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Purification and Characterization of Hen Oviduct α1,2-Mannosidase

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An α-mannosidase capable of hydrolyzing three Man α1,2-residues from pyridylamine-(PA-) labeled $Man_9GlcNAc_2$ was purified from hen oviduct. The purity of the preparation was analyzed by PAGE; its molecular weight was 42,000 by SDS-PAGE or 50,000 by gel filtration. The pH optimum was 6.5. The enzyme was inactivated with EDTA; enzyme activity was restored by the addition of Ca^{2+}. The enzyme acitivity was inhibited by 1-deoxymannojirimycin, but not by swainsonine. The substrate specificity of the purified enzyme was analyzed using PA-oligomannose-type sugar chains. When $Man_9GlcNAc_2$-PA was digested, Manα1-6(Manα1-2Manα1-3)Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA was obtained as an end product, and the enzyme was incapable of hydrolyzing *p*-nitrophenyl α-D-mannoside and Man α1,3- or Man α1,6-residues. Judging from these characteristics, the enzyme was classified as a Man_9-mannosidase or Golgi mannosidase I and speculated to participate in the processing or catabolism of glycoproteins.

Key words: α1,2-mannosidase, Ca^{2+}-dependent enzyme, Golgi mannosidase I, hen oviduct, Man_9-mannosidase.

Several α-mannosidases participate in the biosynthesis and processing of asparagine-linked sugar chains (*1, 2*). The processing of the oligosaccharide $Glc_3Man_9GlcNAc_2$ begins with the hydrolysis of glucose residues with glucosidases I and II in rough endoplasmic reticulum followed by trimming with mannosidases in rough endoplasmic reticulum and in the Golgi apparatus (*1, 2*). Recently, the amino acid sequences of α1,2-mannosidases from human kidney (*3*), yeast (*4*), and rabbit and mouse liver (*5*) have been elucidated from cDNAs. These α1,2-mannosidases have high sequence similarity, and therefore belong to a gene family conserved from yeast to mammals (*6*), though their substrate specificities differs slightly (*2*). Man_9-mannosidases belonging to the α1,2-mannosidase group have been purified from microsomal fractions of animal livers (*7-9*) and have been shown to possess characteristics analogous to that of the Golgi α-mannosidase I (*10, 11*). Elucidation of the characteristics of each α-mannosidase might disclose the correct metabolism of the sugar chains. Our research group previously purified a neutral cytosolic α-mannosidase from quail oviduct and clarified its substrate specificity (*12, 13*). In the course of this work, an α-mannosidase which hydrolyzed high mannose-type oligosaccharides but not *p*-nitrophenyl α-D-mannoside was found. The purification and characteristics of this enzyme are described in this paper.

MATERIALS AND METHODS

Materials—Hen oviduct was purchased from a poultry house. Pyridylamino derivatives of oligomannose-type sugar chains were prepared from quail ovomucoid, ovalbumin, kidney bean glycoprotein I, and ricin as reported (*14, 15*). The structures and designations of the sugar chains are shown in Fig. 1. Hemoglobin was purchased from Sigma (St. Louis), cytochrome *c* from Nacalai Tesque (Kyoto), 1-deoxymannojirimycin (dMNJ) from Genzyme (Boston), and swainsonine from Boehringer (Mannheim). dMNJ-AH-Sepharose 4B was prepared according to Hettkamp, H. *et al.* (*16*).

Protein Assay—The amount of protein was assayed with a BCA protein assay reagent (Bio-Rad, Hercules) using bovine serum albumin as a standard.

Enzyme Assay—M9A-PA was used as a substrate for analysis of the α-mannosidase. Substrate solution (10 μl) containing 25 pmol of M9A-PA was mixed with 20 μl of 200 mM HEPES buffer, pH 7.0, containing 0.2% Triton X-100 and 2 mM $CaCl_2$, and 10 μl of the enzyme solution. The mixture was then incubated at 37°C; the digestion was stopped by heating at 100°C for 2 min. A part of the incubation mixture was analyzed by reversed-phase HPLC. One unit of the enzyme was defined as the amount of the enzyme which hydrolyzed 1 nmol of the substrate per min at 37°C.

