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Partial Purification and Characterization of a Novel Endo-β-mannosidase Acting on N-Linked Sugar Chains from *Lilium longflorum* Thumb

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An enzyme catalyzing the hydrolysis of the Manβ1-4GlcNAc linkage of *N*-linked sugar chains was partially purified and characterized. Endo-β-mannosidase activity was detected using pyridylaminated (PA-) Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc as the substrate in a homogenate of lily flowers (*Lilium longflorum* Thumb). The enzyme was partially purified by ammonium sulfate precipitation, and Q-Sepharose, Superdex 200, hydroxyapatite, Poros PE/M, Mono Q, and Superdex 200 column chromatographies. The optimum pH was 5.0 and the estimated molecular weight of the enzyme was 78,000, as determined by gel filtration. The K_m value found for Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc-PA was 1.4 mM. The enzymatic activity was not influenced by the addition of 10 mM EDTA or 2 mM Ca^{2+}. Experiments on the hydrolysis of several PA-*N*-linked sugar chains revealed that the enzyme hydrolyzed Man$_n$Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc-PA (n = 0-2) into a mixture of Man$_n$Manα1-6Man and GlcNAcβ1-4GlcNAc-PA, indicating that it is an endoglycosidase in nature. However, the enzyme did not hydrolyze β1-4mannohexaose or *p*-nitrophenyl β-mannopyranoside.

Key words: endo-β-mannosidase, enzyme, glycoprotein, lily, *N*-linked sugar chains.

Endo-type enzymes which act on the trimannosyl core structure of *N*-linked sugar chains have been reported and classified into two types. One hydrolyzes the bond between an Asn residue and an reducing end GlcNAc residue (glycopeptidase), and the other the bond between GlcNAc residues (endo-β-*N*-acetylglucosaminidase). These enzymes have been purified and their characteristics have been well studied (*1*), however, an enzyme that acts on the Manβ-residue of the trimannosyl core structure has not been reported so far to our knowledge. In a previous study, careful analysis of *S*-glycoproteins from *Rosaceous* S-RNase revealed that chitobiose was one of the main *N*-linked sugar chains (*2*). The presence of chitobiose residues suggested the existence of an enzyme able to hydrolyze the Manβ1-4GlcNAc linkage, as *N*-linked sugar chains are biosynthesized through *en bloc* transfer of Glc3Man9GlcNAc2 to polypeptide chains (*3*). We attempted to detect this possible enzyme activity by using various pyridylaminated (PA-) sugar chains, and succeeded in doing so when Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc-PA was used as the substrate. Here we describe the partial purification and characterization of a novel endo-β-mannosidase from lily flowers.

MATERIALS AND METHODS

Materials—Lilies (*Lilium longflorum* Thumb) were purchased from a florist. The preparation of PA-GlcNAc (GN), GlcNAcβ1-4GlcNAc-PA (GN2), Manβ1-4GlcNAc-β1-4GlcNAc-PA (M1), Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc-PA (M2B), Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc-PA (M2A), Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA (M3B), Manα1-3Manα1-6Manβ1-4GlcNAc-β1-4GlcNAc-PA (M3C), Manα1-6(Manα1-3)Manα1-6-Manβ1-4GlcNAcβ1-4GlcNAc-PA (M4B), Manα1-6(Man-α1-3)Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA (M5A), and Manα1-6(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA (M2FX) has already been reported (*4*). Manα1-6(Manα1-3)Manα1-6(Manα1-3)Man, Manα1-6(Manα1-3)Man, Manβ1-4Manβ1-4Manβ1-4Manβ1-4Manβ1-4Man (βM6), and Manβ1-4Manβ1-4Man (βM3) were purchased from Funakoshi (Tokyo), and Manα1-6Man, Manα1-3Man, *p*-nitrophenyl α-D-mannopyranoside, and *p*-nitrophenyl β-D-mannopyranoside from Sigma (St. Louis, MO). Bovine immunoglobulin G was obtained from Miles (Kankakee, IL), bovine serum albumin and hen ovalbumin from Seikagaku Kogyo (Tokyo), and cytochrome *c* (horse heart) from Nacalai Tesque (Kyoto). DEAE-Sephacel, a Q-Sepharose column (1.6×8 cm), a Superdex 200 column (1.6×60 cm), and a Mono Q HR 5/5 column (0.5×5 cm) were purchased from Pharmacia (Upp-

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Abbreviations: HPAEC, high pH anion-exchange chromatography; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; MES, 2-morpholinoethanesulfonic acid; PAD, pulse-amperometric detector; PA-, pyridylamino. The abbreviations for sugar chains are listed in Table I.

