α -Mannosidase from Pineapple Fruit: Partial Purification and Action on Glycopeptides

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α -Mannosidase [EC 3.2.1.24, α -D-mannoside mannohydrolase] from the acetone powder of pineapple fruit juice was purified 190-fold by column chromatographic procedures. The partially purified α -mannosidase was detected to be contaminated with little other glycosidases, using *p*-nitrophenyl derivatives of glycosides. The enzyme released mannose from both the carbohydrate moiety of stem bromelain and glycopeptide prepared from the parent protein. The enzyme split about 70% of the total mannose of ovalbumin glycopeptide.

The utilization of specific glycosidases to liberate the individual carbohydrate units is an excellent tool for elucidating the sequence and anomeric configuration of the carbohydrate units of glycoproteins. Thus, α -mannosidase from jack bean meal has been used to identify α -mannosidic linkage of glycopeptides of ovalbumin,¹⁾ α_1 -acid glycoprotein²⁾ and stem bromelain³⁾; α -mannosidase from *Turbo cornutus* was utilized for glycopeptides of ovalbumin,⁴⁾ Taka-amylase⁴⁾ and stem bromelain⁵⁾; and α -mannosidase from hog kidney⁶⁾ was used for glycopeptides of ovalbumin, Taka-amylase and stem bromelain. Since the glycosidase employed for this purpose needs to be free from unnecessary glycosidase contaminating, in the present work we have attempted to purify α -mannosidase from pineapple fruit, with special regard to the contamination of other glycosidases. This paper describes the presence of activities of several glycosidases, *i.e.* α -glucosidase, α -galactosidase, β -galactosidase, α -mannosidase, β -mannosidase, β -xylosidase and β -N-acetylglucosaminidase in the acetone-dried powder prepared from pineapple fruit juice. One of the major glycosidases, α -mannosidase, was partially purified and the action of the purified α -mannosidase on some natural substrate is also described.

MATERIALS AND METHODS

Acetone-dried powder of pineapple fruit juice (Lot No. RG 163-18) was donated by Dole Co., Honolulu Hawaii. *p*-Nitrophenyl-derivatives of α -mannose, α -glucose, α -galactose, β -galactose, β -mannose, β -xylose and β -N-acetylglucosamine were purchased from Pierce Chemical Co. DEAE-cellulose (DE-52) was a product of Whatman Co., and Sephadex G-100, a product of Pharmacia Co.

Cellulose AP⁷⁾ was a gift from Dr. T. Yamamoto of Osaka City University. One g of Cellulose AP suspended in 10 ml of 2 mM acetate buffer, pH 5.2, was centrifuged and the supernatant was used as the Cellulose AP preparation.

Stem bromelain, a glycoprotein, was purified by the method of Murachi *et al.*⁸⁾ and its glycopeptide was prepared by the pronase digestion as reported by Murachi *et al.*⁹⁾ Ovalbumin glycopeptide was prepared from twice-crystallized ovalbumin by the method used for the preparation of stem bromelain glycopeptide.

Assay of glycosidases. The determination of α -mannosidase activity was carried out as follows. The incubation mixture was composed of 0.2 ml of enzyme solution and 0.5 ml of 0.05 M acetate buffer, pH 4.4, containing 0.4 mg of *p*-nitrophenyl α -mannoside. After incubation for 30 min at 37°C, 4 ml of 5% Na₂CO₃ was added to the incubation mixture and the liberated *p*-nitrophenol was measured using the extinction at 410 nm. One unit of α -mannosidase was defined as the amount of enzyme which liberated 1 μ mole of *p*-nitrophenol from the substrate per min under the assay conditions. The specific activity of the enzyme was expressed as the units per mg of protein. The measurement of other glycosidases contained in

α -mannosidase preparation was carried out in the same way using *p*-nitrophenyl glycosides as described above.

Amount of stem bromelain glycopeptide was determined on a Hitachi Model KLA-3B Amino Acid Analyzer after hydrolysis of peptide with HCl. The mannose content of ovalbumin glycopeptide was determined by orcinol- H_2SO_4 method of Winzler.¹⁰⁾ The protein concentration of enzyme solution was estimated by the Kalckar's method,¹¹⁾ using the equation: Protein (mg/ml) = $1.45 A_{280} - 0.74 A_{260}$.

Liberated sugars were separated by descending paper chromatography on Toyo Roshi filter paper No. 51 with ethylacetate-acetic acid-water (3:1:3, upper phase) as solvent.