The following procedure was used to study the reactivation of EDTA-treated enzyme with several metal ions. A mixture of the enzyme solution (10 μl) and 20 μl of the buffer described above containing 2 mM EDTA was preincubated at 37°C for 30 min followed by the addition of 10 μl of a 10 mM metal ion solution and 10 μl of the substrate solution (M9A 25 pmol/10 μl). The mixture was then incubated for 2 h at 37°C, and the enzymatic reaction was terminated by heating at 100°C for 2 min.

p-Nitrophenol released from PNP α-Man was measured at 400 nm. When the oligosaccharides not labeled with fluorescence were used as substrates, products were analyzed after pyridylamination of the sugars released in the

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Abbreviations: dMNJ, 1-deoxymannojirimycin; PA-, pyridylamino; PNP α-Man, *p*-nitrophenyl α-D-mannoside; M4-M9, $Man_4GlcNAc_2$-$Man_9GlcNAc_2$; M4′-M9′, $Man_4GlcNAc$-$Man_9GlcNAc$.

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reaction mixture (*14*). All assays were adjusted to less than 20% hydrolysis of the substrate.

High Performance Liquid Chromatography—Size-fractionation HPLC was done using a TSKgel Amide-80 column (0.46×7.5 cm), and reversed-phase HPLC using a Cosmosil 5C18-P column (0.46×15 cm) as reported previously (*15*).

Purification of α1,2-Mannosidase—A homogenate was prepared from 300 g of the magnum section of hen oviducts in 500 ml of 10 mM HEPES buffer, pH 7.3, containing 0.5% Triton X-100 with a Plotter-Elvehjem-type Teflon homogenizer. The homogenates were centrifuged at 5,000×*g* for