TABLE I. **Sugar chain structures and abbreviations used in the present study.**

Abbreviation	Structure
GN	GlcNAc-PA
GN2	GlcNAcβ1-4GlcNAc-PA
M1	Manβ1-4GlcNAcβ1-4GlcNAc-PA
M2A	Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc-PA
M2B	Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc-PA
M3B	Manα1-6 Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc-PA
M3C	Manα1-3Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc-PA
M4B	Manα1-6 Manα1-3Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc-PA
M5A	Manα1-6 Manα1-3Manα1-6 Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc-PA
M2X	Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc-PA Xylβ1-2
M2FX	Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc-PA Xylβ1-2 Fucα1-3
βM3	Manβ1-4Manβ1-4Man
βM6	Manβ1-4Manβ1-4Manβ1-4Manβ1-4Manβ1-4Man

sala, Sweden), a HA 1000 hydroxyapatite column (0.75×7.5 cm) from Tosoh (Tokyo), a Poros PE/M column (0.46×10 cm) from PerSeptive Biosystems (Framingham, MA), a Shodex Asahipak NH2-P column (0.46×7.0 cm) from Showa Denko (Tokyo), a Cosmosil 5C18-P column (0.46×15 cm) from Nacalai Tesque, and a Carbopac PA-1 column (0.2×25 cm) from Dionex (Sunnyvale, CA).

Assaying of Enzyme Activities—Endo-β-mannosidase activity was measured using M2B as the substrate unless otherwise specified. The enzyme solution and 200 pmol of M2B in 16 μl of 0.16 M ammonium acetate buffer, pH 5.0, were incubated at 37°C for 30 min. The enzymatic reaction was terminated by heating at 100°C for 3 min. The PA-chitobiose released was quantified by size-fractionation HPLC. One unit of enzyme activity was defined as the amount of enzyme that released 1 nmol of PA-chitobiose from M2B per minute under the conditions used. α-Mannosidase activity was measured using *p*-nitrophenyl α-mannopyranoside as the substrate. The enzyme solution and 2 mM *p*-nitrophenyl α-mannopyranoside in 14 μl of 0.11 M ammonium acetate buffer, pH 5.0, were incubated at 37°C for 30 min. The *p*-nitrophenol released was quantified by measuring the absorbance at 400 nm after the addition of 110 μl of an aqueous 0.25 M Na_2CO_3 solution.

Purification of Endo-β-mannosidase—Step 1. Preparation of a crude enzyme solution: Lily flowers (800 g) frozen with liquid nitrogen were powdered with a Waring blender. The powder was then suspended in 2,500 ml of 0.11 M sodium acetate buffer, pH 6.0, and stirred at 4°C for 3 h. The homogenate was centrifuged at 8,000 rpm for 20 min. The resulting supernatant (2,900 ml) was fractionated by ammonium sulfate precipitation, and the precipitate formed between 20 and 60% ammonium sulfate saturation was collected. This precipitate was dialyzed against 0.01 M ammonium acetate buffer, pH 6.0, and the precipitate formed was removed by centrifugation at 9,500 rpm for 30 min. The pH of the solution was brought to 3.8 with an aqueous acetic acid solution, and then the solution (130 ml) was loaded on a DEAE-Sephacel column (6.5×1.5 cm) equilibrated with 0.01 M ammonium acetate buffer, pH 3.8. The resin was washed with 400 ml of the same buffer. The washings and the path-through fraction were combined and concentrated to 50 ml with an Amicon YM 10 membrane. The enzyme was further purified as described below, and an aliquot of each fraction was assayed for α-mannosidase activity and PA-chitobiose releasing activity from M2B. The column chromatographies were carried out with an FPLC system (Pharmacia) at 25°C.