Hydrolysis of stem bromelain glycopeptide by α -mannosidase. After stem bromelain glycopeptide (0.8 μ mole) had been dissolved in 1.0 ml of 0.1 M acetate buffer, pH 4.4, 0.2 ml of α -mannosidase solution (2.8 units) was added to the glycopeptide solution, the reaction mixture was incubated at 37°C in the presence of approximately 5 μ l of toluene. Aliquots of the reaction mixture (0.3 ml) were withdrawn at the onset of the incubation and after 24 hr, 48 hr, 96 hr and 240 hr, and were assayed as to reducing power according to the method of Somogyi.¹²⁾ In the control run, the same amounts of substrate and enzyme as those in the test run were incubated separately and mixed at the end of the corresponding incubation periods.

Hydrolysis of ovalbumin glycopeptide by α -mannosidase. Ovalbumin glycopeptide (corresponding to 1.16 mg of mannose) was incubated with 0.2 ml of the enzyme solution (2.8 units). Further analyses were carried out in the same way as described about stem bromelain glycopeptide.

Hydrolysis of stem bromelain (glycoprotein) by α -mannosidase. One μ mole of stem bromelain was dissolved in 0.5 ml of 0.05 M acetate buffer, pH 4.4, and was incubated at 37°C with 0.2 ml of α -mannosidase solution (1.0 unit) with one drop of toluene. After 48 hr-incubation the reaction mixture was heated at 100°C for 5 min, and was centrifuged at 3000 rpm for 5 min to remove the insoluble material. The supernatant was passed successively through columns of 2 ml Amberlite IR-120 (H^+ form) and of 2 ml Dowex-1 (CO_3^{2-} form) in order to desalt. The effluent was dried and the residue was chromatographed on paper. Mannose thus separated was eluted from the paper, and its quantity was determined as described before.¹⁰⁾

RESULTS AND DISCUSSION

Purification of α -mannosidase from pineapple fruit

Ten g of acetone-dried powder prepared from

pineapple fruit juice was suspended in 100 ml of cold 0.02 M potassium phosphate buffer, pH 7.2, and 100 mg of $\text{Na}_2\text{S}_4\text{O}_6$ was added to the suspension to block the essential sulfhydryl group of major proteolytic enzyme in fruit juice. The mixture was allowed to stand for 1 hr at 4°C. Because it was an extremely viscous solution, Cellulose AP, a mixture of cellulase, hemicellulase and pectinase, was added in order to reduce the viscosity. After the addition of 3 ml of 1% Cellulose AP containing 0.5 mM EDTA and 0.5 mM $\text{Na}_2\text{S}_4\text{O}_6$, the mixture was stirred for 30 min at 0°C and centrifuged at 6000 rpm for 30 min to remove the insoluble material. Table I shows the activities of several glycosidases in the supernatant fluid. In Table I, activities of several glycosidases in the pineapple fruit extract (Lot No. RG 163-18) were also compared with those obtained by Li and Lee.¹³⁾

TABLE I. ACTIVITIES OF SEVERAL GLYCOSIDASE IN CRUDE EXTRACT OF PINEAPPLE FRUIT (Lot No. RG 163-18)

The activities of glycosidases varied considerably in different lots of pineapple fruit extract. In some cases the activity of β -xylosidase was about three times higher than that shown in the table.

Glycosidase	Activity (fruit) (unit/ml of enzyme solution)	Relative activity (fruit) (%)	Relative activity (stem) ¹³⁾ (%)
α -Mannosidase	1.31	100.0	100.0
α -Galactosidase	2.24	171.0	61.8
β -Galactosidase	0.83	63.3	77.6
β -Mannosidase	0.08	6.18	0.25
β -Xylosidase	0.12	9.1	—
β -N-Acetylglucosaminidase	0.11	8.4	56.7
α -Glucosidase	0.06	4.5	—

After the crude extract was applied to a 7.6×7.0 cm column of DEAE-cellulose equilibrated beforehand with 0.02 M potassium phosphate buffer containing 0.1 mM $\text{Na}_2\text{S}_4\text{O}_6$, pH 7.2, the column was eluted with 500 ml of the same buffer. Fractions containing glycosidases were pooled (400 ml) and concentrated to 40 ml by lyophilization. The concentrated effluent was brought to 60% saturation by the

FIG. 1. Second DEAE-cellulose Column Chromatography of the Resulting Solution of Ammonium Sulfate Treatment.

