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PURIFICATION OF β-MANNOSIDASE FROM A SNAIL, *ACHATINA FULICA*, AND ITS ACTION ON GLYCOPEPTIDES

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SUMMARY

1. β-Mannosidase (β-D-mannoside mannohydrolase, EC 3.2.1.25) was extracted and purified from viscera of the snail, *Achatina fulica*, about 100-fold over the crude extract. The purified β-mannosidase was free from α-mannosidase (α-D-mannoside mannohydrolase, EC 3.2.1.24) and β-acetylglucosamininase (β-2-acetamido-2-deoxy-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30) activities.

2. Specific cleavage of β-mannosidic linkage by the purified β-mannosidase was demonstrated using a trimannoside, α-Man-(1 → 4)-β-Man-(1 → 4)-Man, as substrate. This mannoside could be degraded to mannose stepwise by the successive actions of α-mannosidase from hog kidney and the purified β-mannosidase.

3. The purified β-mannosidase released mannose readily from a glycopeptide, Man–$(GlcNAc)_2$–Asn–(Ser), which had been isolated from the α-mannosidase digest of Taka-amylase glycopeptide.

INTRODUCTION

α-Mannosidases (α-D-mannoside mannohydrolase, EC 3.2.1.24) purified from various sources, both plant and animal, have been used to determine anomeric configurations of mannose residues in carbohydrate moieties of glycoproteins[1]. Results have shown that most mannose residues in glycoproteins have the α-anomeric configuration. However, in these experiments, the release of mannose from glycoproteins or glycopeptides prepared, therefrom, by pronase digestion is usually incomplete. For example, α-mannosidase from hog kidney was capable of releasing only about 60% of the mannose residues from glycopeptide prepared from ovalbumin[2]. Similar results have been obtained with α-mannosidases from Jack bean meal[3] or from the liver of *Turbo cornutus*[4]. It has been claimed that the limited action of α-mannosidase may be due to aglycon specificity of the enzymes.

To see if mannosidic linkages resistant to α-mannosidase action might be of the β-type, we purified β-mannosidase (β-D-mannoside mannohydrolase, EC 3.2.1.25) from viscera of the snail, *Achatina fulica*, and found, using the purified β-mannosidase, that one of the mannose residues in ovalbumin or in Taka-amylase (α-1,4-glucan-4-

glucanohydrolase, EC 3.2.1.1) has the β-anomeric configuration. Results of studies on ovalbumin glycopeptide have already been presented[5]. In this paper we report the purification procedures of β-mannosidase and its action on Taka-amylase glycopeptide.

MATERIALS AND METHODS

Enzyme substrates

Phenyl-β-D-mannoside, *p*-nitrophenyl-α-D-mannoside and *p*-nitrophenyl-β-D-*N*-acetylglucosaminide were synthesized in our laboratory (see refs. 6–9). The phenyl-β-mannoside used was highly pure, showing a single peak on gas–liquid chromatography (for the details, see ref. 5), and was cleaved quantitatively by the purified β-mannosidase into mannose and phenol.

A trimannoside, α-Man-(1 → 4)-β-Man-(1 → 4)-Man, from ivory nut mannan[10] was a gift from Prof. G. O. Aspinall of Trent University, Ontario, Canada, and Taka-amylase glycopeptide was from Prof. Y. Matsushima of Osaka University.

Enzyme sources

Snails, *Achatina fulica*, were collected in the Okinawa Islands. The acetone powder of their viscera was used to extract β-mannosidase. All enzyme purification operations were carried out at 0–5 °C.

α-Mannosidase was prepared from hog kidney by the method of Okumura and Yamashina[2].

Enzyme assays

β-Mannosidase was assayed at pH 4.5 in a buffer prepared from 0.05 M citric acid and 0.025 M K_2HPO_4 containing 5 mM phenyl-β-mannoside. Liberated phenol was determined by the method of Kerr *et al.*[11]. α-Mannosidase and β-acetylglucosaminidase (β-2-acetamido-2-deoxy-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30) were assayed using *p*-nitrophenyl-α-mannoside and *p*-nitrophenyl-β-*N*-acetylglucosaminide, respectively, under the conditions described by Conchie *et al.*[12]. One unit of glycosidase is defined as the amount of enzyme which will liberate one μmole of phenol or *p*-nitrophenol per min under the assay conditions.