Step 2. Q-Sepharose chromatography: A part of the crude enzyme solution (5.4 ml) was placed on a Q-Sepharose column equilibrated with 0.05 mM ammonium acetate buffer, pH 6.0. The enzyme was eluted with a linear gradient of 0.05 to 0.5 mM ammonium acetate in 30 min at the flow rate of 2.0 ml/min. The fraction containing endo-β-mannosidase activity was collected.

Step 3. Superdex 200 chromatography: The fraction obtained in Step 2 was purified on a Superdex 200 column equilibrated with 0.1 M ammonium acetate buffer, pH 5.0, containing 0.1 M NaCl. The enzyme was eluted with the same buffer at the flow rate of 1.0 ml/min.

Step 4. Hydroxyapatite chromatography: The fraction obtained in Step 3 was collected and purified on a hydroxyapatite HA 1000 column equilibrated with 0.01M sodium phosphate buffer, pH 6.0. The enzyme was eluted with a linear gradient of the phosphate buffer, from 0.01 to 0.5 M, in 30 min at the flow rate of 1.0 ml/min.

Step 5. Hydrophobic chromatography: The fraction obtained in Step 4 was purified on a Poros PE/M column equilibrated with 10 mM sodium phosphate buffer, pH 6.0, containing 1.0 M ammonium sulfate. The enzyme was eluted with a linear gradient of ammonium sulfate, from 1.0 to 0 M, in 5 min at the flow rate of 5 ml/min.

Step 6. Mono Q chromatography: The fraction obtained in Step 5 was purified on a Mono Q HR 5/5 column equilibrated with 0.01 M sodium phosphate buffer, pH 6.0. The enzyme was eluted with a linear gradient of NaCl, from 0 to 0.3 M, in 30 min at the flow rate of 1.0 ml/min.

Step 7. Superdex 200 chromatography: The fraction obtained in Step 6 was purified on a Superdex 200 column as described in Step 3. The fraction containing endo-β-mannosidase activity was collected and used as the purified enzyme.

Polyacrylamide Gel Electrophoresis (PAGE)—SDS-PAGE was performed on a 10% polyacrylamide gel by the method of Laemmli (*5*). Proteins were stained with Coomassie Brilliant Blue R 250.

pH-Dependence of Enzyme Activities—M2B was used as the substrate and the initial hydrolysis rates were measured as described above under "Assays of Enzyme Activities," except that the buffers used were ammonium acetate buffer (0.06 M, pH 4.0-5.5), MES buffer (0.06 M, pH 5.5-6.5), and HEPES buffer (0.06 M, pH 6.5-8.0).

High-Performance Liquid Chromatography (HPLC)—A Beckman model 332 chromatograph equipped with a

Hitachi model 650-10M fluorescence spectrophotometer was used. Size-fractionation HPLC was performed on a Shodex Asahipak NH2-P column at the flow rate of 0.6 ml/min at 25°C. The eluent used was 3% (v/v) acetic acid in a 400:85 (v/v) mixture of acetonitrile:water adjusted to pH 7.3 with triethylamine. The excitation and emission wavelengths were 315 and 380 nm, respectively. Reversed-phase HPLC was performed on a Cosmosil 5C18-P column at the flow rate of 1.5 ml/min at 25°C. The eluents used were 0.1 M ammonium acetate buffer, pH 4.0 (Eluent A), and 0.1 M ammonium acetate buffer, pH 4.0, containing 0.5% 1-butanol (Eluent B). The column was equilibrated with 6% Eluent B. After injecting a sample, the proportion of Eluent B was increased linearly to 30% in 30 min. Elution was monitored as to fluorescence, the excitation and emission wavelengths being 320 and 400 nm, respectively.