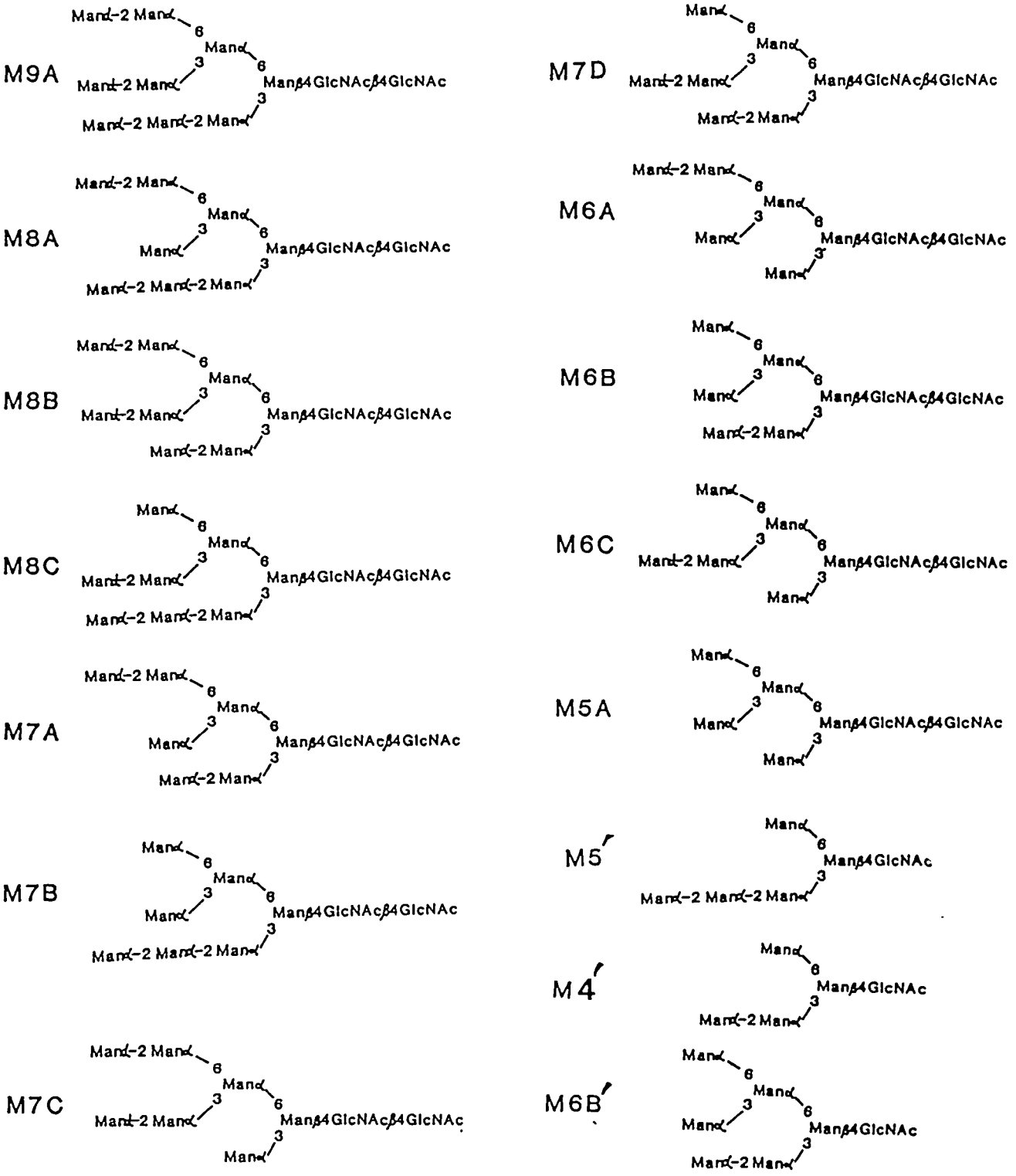

Fig. 1. **Structures and designations of sugar chains used in the present study.**

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60 min, and the supernatant was used as a crude enzyme solution. The precipitate with ammonium sulfate at a concentration between 25 and 55% saturation was dissolved in 200 ml of 10 mM HEPES buffer, pH 7.3, containing 0.5% Triton X-100, and the solution was dialyzed against 10 mM HEPES buffer, pH 7.0, containing 0.5% Triton X-100 and 0.3 M NaCl. One-fifth of the dialysate was placed on a Sephacryl S-200 column (3.2×123 cm) equilibrated with the same buffer as that used for dialysis. The enzyme was further purified by the following chromatographies: ion exchange on a Mono Q HR 5/5 column with a linear gradient of NaCl concentration from 0 to 50% in 10 mM HEPES buffer, pH 7.3, containing 1 M NaCl; gel filtration on a Biogel P-60 column (1.6×90 cm) in 10 mM HEPES buffer, pH 7.3, containing 0.1% Triton X-100 and 0.3% NaCl; and affinity chromatography on a dMNJ-AH Sepharose 4B column (0.8×6 cm) in 10 mM HEPES buffer, pH 7.3, the enzyme being eluted with 10 mM HEPES buffer, pH 7.3, containing 1 M NaCl. All the chromatographies were done at 4°C. Ultrafiltration with Amicon, Diaflo membrane, or ultracent-30 was used to concentrate the enzyme.

PAGE was done according to the method of Williams and Reisfeld (*17*) using 5% gel, or that of Laemmli (*18*) in the presence of SDS using 7.5% gel after reduction with 2-mercaptoethanol.