Other materials

Sephadex was obtained from Pharmacia, Sweden, and Bio-Gel from Bio-Rad Lab., U.S.A. DEAE-cellulose was a product of Brown Co., U.S.A. Hydroxylapatite was prepared according to the method of Siegelman *et al.*[13].

Analytical methods

Neutral sugar was determined by the orcinol–H_2SO_4 reaction using mannose as standard[11]. Glucosamine was determined by the Elson–Morgan reaction, as modified by Svennerholm[15], after hydrolysis in 2 M HCl at 100 °C for 16 h. To determine compositions of the isolated glycopeptides, glucosamine was determined together with amino acids in a Hitachi amino acid analyzer after hydrolysis in 6 M HCl at 100 °C for 16 h. The destruction of glucosamine occurring during hydrolysis was about 20%, which was estimated by determining the destruction of glucosamine occurring during

heating a mixture, composed of *N*-acetylglucosamine, serine and asparagine with a molar ratio of 2:0.8:1, under the hydrolysis conditions. Addition of mannose to this mixture had no effect on glucosamine destruction.

Enzymatic release of mannose from glycopeptides was followed by determining the reducing power according to the method of Park and Johnson[16]. The amount of released mannose was also determined by gas–liquid chromatography after conversion to mannitol, essentially according to Sweeley *et al.*[17]. Degradation of the trimannoside with α- and β-mannosidases was followed by the gas-chromatographic determination.

The ninhydrin reaction to monitor chromatographic fractions was carried out according to Yemm and Cocking.[18] Protein was determined by the method of Lowry *et al.*[19] using ovalbumin as standard.

Polyacrylamide-gel electrophoresis to examine the purity of the enzyme preparation was carried out in the system described by Davis[20] using 7.5% gel of pH 9.4 prepared in Davis' Tris–glycinate buffer.

RESULTS

Purification of β-mannosidase

Extraction and partial fractionation of β-mannosidase from the acetone powder of snail viscera. A 10-g portion of the powder was homogenized with 200 ml of 0.05 M phosphate buffer, pH 7.24, in a Waring Blendor for 10 min. The homogenate was centrifuged at 13 000 × *g* for 20 min, then the precipitate was re-extracted with 50 ml of the buffer. The combined supernatant was submitted to freezing with dry ice–acetone then thawing at room temperature. The resulting precipitate was removed by centrifugation, then the supernatant was made 32% saturation with $(NH_4)_2SO_4$. The supernatant obtained after centrifugation at 13 000 × *g* for 20 min was then brought to 59% $(NH_4)_2SO_4$ saturation. The precipitate collected by centrifugation was dissolved in water to give a protein concentration of 0.3–0.4%. This solution was made 0.04 and 0.01 M with $(NH_4)_2SO_4$ and zinc acetate, respectively, then adjusted to pH 6.1 with 1 M NaOH. Cold 50% acetone made up of equal volumes of acetone and 0.01 M zinc acetate–acetic acid buffer, pH 5.9, was added with stirring to give 40% acetone concentration. The resulting precipitate was removed by centrifugation at 13 000 × *g* for 20 min, then the supernatant was brought to 70% acetone concentration by adding cold acetone. The precipitate collected by centrifugation was dissolved in 0.01 M phosphate buffer, pH 7.24, then dialyzed against water. The dialysate was lyophilized after centrifugation to remove insoluble materials.

Chromatographic fractionation. The material from the previous step (90 mg protein) was dissolved in 6 ml of 0.005 M phosphate buffer, pH 6.8, then applied to a column of hydroxylapatite (1.6 cm × 16 cm). About 70% of the β-mannosidase activity applied passed through the column; the rest was eluted with 0.2 M buffer together with β-acetylglucosaminidase activity.