High pH Anion-Exchange Chromatography (HPAEC)—A Dionex DX-500 chromatograph was used. Sugar chains

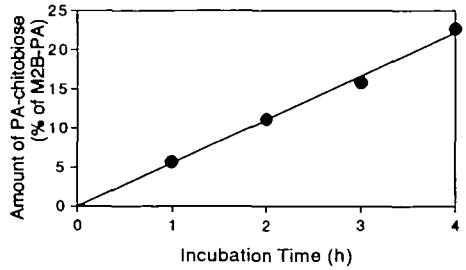

Fig. 1. **Hydrolysis of M2B with the crude enzyme preparation.** The PA-chitobiose released was quantified by size-fractionation HPLC with a fluorescence detector. The enzyme activity was measured as described under "MATERIALS AND METHODS" using M2B.

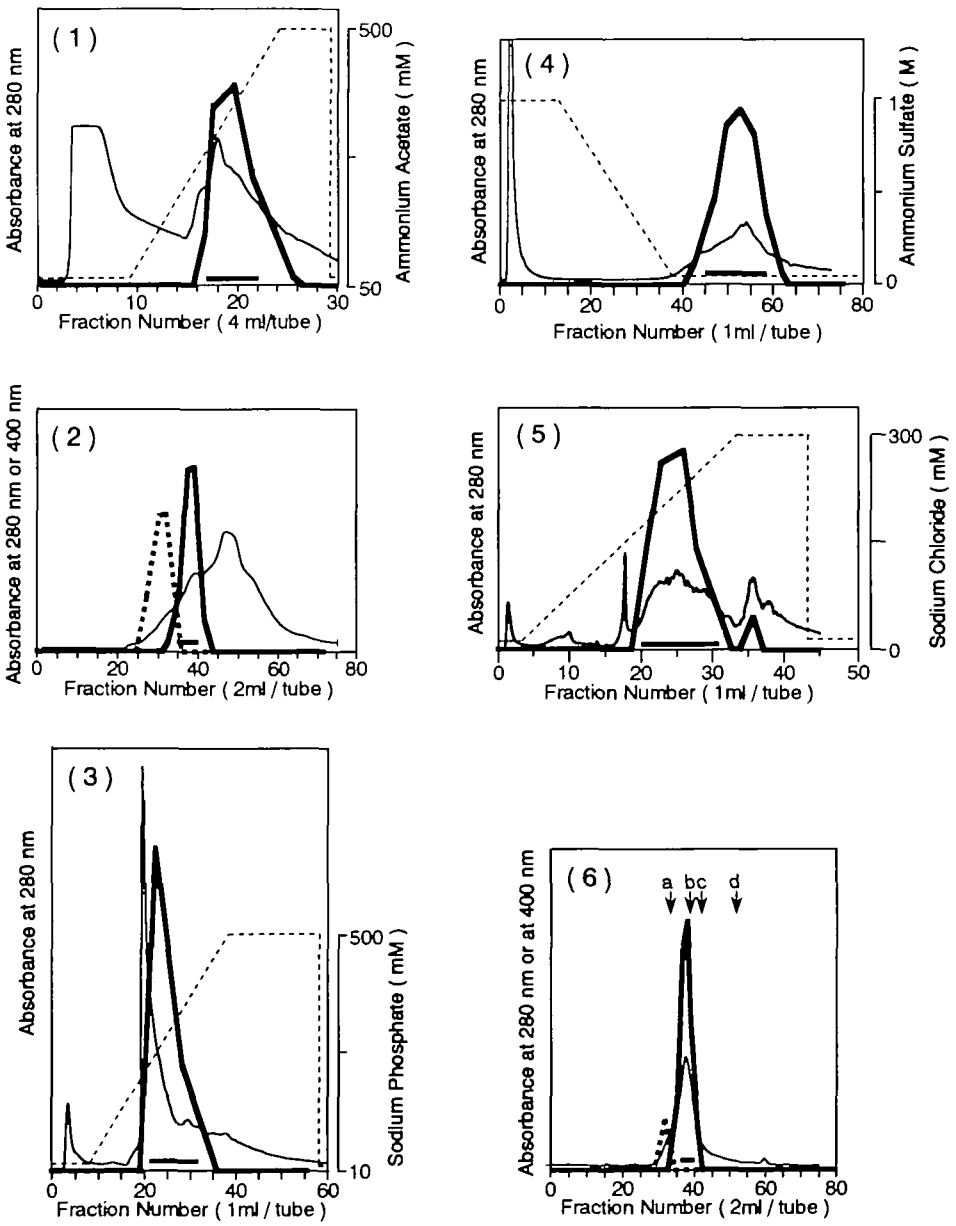

Fig. 2. **Purification of endo-β-mannosidase from lily flowers.** Chromatographies were carried out as described under "MATERIALS AND METHODS." (—), absorbance at 280 nm; (—), PA-chitobiose releasing activity with M2B as the substrate; (·····), α-mannosidase activity; (- - -), salt concentration. The fractions indicated by the bars were collected. (1) Q-Sepharose chromatography of the crude enzyme solution; (2) Superdex 200 chromatography; (3) hydroxyapatite chromatography; (4) Poros PE/M chromatography; (5) Mono Q chromatography; (6) Superdex 200 chromatography. Arrows indicate the elution positions of: a, immunoglobulin G; b, bovine serum albumin; c, ovalbumin; d, cytochrome *c*.

were analyzed on a Carbopac PA-1 column. Elution was monitored with a pulse-amperometric detector (PAD). Gradient elution was carried out as follows. Two eluents, A and B, were used: Eluent A was 0.1 M NaOH aqueous solution, and Eluent B was 0.1 M sodium acetate in an aqueous 0.1 M NaOH solution. The column was equilibrated with 10% Eluent B. After injecting a sample, the percentage of Eluent B was increased linearly to 50% in 30 min at the flow rate of 1.0 ml/min.

RESULTS AND DISCUSSION

Detection of Endo-β-mannosidase Activity—Our aim was to detect endo-β-mannosidase activity in a homogenate prepared from lily flowers. The enzyme activity was analyzed by measuring GN2 released from M2B, which was selected as the substrate. Size-fractionation HPLC was employed for the analysis. GN2, the hydrolysis product, linearly increased with the reaction time without the production of Manβ1-4GlcNAcβ1-4GlcNAc-PA (Fig. 1), suggesting the presence of an enzyme that catalyzes the hydrolysis of the Manβ1-4GlcNAc linkage.