The supernatant obtained from step of ammonium sulfate precipitation (105 ml) was applied on a column of DEAE-cellulose (1.2×16.5 cm) equilibrated with 0.002 M potassium phosphate buffer, pH 6.6 (I). After washing the column with 150 ml of the same buffer, the enzyme was eluted with 0.2 M potassium phosphate buffer, pH 7.2 (II). Glycosidase activities in 20 μ l aliquots were assayed using *p*-nitrophenyl glycosides as a substrate. ○, α -mannosidase; △, α -galactosidase; ▲, β -galactosidase; ×, β -N-acetylglucosaminidase; ■, β -xylosidase; ●, β -mannosidase; □, α -glucosidase; ---, protein.

addition of ammonium sulfate, followed by centrifugation for 20 min at 6000 rpm. The precipitate was dissolved in 20 ml of water and was dialyzed at 4°C for 2 days against a large volume of water. The dialysate was subjected to the second Cellulosin AP treatment as described above.

The obtained mixture was applied on a column of DEAE-cellulose (1.2×16.5 cm) which had been equilibrated with 0.002 M potassium phosphate buffer, pH 6.6. The column was washed with 150 ml of the same buffer, and then was eluted with 0.2 M potassium phosphate buffer, pH 7.2. A typical elution profile is shown in Fig. 1. The major α -mannosidase fractions were pooled and concentrated to 2 ml by lyophilization.

The eluate was applied to a Sephadex G-100 column (2.2×37 cm) equilibrated with 0.05 M acetate buffer, pH 5.2. The column was eluted with the same buffer and 5 ml per each fraction were collected (Fig. 2). Fractions

TABLE II. OVERALL PURIFICATION PROCEDURE OF PINEAPPLE FRUIT α -MANNOSIDASE

Step	Total volume (ml)	Protein (mg/ml)	Activity (units/ml)	Specific activity (units/mg protein)
1. Extraction	100	72.0	1.31	0.018
2. First DEAE-cellulose column chromatography	220	6.20	0.45	0.073
3. Ammonium sulfate precipitation	105	5.70	0.62	0.108
4. Second DEAE-cellulose column chromatography	54	1.62	0.30	0.185
5. First Sephadex G-100 column chromatography	20	0.25	0.77	3.08
6. Second Sephadex G-100 column chromatography	17	0.24	0.82	3.42

from 9 to 12 containing the main α -mannosidase activity were pooled and concentrated to 2 ml by lyophilization for subsequent fractionation. This gel filtration step removed

FIG. 2. First Sephadex G-100 Column Chromatography of the Eluate of the DEAE-cellulose Column. The eluate of the DEAE-cellulose column was applied to a Sephadex G-100 column (2.2×37 cm) equilibrated with 0.05 M acetate buffer, pH 5.2. The column was eluted with the same buffer. Glycosidase activities in $20 \mu\text{l}$ aliquots were assayed using *p*-nitrophenyl glycosides as a substrate. $\circ$, α -mannosidase; $\triangle$, α -galactosidase; $\blacktriangle$, β -galactosidase; $\times$, β -N-acetylglucosaminidase; $\blacksquare$, β -xylosidase; $\bullet$, β -mannosidase; $\square$, α -glucosidase; -----, protein.

FIG. 3. Second Sephadex G-100 Column Chromatography of the Filtrate of the First G-100 Column. The conditions were the same as Fig. 2. $\circ$, α -mannosidase; $\triangle$, α -galactosidase; $\blacktriangle$, β -galactosidase; $\times$, β -N-acetylglucosaminidase; $\blacksquare$, β -xylosidase; $\bullet$, β -mannosidase; $\square$, α -glucosidase; -----, protein.

most contaminating α -galactosidase, β -xylosidase and β -N-acetylglucosaminidase.

The obtained enzyme preparation was further purified by rechromatography on Sephadex G-100 under the identical conditions. The elution profile is shown in Fig. 3. Almost all of other glycosidases were removed by the repeated chromatography. The most active fractions (fraction from 22 to 26 in Fig. 3) were combined, lyophilized and stored at -20°C . This preparation was designated as purified α -mannosidase.

Overall purification procedure and the yield of α -mannosidase activity are summarized in Table II. The enzyme was purified 190-fold with a recovery of 9.3%.

Properties of the purified α -mannosidase

The purified α -mannosidase preparation contained only a very small amount of β -xylosidase and β -mannosidase activity as shown in Table III.