Each one third of the effluent (about 10 ml) was applied to a column of DEAE-cellulose (1.6 cm × 16 cm) equilibrated with 0.005 M phosphate buffer, pH 6.8, then eluted with phosphate buffer of stepwise increasing concentrations, as shown in Fig. 1. Fractions in the peak of β-mannosidase activity eluted with 0.02 M buffer were collected, concentrated by ultrafiltration using a membrane filter (Diaflo Ultrafiltration, Model 200, with membrane PM-10, Amicon Corp., U.S.A.) and dialyzed. This prepa-

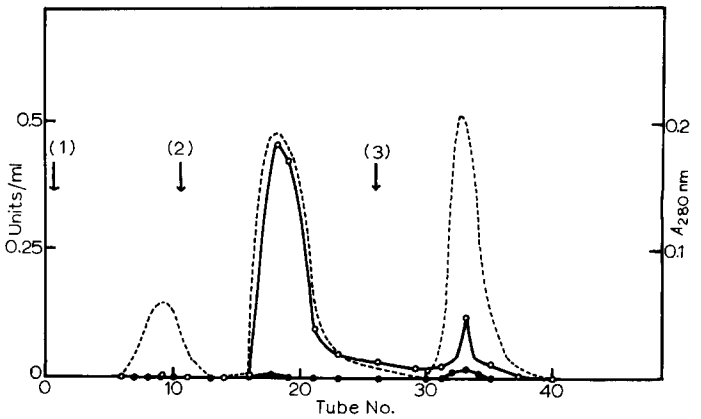

Fig. 1. Chromatographic purification of β-mannosidase on DEAE-cellulose. A 10-ml solution of the β-mannosidase preparation from hydroxylapatite chromatography in 0.005 M phosphate buffer, pH 6.8, was applied to a column of DEAE-cellulose (1.6 cm × 16 cm) equilibrated with the same buffer. Elution was carried out with phosphate buffer of stepwise increasing concentrations; (1) 0.005 M, (2) 0.02 M and (3) 0.1 M. Flow rate was 15 ml/h, and 4-ml fractions were collected. Aliquots were used for enzyme assays. Protein was determined by the absorbance at 280 nm. ○—○, β-mannosidase activity; ●—●, β-acetylglucosaminidase activity; — — —, absorbance at 280 nm.

ration, designated as purified β-mannosidase, was still impure showing at least four protein bands on disc electrophoresis, but was practically free from α-mannosidase and β-acetylglucosaminidase activities. Specific activity of the purified β-mannosidase was 4.73 units/mg protein under the assay conditions, but was 6.67 on calculation from a Lineweaver–Burk plot. The second peak of β-mannosidase activity overlapped a small peak of β-acetylglucosaminidase activity, and was not used in the present study. Purification procedures are summarized in Table I.

Properties of the purified β-mannosidase

pH optimum. The pH profile of activity was obtained using two buffer systems, *i.e.* one prepared from 0.25 M citric acid and 0.125 M K_2HPO_4 for pH values from

TABLE I

SUMMARY OF PURIFICATION OF β-MANNOSIDASE FROM SNAIL VISCERA

Fraction from	*Protein (mg)*	*Total activity (units)*	*Specific activity (units/mg protein)*	*Yield (%)*	*α-Mannosidase activity (units)*	*β-Acetylglucosaminidase activity (units)*
0.05 M phosphate buffer extract	2000	82	0.041	100	37	366
$(NH_4)_2SO_4$ fractionation	753	78	0.103	95	32	266
Acetone fractionation	93	50	0.54	61	1.0	140
Lyophilization	93	40	0.43	49	0.4	122
Hydroxylapatite chromatography	19	27	1.41	33	0.0095	0.368
DEAE-cellulose chromatography	5.6	26.5	4.73	32	0.0023	0.096

2.5 to 5.0 and the other prepared from 0.25 M citric acid and 1 M KOH for pH values from 4.0 to 6.5. Optimum pH was at 4.5, but the peak of activity was broad in a pH range from 4 to 5. The results were similar for two buffer systems.

Michaelis constant (K_m). Using various concentrations of phenyl-β-mannoside under the assay conditions, the K_m value was about 6.5 mM.

