these glycosidases.

α -Mannosidase was purified with a recovery of 16% to an electrophoretically homogeneous form. The enzyme was largely inactivated at the final step of purification. This inactivation appears to depend on the degree of purification and dilution of the enzyme. The addition of a small amount of Zn^{2+} in the buffer may protect the activity at the advanced steps of purification. Inactivation of the enzyme will be hastened by trace amounts of certain bivalent ions such as Cd^{2+} and Cu^{2+} , as well as EDTA.¹⁰⁾ Watermelon α -mannosidase has similar properties to the α -mannosidase from jack beans¹²⁾ in the pH optimum and K_m value toward *p*-nitrophenyl α -D-mannoside. The α -mannosidase preparation liberated mannose from ovalbumin glycopeptide. This indicates that the enzyme can hydrolyze α -mannosidic linkages in various natural substrates.

β -N-Acetylhexosaminidase was purified with a recovery of 42% to an electrophoretically homogeneous form. The enzyme has similar properties to the β -N-acetylhexosaminidase from jack beans¹²⁾ in the pH optimum and

FIG. 10. Liberation of Mannose from Ovalbumin Glycopeptide by α -Mannosidase.

Samples, which consisted of 350 μ g of ovalbumin glycopeptide, 2.0 units of α -mannosidase, 100 μ g of bovine serum albumin and 1 nmol of $ZnSO_4$ in 0.5 ml of sodium acetate buffer, pH 4.5, were placed in four small screw-cap tubes, and incubated at 37°C in the presence of toluene. After 10, 24, 44, and 68 hr, one of the samples was used for gas chromatographic analysis of liberated mannose and to the other, 0.25 ml of 0.05 M sodium acetate buffer, pH 4.5, containing 1.0 unit of α -mannosidase, 50 μ g of albumin, and 0.5 nmol of $ZnSO_4$ was added. The percent liberation of mannose was calculated on the basis of the total mannose content of the ovalbumin glycopeptide.

Km value toward *p*-nitrophenyl β -D-*N*-acetylglucosaminide.

In our previous study,³⁾ α -galactosidase was purified from watermelon fruit by procedures including chromatographies on DEAE-Sephadex A-50, CM-Sephadex C-50, and Sephadex G-100. The enzyme liberates galactose from melibiose, raffinose, stachyose, and ceramide trihexoside, and abolishes the hemagglutination-inhibiting activities of human ovarian cyst B-glycoprotein and blood group B-type ghosts with release of the galactose unit. In this study, a sufficient amount (130 units) of purified α -galactosidase was also obtained from the α -galactosidase preparation after DE-53 chromatography (Fig. 1) by similar procedures described above (data not shown). Ahmed *et al.*¹⁴⁾ prepared partially purified α -galactosidase from watermelon seeds. Watermelon fruit is much better than the seeds (Table I).

Watermelons, which are readily available and big, are useful as a source of α -mannosidase, α -galactosidase, and β -*N*-acetylhexosaminidase. The purified glycosidases obtainable by this simple procedure may be valuable for structural and functional studies on various glycoconjugates.

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