Purification of Endo-β-mannosidase—Endo-β-mannosidase was purified from lily flowers as described under "MATERIALS AND METHODS" (Fig. 2). The purification steps are summarized in Table II. Endo-β-mannosidase was separated from α-mannosidase by gel filtration. Although the yield of the purified enzyme was low, the relative instability of the enzyme resulting in its inactivation during the purification must be taken into consideration. The enzyme preparation obtained in the present study contained several proteins, as judged on SDS-PAGE (data not shown).

Characterization of the Purified Endo-β-mannosidase—The molecular weight of the purified enzyme was found to be 78,000 on Superdex 200 gel filtration (Fig. 2-6), and the optimal pH was 5.0 when M2B was used as the substrate. The K_m value of the enzyme was 1.4 mM for M2B. The enzyme was neither activated on incubation with 2 mM Ca^{2+} nor inactivated with 10 mM EDTA. The enzyme was unstable at alkaline pH, and inactivated by incubation at pH 8.0 at 37°C for 2 h. Enzymatic reactions were analyzed by means of pulse-amperometric detection in order to determine all the reaction products, including non-fluorescing areas. When M2B was used as the substrate, GN2 and Manα1-6Man were detected in the digest; however, Man, M1, and GN were not detected, indicating that the enzyme only hydrolyzed the Manβ1-4GlcNAc linkage (Fig. 3). When M3C and M4B were used as substrates, PA-chitobiose and oligomannosides were detected. Although we have no standard oligomannosides for these cases, the elution positions on HPAEC were reasonable. Man and M1 were not detected (Fig. 4, A and B). The reactions were also confirmed by reversed-phase HPLC using a fluorescence detector, and the same results were obtained (data not shown). These findings indicate that the enzyme is of the endo-type, and that it hydrolyzes the Manβ1-4GlcNAc linkage.