Heat stability. The purified β-mannosidase was invariably stable if its aqueous solution (the dialysate as described above) was kept frozen at −20 °C. To see the heat stability, the enzyme solutions in buffers of various pH values from 3.5 to 7.0 prepared from 0.01 M citric acid and 0.01 M K_2HPO_4 containing 0.03 unit/ml were heated at 55 °C for 5 min. At pH values from 5 to 7 only 10–20% of the activity, at pH 4.5 about 40% and at pH values lower than 4.5 more than 80% were lost.

Action of the purified β-mannosidase on a trimannoside

A trimannoside, α-Man-(1 → 4)-β-Man-(1 → 4)-Man, was used to test the specificity and activity of the purified β-mannosidase on naturally occurring mannoside.

No increase in reducing power was observed when the trimannoside, corresponding to 230 μg mannose, and 0.12 unit of the purified β-mannosidase per ml of the citrate–phosphate buffer, pH 4.5, was incubated at 37 °C for 12 h.

For the successive degradation using α- and β-mannosidases, the trimannoside corresponding to 460 μg mannose was initially incubated with 0.4 unit of α-mannosidase from hog kidney in 1.0 ml of citrate–phosphate buffer, pH 4.5; the buffer used for β-mannosidase assay, at 37 °C. After 10 h, 0.8 ml of the reaction mixture was withdrawn and mixed with an equal volume of the purified β-mannosidase (0.18 unit) solution in the same buffer.

Incubation of both the initial and new (with added β-mannosidase) reaction mixtures was continued up to a total of 15 h. Gas-chromatographic analyses were performed at 8 and 15 h on the initial reaction mixture and at 15 h on the new reaction mixture.

One of the three mannose residues was released on incubation with α-mannosidase alone, the same value for released mannose being obtanied at 8 and 15 h. The produced dimannoside, β-Man-(1 → 4)-Man, was then degraded quantitatively by β-mannosidase after a total of 15 h. Thus, the specificities and purities of our α- and β-mannosidases could be fully established.

The purified β-mannosidase had no action towards intact glycopeptides from Taka-amylase or ovalbumin (see ref. 5), indicating that these glycopeptides have no β-mannosidic linkage at the non-reducing positions.

Determination of the anomeric configuration of mannose residues in Taka-amylase glycopeptide

Sequences of the sugar residues in Taka-amylase glycopeptide have been pro-

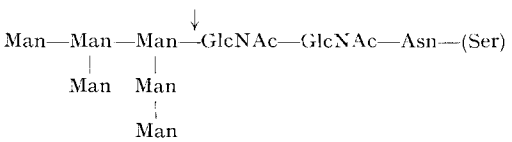

Fig. 2. Structure of Taka-amylase glycopeptide proposed by Yamaguchi *et al.*[21]. The position of the β-mannosidic linkage as revealed by the present study is indicated by the arrow.

posed by Yamaguchi *et al.*[21], as shown in Fig. 2. The anomeric configurations of the mannose residues have also been investigated by the same authors using a crude α-mannosidase preparation from the liver of *Turbo cornutus*[22], and by Lee[23] using a newly isolated plant α-mannosidase; with the tentative conclusion that all the mannoside linkages are of the α-type. However, we suspected that β-mannosidase contaminating the α-mannosidase preparations might have worked under the conditions used.

Taka-amylase glycopeptide corresponding to 2.0 mg mannose was first reacted with 6 units of hog kidney α-mannosidase in 4 ml of 0.2 M Na_2HPO_4–0.1 M citric acid buffer, pH 4.6, containing 5 mM zinc acetate at 37 °C for 50 h, when the increase in reducing power had reached a plateau. The value, confirmed by gas-chromato-