TABLE I. **Summary of purification of α1,2-mannosidase from hen oviduct.**

Step	Total activity (unit)	Protein (mg)	Specific activity (unit/mg)	Purification (-fold)	Recovery (%)
1. Crude enzyme	4.9	1,200	4.0	1.0	100
2. Ammonium sulfate (25-55%)	3.3	400	8.3	2.1	67
3. Sephacryl S-200	0.26	0.20	1,300	330	5.4
4. Mono Q	0.13	0.04	3,300	830	2.7
5. Bio-Gel P-60	0.05	0.02	2,700	680	1.1
6. dMNJ-AH-Sepharose	0.04	0.01	4,000	1,000	0.8

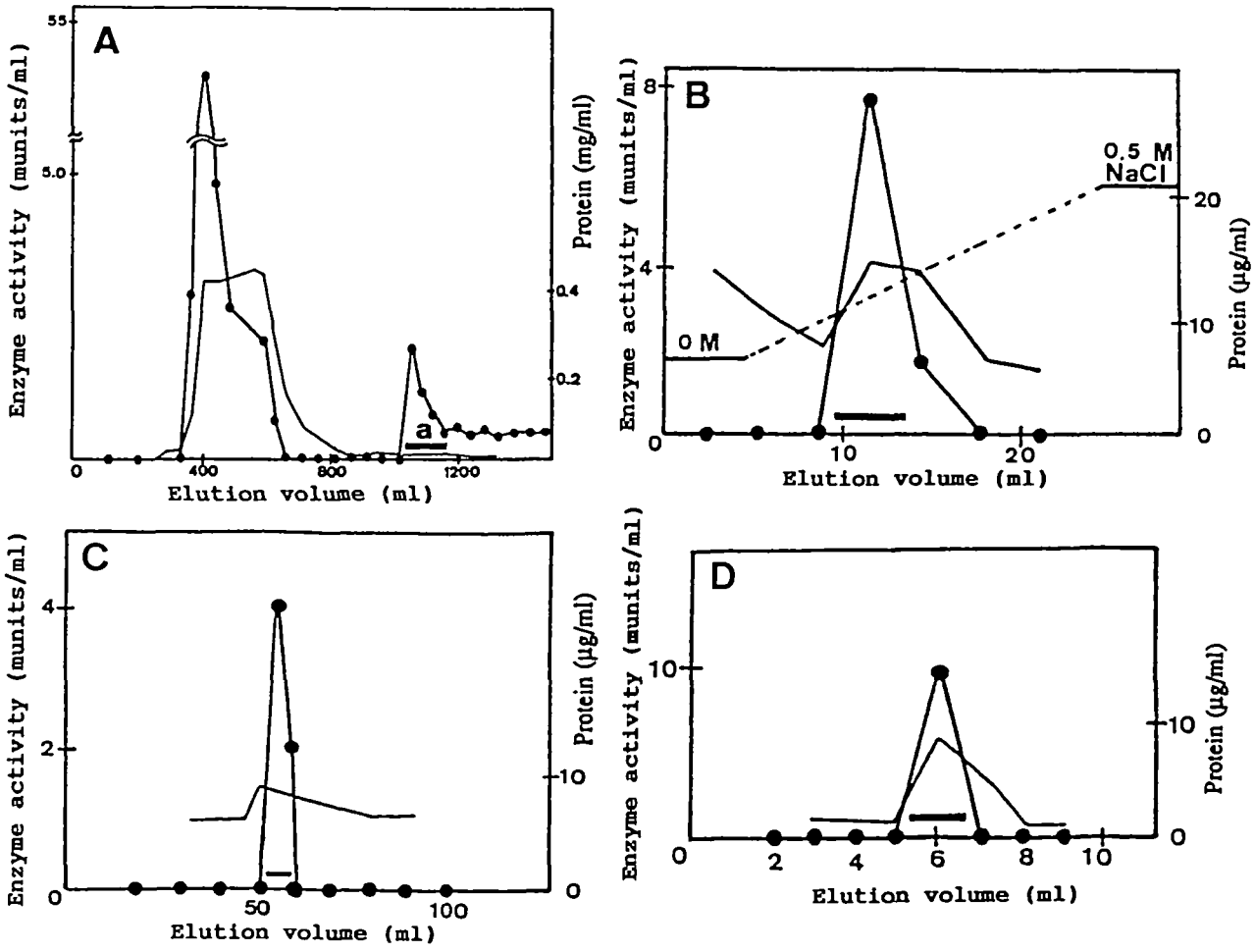

Fig. 2. **Purification of α1,2-mannosidase from hen oviduct.** A, Sephacryl S-200 gel-filtration (3.2×123 cm); B, Mono Q HR 5/5 (0.5×5 cm); C, Biogel P-60 (1.6×90 cm); and D, dMNJ-AH Sepharose 4B affinity chromatography (0.8×6 cm). Fractions indicated by bars were collected. –●–, M9A-PA hydrolysis activity; —, amount of protein; ····, concentration of NaCl.