TABLE III. ACTIVITIES OF SEVERAL GLYCOSIDASES REMAINED IN PURIFIED α -MANNOSIDASE

Glycosidase	Relative activity
α -Mannosidase	100.0
α -Glucosidase	$<0.1^a$
α -Galactosidase	<0.1
β -Galactosidase	0.8
β -Mannosidase	<0.1
β -Xylosidase	0.9
β -N-Acetylglucosaminidase	<0.1

^a) These values are expressed as activity relative to that of α -mannosidase.

The optimal pH of the α -mannosidase activity was between 4.0 and 4.4 as illustrated in Fig. 4. This value is similar to that of α -mannosidase from pineapple stem,¹³⁾ hog kidney⁶⁾ and *Turbo cornutus*.⁴⁾ After lyophilization, the purified enzyme could be stored at -20°C for several months without an appreciable loss of activity, and was not activated by sodium chloride. These two features are the difference between the present α -mannosidase and the enzyme preparations both from *Turbo cornutus*⁴⁾ and from *Charonia lampas*.¹⁴⁾ The latter two preparations are known to be

FIG. 4. Effects of pH on α -Mannosidase Activity. ●—●, 0.05 M citrate phosphate buffer; ■—■, 0.05 M acetate buffer.

stored at -20°C but to be inactivated by lyophilization.

Action of the purified α -mannosidase on glycopeptides and glycoprotein

Stem bromelain, a pineapple thiol protease, is a glycoprotein which contains 3 moles of mannose, 2 moles of N-acetylglucosamine, 1 mole of xylose and 1 mole of fucose residues per mole of protein.⁵⁾ It was expected that specific α -mannosidase could release 2 moles of α -mannose from 1 mole of the glycoprotein.

FIG. 5. Hydrolysis of Stem Bromelain Glycopeptide by α -Mannosidase.

After stem bromelain glycopeptide (0.8 μ mole) had been dissolved in 1.0 ml of 0.1 M acetate buffer, pH 4.4, 0.2 ml of α -mannosidase solution (2.8 units) was added to the glycopeptide solution. The reaction mixture (0.3 ml) was assayed with regard to the amounts of liberated sugar (see text).

The purified α -mannosidase released approximately 2 moles of mannose per mole of stem bromelain glycopeptide after 10 day-incubation (Fig. 5). This value was coincident with the value expected from the structure of glycopeptide, and was also consistent with the values obtained for α -mannosidases from *Turbo cornutus*⁵⁾ and hog kidney.⁵⁾ The purified α -mannosidase also released 0.45 mole of mannose per mole of stem bromelain glycoprotein after 48 hr-incubation.

FIG. 6. Hydrolysis of Ovalbumin Glycopeptide by α -Mannosidase.

Ovalbumin glycopeptide (corresponding to 1.16 mg of mannose) was incubated with 0.2 ml of the enzyme solution (2.8 units). Further analyses were carried out in the same way as described about stem bromelain glycopeptide (see text).

As shown in Fig. 6, α -mannosidase hydrolyzed about 70% of mannosidic linkages of the ovalbumin glycopeptide after 96 hr-incubation. α -Mannosidase from *Turbo cornutus*⁴⁾ released 64~66% of mannose from the same substrate after 5 day-incubation; the enzyme from jack bean meal¹⁾ 65% of mannose after 8 hr; and the one from hog kidney⁶⁾ 50% of mannose of ovalbumin glycopeptide after 90 hr.

Recently Li and Lee purified and partially characterized α -mannosidase from pineapple stem.¹³⁾ We found the pineapple fruit α -mannosidase, like α -mannosidase of stem,¹³⁾ to be most active around pH 4.0 (Fig. 4) and a basic protein. Since both pineapple stem

and fruit are known to contain multiple forms of proteases^{18,16)} and cellulases,¹⁷⁾ however, the stem and fruit are expected to contain also a number of α -mannosidase isoenzymes which are extremely close in their properties such as amino acid composition, partial amino acid sequence, molecular weight, *etc.* Therefore unless the characteristics of both stem α -mannosidase (Li and Lee) and the fruit enzyme are extensively understood, but it is difficult to decide at the moment whether these two α -mannosidases are identical or not.

A major portion of proteins contained in the acetone-dried powder of both stem and fruit juice is a mixture of proteases, and α -mannosidase is one of the minor components of the powder. Basic α -mannosidase of the fruit can be readily separated by ion exchange chromatography from the major protease components, because most of them are acidic in nature. On the contrary, such simple means can hardly separate basic α -mannosidase from the major protease components, most of which are also basic, of the stem juice, because of their similarity in reaction with ion exchangers. Thus there is a distinct advantage in using the fruit acetone powder as a starting material.

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