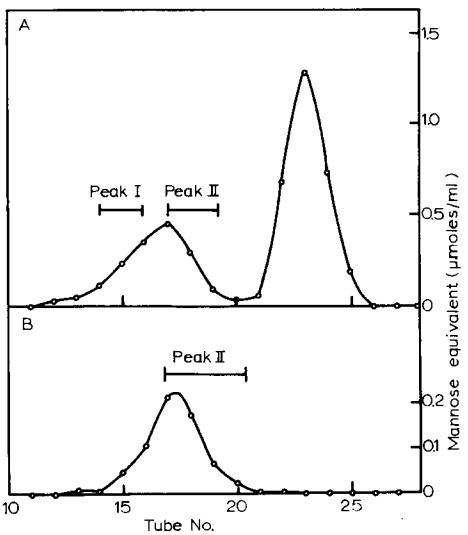

Fig. 3. A. Fractionation of the α-mannosidase digest of Taka-amylase glycopeptide by gel filtration. The digest prepared as described in the text was applied to a column of Bio-Gel P-4 (0.9 cm × 90 cm), then eluted with water at a flow rate of 15 ml/h. Fractions of 2.2 ml were collected, and aliquots were used for the orcinol–H_2SO_4 reaction. B. Fractions in the Peak II of A were collected and concentrated, and the sample corresponding to 280 μg mannose was rechromatographed as in A.

graphic analysis, corresponded to the release of 4 moles of mannose per mole of the glycopeptide containing 6 moles of mannose. However, these values represent only average numbers since the glycopeptide is heterogeneous as shown below.

The digest was applied to a column of Bio-Gel P-4 to separate the glycopeptide produced from the mannose released with the results shown in Fig. 3A. The first peak was apparently heterogenous, and the separated peaks, Peaks I and II, showed different compositions on analysis, as shown in Table II. Peak I contained approximately 3 mannose and 2 glucosamine residues per mole of aspartic acid, and Peak II 1 mannose and 2 glucosamine residues. These glycopeptides were no longer susceptible to α-mannosidase.

To remove contaminating Peak I from Peak II, fractions in Peak II were col-

TABLE II

AMINO ACID AND CARBOHYDRATE COMPOSITIONS OF GLYCOPEPTIDES ISOLATED FROM THE α-MANNOSIDASE DIGEST OF TAKA-AMYLASE GLYCOPEPTIDE

Analytical methods are described in the text. Values are expressed as molar ratios to aspartic acid. Nearest integers are shown in parentheses.

α-Mannosidase digest	*Mannose*	*Glucosamine*	*Aspartic acid*	*Serine*
Peak I	2.8 (3)	1.68* (2)	1.00	0.80
Peak II	1.1 (1)	1.49* (2)	1.00	0.87

* Under the hydrolysis conditions used about 20% destruction of glucosamine occurred, but figures shown are uncorrected for destruction.

lected, concentrated and submitted to rechromatography on Bio-Gel P-4 under the same conditions as used for the initial chromatography (Fig. 3B). Of the fractions in the Peak, those indicated by a bar were collected, because the fractions at the front of the peak were considered to be contaminated with Peak I materials. Recovery of purified peak II glycopeptide was about 80% of the initial Peak II.

Purified peak II glycopeptide corresponding to 152 μg mannose was incubated with 0.32 unit of the β-mannosidase in 3 ml of citrate–phosphate buffer, pH 4.5, at 37 °C. The increase in reducing power reached a plateau after 1.5 h which corresponded to 100% release of the mannose from Peak II glycopeptide. The amount of released mannose was confirmed by gas-chromatographic analysis.

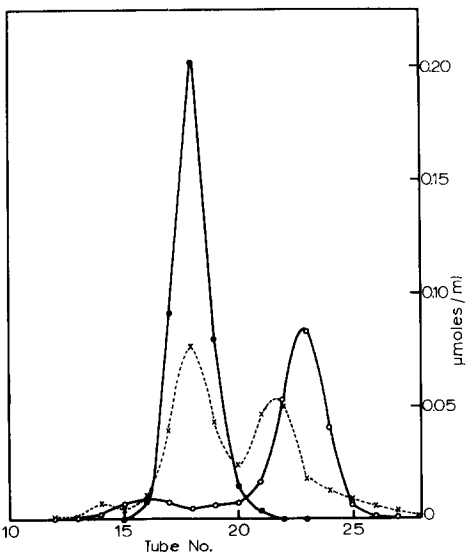

Fig. 4. Fractionation of the β-mannosidase digest by gel filtration. The digest (prepared as described in the text), corresponding to 102 μg mannose, was applied to a column of Bio-Gel P-4 (0.9 cm × 90 cm), then eluted with water at a flow rate of 15 ml/h. Fractions of 2.2 ml were collected, and aliquots were used for the orcinol–H_2SO_4 reaction (○—○), the ninhydrin reaction (×---×), and the Elson–Morgan reaction (●—●), which was carried out after hydrolysis of aliquots of the fractions in 2 M HCl at 100 °C for 16 h. Values are expressed as equivalents of mannose, aspartic acid and glucosamine, respectively.