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RESULTS AND DISCUSSION

Purification of α1,2-Mannosidase—During the course of studies on the purification of α-mannosidases, we detected an α-mannosidase activity which was retarded on Sephacryl S-200 (Fraction a in Fig. 2A). The α-mannosidase activity appearing between elution volume 390 and 600 ml seems to contain other cellular mannosidases. Fraction a was further purified by ion exchange chromatography using Mono Q (Fig. 2B), gel filtration on Bio-Gel P-60 (Fig. 2C), and affinity chromatography on dMNJ-AH-Sepharose 4B (Fig. 2D). The results of the purification are summarized in Table I. Gel filtration on Bio-Gel P-60 was not so effective for the purification, but used for the estimation of the molecular size. The purity of the enzyme was confirmed by SDS- and native-PAGE (Fig. 3, A and B, respectively). The presence of the enzyme activity at the stained position on native PAGE (Fig. 3B) was confirmed by measuring the activity of the sliced gels. The molecular weight of the enzyme was 42,000 by SDS-PAGE and 50,000 by gel filtration on Bio-Gel P-60, indicating a single polypeptide chain. The optimum pH of the enzyme was 6.5 when M9A-PA was used as a substrate.

Effects of Metal Ions and Inhibitors on Enzyme Activity—The enzyme was completely inactivated with 1.7 mM EDTA; 94% of the activity was restored by adding 2 mM of Ca^{2+}, 26% by adding 2 mM Mn^{2+}, and 28% by adding 2 mM Co^{2+}, but the activity was inhibited by adding 2 mM of each of Mg^{2+}, Zn^{2+}, or Cd^{2+}. dMNJ, an inhibitor for Golgi processing mannosidase I and Man_9-mannosidase, reduced the activity to 13% at 5 μM, but swainsonine did not inhibit the enzyme up to a concentration of 500 μM.

Substrate Specificity of the Enzyme—The hydrolysis products of M9A-PA were separated by size-fractionation

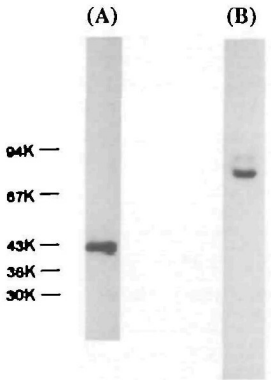

Fig. 3. **Polyacrylamide gel electrophreses of the purified enzyme in the presence (A) and absence (B) of SDS.**

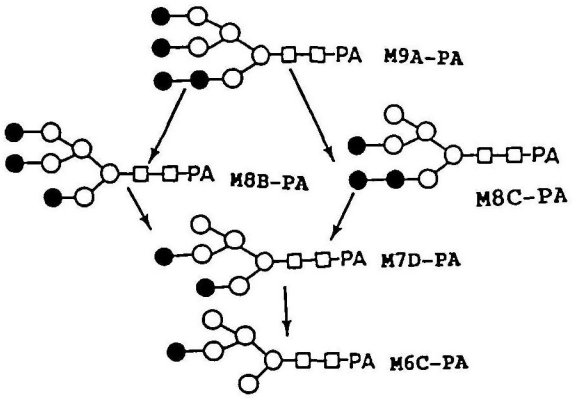

Fig. 5. **Probable course of hydrolysis of M9A-PA with purified α1,2-mannosidase.** ●, Man α1,2-residue; ○, Man residue; □, GlcNAc residue.