Substrate Specificity of the Purified Endo-β-mannosidase—In order to examine the substrate specificity of the enzyme, the initial rates of hydrolysis of the PA-sugar chains were measured using the purified endo-β-mannosidase, and the product, PA-chitobiose, in the digests was analyzed by size-fractionation HPLC with a fluorescence detector (Table III). M2B was the best substrate among those tested; however, M3C and M4B were also hydrolyzed. A small amount of GN2 was obtained from M3B, but Manα1-6(Manα1-3)Man was not detected on HPAEC (data not shown). Judging from the detection of a small amount of α-mannosidase activity, as revealed using *p*-nitrophenyl α-mannopyranoside (Table III), there was contamination by an α-mannosidase that was not well

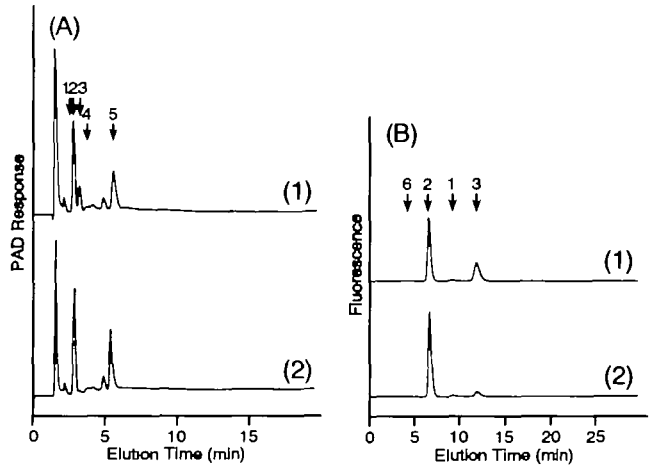

Fig. 3. **Analysis of GN2 releasing activity of the purified enzyme with M2B as the substrate.** The purified enzyme was incubated with M2B as described under "MATERIALS AND METHODS" for 2 h (A-1 and B-1), 6 h (A-2), or 4 h (B-2). A part of the solution was analyzed by HPAEC with a PAD (A), and another by reversed-phase HPLC with a fluorescence detector (B). Arrows indicate the elution positions of: 1, M1; 2, GN2; 3, M2B; 4, Man; 5, Manα1-6Man; 6, GN.

TABLE II. **Summary of the purification of endo-β-mannosidase from *L. longflorum*.**

Purification step	Protein (mg)*	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification (-fold)
Crude enzyme	110,000	1,800	0.016	100	1
Ammonium sulfate	18,000	350	0.020	19	1.2
DEAE-Sephacel	9,900	250	0.025	14	1.5
Q-Sepharose	740	71	0.095	3.9	5.8
Superdex 200	74	38	0.51	2.1	31
Hydroxyapatite	20	19	0.95	1.0	58
Poros PE/M	1.9	3.0	1.6	0.2	96
Mono Q	0.35	1.2	3.6	0.07	220
Superdex 200	0.09	0.4	4.1	0.02	250

*The absorbance at 280 nm was used as an approximation of the protein concentration during purification with bovine serum albumin as the standard.