The digest was then applied to a column of Bio-Gel P-4 and eluted with water producing the results shown in Fig. 4. Of the peaks that were positive in the ninhydrin reaction, the first peak contained glucosamine (as shown by the Elson–Morgan reaction on the hydrolysates of the fractions), and the second peak no glucosamine. The latter peak was considered to be derived from the enzyme preparation. The third peak was the released mannose, and the pooled fractions in this peak contained mannose in the amount expected from 100% release of mannose from Peak II glycopeptide. Small values of the orcinol–H_2SO_4 reaction in the first peak were considered due to the enzyme preparation.

Fractions in the first peak were collected and submitted to analysis. Analysis of the hydrolysate using the amino acid analyzer showed that this peak contained 1.56 moles of glucosamine and 0.83 mole of serine per mole of aspartic acid. If corrected for the destruction of glucosamine occurring during hydrolysis under the conditions used, this glycopeptide contained 2 moles of glucosamine per mole of aspartic acid, corresponding to the innermost structure of the parent Taka-amylase glycopeptide (see Fig. 2). Thus, we concluded that the innermost mannosidic linkage is of the β-type, as shown by an arrow in Fig. 2.

The Peak I glycopeptide (see Fig. 3A) was practically insusceptible to the β-mannosidase, with only about 10% of the total mannose residues released. This release would be due to contamination of Peak II.

DISCUSSION

While β-mannosidic linkage is known to occur in plant mannans[10], galactomannans[24,25], glucomannans[24,25], algal mannans[26] and a bacterial polysaccharide[27], it has never been found in glycoproteins containing mannose as one of the most common constituents. For glycoproteins, the presence of only the α-mannosidic linkage has been demonstrated using α-mannosidases from various sources. Use of β-mannosidase for this type of study has not been attempted since sources for the preparation of β-mannosidase are rather limited. Muramatsu *et al.*[4] have purified β-mannosidase from a marine gastropod, *Turbo cornutus*, and their purified preparation was successfully used to determine the anomeric configuration of mannose residues in *Salmonella* antigenic polysaccharides[27].

We found that viscera of the snail, *Achatina fulica*, is a good source of β-mannosidase, its activity in the crude extract being even higher than α-mannosidase activity. The purified β-mannosidase preparation had a specific activity higher than that from *Turbo cornutus*[4].

Taka-amylase glycopeptide was used in the present study, since the sugar sequences of this glycopeptide have extensively been studied. However, the heterogeneity of the carbohydrate moiety was disclosed on fractionation of the α-mannosidase digest of the glycopeptide. This was similar what was found for ovalbumin glycopeptide, and the heterogeneous nature of the carbohydrate moiety seems to be common to many glycoproteins.[28] The heterogeneity of Taka-amylase glycopeptide was, however, not as marked as that found for ovalbumin glycopeptide. The molar ratio of two peaks, Peaks I and II, obtained from the α-mannosidase digest was roughly 1:3, as estimated from the amount of aspartic acid in each peak.

Insusceptibility of Peak I glycopeptide to the β-mannosidase suggests that one

of the *N*-acetylglucosamine residues resides at the non-reducing terminus. This is similar to structures proposed for three out of five glycopeptides from ovalbumin[28]. To determine whether β-mannosidic linkage is common to all the glycopeptides from Taka-amylase, use of acetylglucosaminidase prior to the use of α-mannosidase would be required. Further, it would be interesting to see if β-mannosidic linkage is present in glycoproteins other than ovalbumin and Taka-amylase.

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