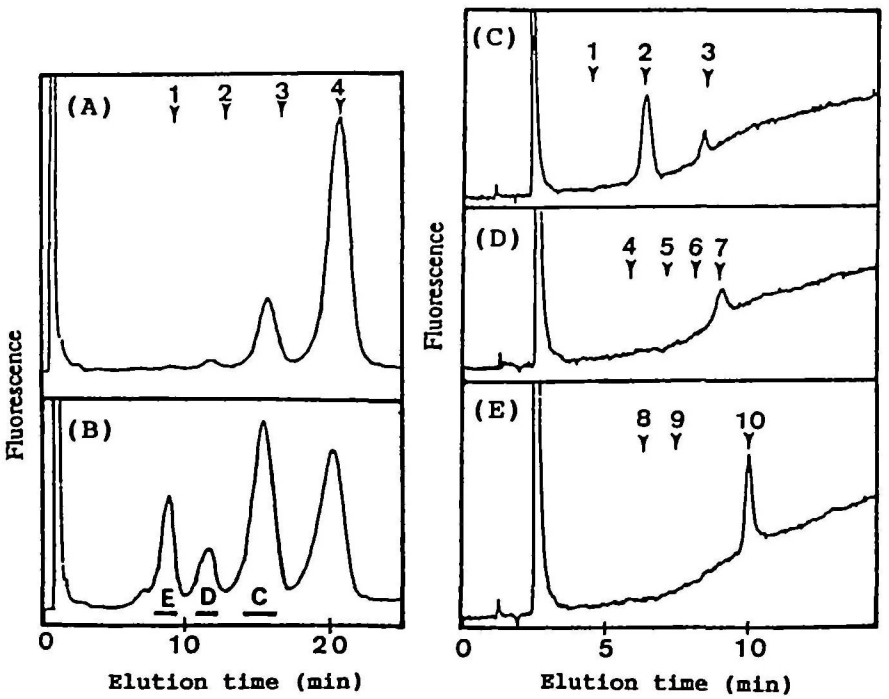

Fig. 4. **Analysis of α1,2-mannosidase digests of M9A-PA.** M9A-PA was digested with the purified enzyme for 2 h (A) and 8 h (B), and the digests were analyzed by size-fractionation HPLC. Elution positions of standard PA-sugar chains for A and B: Arrowhead 1, M6; 2, M7; 3, M8; 4, M9. Peaks C, D, and E, indicated by bars, were collected and analyzed on a reversed-phase column as shown in Fig. 5, C, D, and E, respectively. Elution positions of standards for Fig. 5, C, D, and E: Arrowhead 1, M8A; 2, M8B; 3, M8C; 4, M7A; 5, M7B; 6, M7C; 7, M7D; 8, M6A; 9, M6B; 10, M6C.

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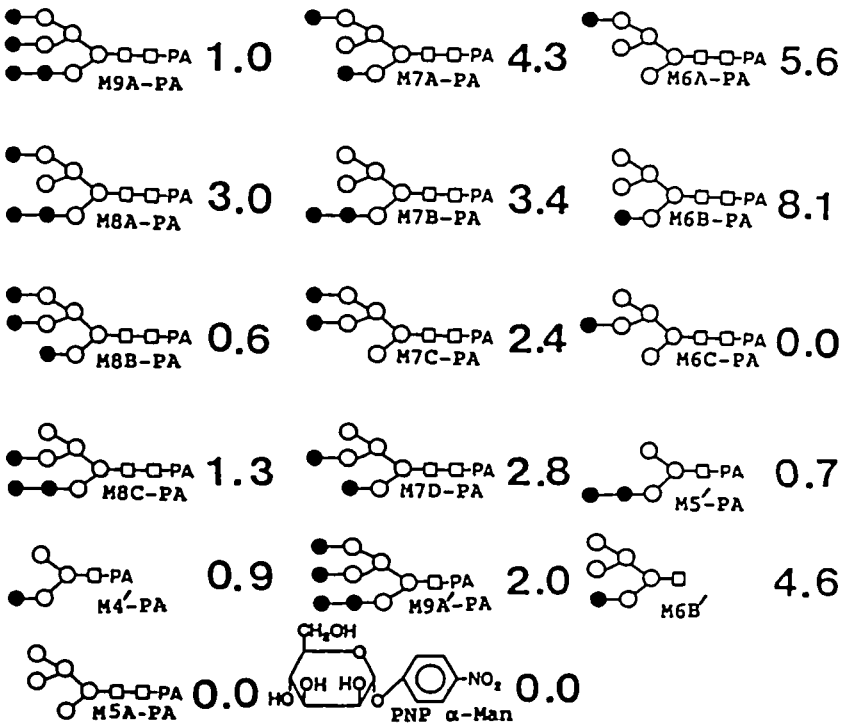