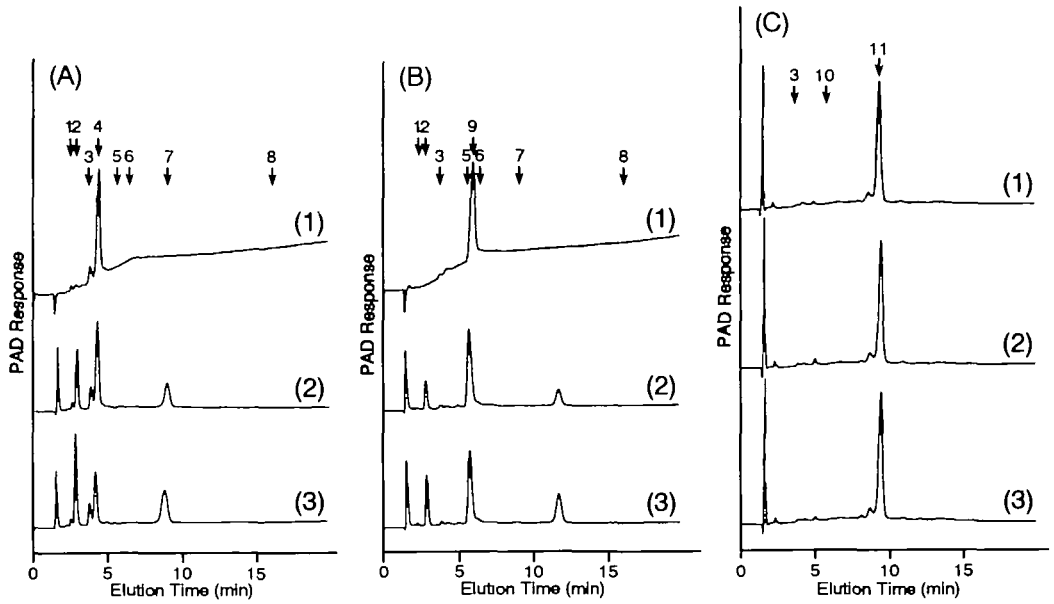

Fig. 4. **Hydrolysis of M3C (A), M4B (B), and βM6 (C) with the purified enzyme.** Each substrate (500 pmol) and the enzyme were incubated for 0 h (1), 2 h (2), or 4 h (3) as described under "MATERIALS AND METHODS." A part of the solution was analyzed by HPAEC with a PAD. Arrows indicate the elution positions of: 1, M1; 2, GN2; 3, Man; 4, M3C; 5, Manα1-6Man; 6, Manα1-3Man; 7, Manα1-6(Manα1-3)Man; 8, Manα1-6(Manα1-3)Manα1-6(Manα1-3)Man; 9, M4B; 10, βM3; 11, βM6.

TABLE III. **Relative initial rates of hydrolysis of PA-sugar chains with the purified endo-β-mannosidase.** The sugar chain abbreviations are listed in Table I. The enzyme activity was measured using 500 pmol of the substrate as described under "MATERIALS AND METHODS."

Substrate	Relative hydrolysis rate
M2B	100
M3C	37
M4B	22
M1A	8
M3B	4
M2A	0
M5A	0
M2FX	0
M2X	0
βM6	0
p-Nitrophenyl α-mannopyranoside	0.6
p-Nitrophenyl β-mannopyranoside	<0.1

separated on Superdex 200 chromatography (Fig. 2-6). Therefore, the weak hydrolysis of M3B may be due to hydrolysis by the endo-β-mannosidase and the contaminating α-mannosidase. M5A, M2X, and M2FX were not hydrolyzed. These findings indicate that the Manα1-3Manβ and Xylβ1-2Manβ structures inhibited the hydrolysis. M1 was slowly hydrolyzed, and the hydrolysis seemed to be due to the endo-β-mannosidase and not a β-mannosidase contaminating the preparation, as *p*-nitrophenyl β-mannopyranoside and βM6 were not hydrolyzed by the enzyme preparation (Table III). βM6 was not hydrolyzed, indicating that the purified enzyme was not a β-mannanase, as has already been reported (*6*). Judging from the substrate specificity of the endo-β-mannosidase, *N*-linked sugar chains are degraded first by an α-mannosidase(s) which hydrolyzes Manα residues linked to the Manα1-3Manβ branch, as such Manα residues are easily hydrolyzed by α-mannosidases such as jack bean α-mannosidase (*7*). The combination of an α-mannosidase and an endo-β-mannosidase seems to produce the *N*-linked chitobiose structure in *Rosaceous* S-RNase (*2*).

The endo-β-mannosidase belongs to a new class of carbohydrases, and it is suggested that it plays a role in the catabolism of *N*-linked sugar chains in plant cells, leading to oligosaccharides (*8, 9*).

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