Fig. 6. **Initial hydrolysis rates of sugar chains and PNP α-Man relative to that of M9A-PA with purified α1,2-mannosidase.** ●, Man α1,2-residue; ○, Man residue; □, GlcNAc residue.

HPLC as shown in Fig. 4, A and B. Fractions C, D, and E, purified from the 8-h digest of M9A-PA and corresponding to the elution positions of M8, M7, and M6, respectively, were collected (Fig. 4B). The exact structures of the isomers in each fraction were analyzed by reversed-phase HPLC as shown in Fig. 4, C, D, and E, respectively, by comparing the elution positions with those of the authentic PA-sugar chains. The results show that Fraction C was a mixture of M8B-PA and M8C-PA, Fraction D was M7D-PA, and Fraction E was M6C-PA. The ratio of M8B-PA to M8C-PA was 5. From these results, the course of the hydrolysis of M9A-PA to M6C-PA with the purified enzyme is speculated in Fig. 5. The intermediate product of $Man_8GlcNAc_2$ by the enzyme was different from that reported for pig liver Man_9-mannosidase (*7*), though prolonged digestion for 17 h gave the same product, M6C-PA. The initial hydrolysis rates of PA-labeled oligomannose-type sugar chains relative to that of M9A-PA are summarized in Fig. 6. The enzyme hydrolyzed M6B-PA fastest, M6A-PA next, and M8B-PA and M5′-PA slowest among the PA-sugar chains tested. M6C-PA, M5A-PA, and PNP α-Man were not hydrolyzed. The enzyme was specific to the Man α1,2-residue, but inert to the Man α1,3- and Man α1,6-residues. The non-hydrolysable Man α1,2-residue in M9A-PA, *i.e.* Man α1,2-residue in M6C-PA, interfered with the hydrolysis of the other Man α1,2-residues judging from a comparison of the hydrolysis rates of the following sets of sugar chains: M8A-PA and M9A-PA; M7A-PA and M8B-PA; M7B-PA and M8C-PA; M6B-PA and M7D-PA; M6A-PA and M7C-PA (Fig. 6). The results suggest that the substrate specificities of the present enzyme and dMNJ-resistant ER α1,2-mannosidase (*19*) seem to be complementary, since the former enzyme cleaves the rest of the three Man α1,2-residues from the M8A structure produced by the latter. Comparison of the initial hydrolysis rates of M9A-PA and M9A′-PA, and M6B-PA and M6B′ indicated that GlcNAc residue at the reducing end had little effect—contrary to the Co^{2+}-activated neutral quail α-mannosidase (*13*), in which deletion of the reducing end GlcNAc residue raised the hydrolysis rate about 16-fold.

On the basis of the characteristics, elucidated in this study—*i.e.*, the hydrolysis of Man α1,2-residues, molecular weight, and behavior toward inhibitors and metal ions, the α-1,2 mannosidase is similar to the Golgi α1,2-mannosidase I (*4, 5, 11*), but judging from the substrate specificity, it more closely resembled to the Man_9-mannosidases from pig (*7, 8*) and calf (*9*).

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