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Purification and Characterization of Neutral α-Mannosidase from Hen Oviduct: Studies on the Activation Mechanism of Co^{2+}

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Neutral α-mannosidase was purified to homogeneity from hen oviduct. The molecular mass of the enzyme was 480 kDa on gel filtration, and the 110-kDa band on SDS-PAGE in the presence of 2-mercaptoethanol indicated that it is composed of four subunits. The activated enzyme hydrolyzed both *p*-nitrophenyl α-D-mannoside and high mannose-type sugar chains. This substrate specificity is almost the same as that reported for the neutral α-mannosidase from Japanese quail oviduct [Oku and Hase (1991) *J. Biochem.* 110, 982-989]. Manα1-6(Manα1-3)Manα1-6(Manα1-3)Manβ1-4GlcNAc (K_m=0.44 mM) was hydrolyzed four times faster than Manα1-6(Manα1-3)Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc, and Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAc was obtained as the end product from $Man_9GlcNAc$ on digestion with the activated α-mannosidase. The enzyme was activated 24-fold on preincubation with Co^{2+}. The activation with other metal ions, like Mn^{2+}, Ca^{2+}, Fe^{2+}, Fe^{3+}, and Sr^{2+}, was less than 5-fold, and Zn^{2+}, Cu^{2+}, and Hg^{2+} inhibited the enzyme activity. The optimum pHs for both the enzyme activity and activation with Co^{2+} were around 7. The cobalt ion contents of the purified, EDTA-treated, and Co^{2+}-activated enzymes were 1.5, 0.0, and 3.9, respectively, per molecule. Since the Co^{2+}-activated enzyme gradually lost its activity on incubation with EDTA and the activity was restored promptly on the addition of Co^{2+}, the binding of Co^{2+} to the enzyme seems to be essential for its activation. The results obtained with protease inhibitors together with those of the SDS-PAGE before and after activation, showed that the proteolytic cleavage reported for the activation of monkey brain α-mannosidase seems not to be involved.

Key words: cobalt ion, cytosol, hen oviduct, α-mannosidase, oligosaccharide.

Cellular α-mannosidases are classified into several groups according to their subcellular locations, functions, substrate specificities, and physicochemical properties (*1, 2*). A group of α-mannosidases, designated as neutral α-mannosidases, isolated from the cytosol exhibit some common characteristics; *i.e.*, a molecular mass of 300-500 kDa and an optimal pH of around 7.

Detailed studies by our group of neutral α-mannosidases from Japanese quail oviduct (*3, 4*) and bovine liver (*5*) revealed the following three characteristics of neutral/cytosolic α-mannosidase. (1) The enzyme hydrolyzes certain α1-2, α1-3, and α1-6 linked mannose residues of $Man_9GlcNAc$, leaving Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAc as an end product. (2) Sugar chains with a single GlcNAc residue at the reducing end are hydrolyzed more preferably than those with two GlcNAc residues. This substrate specificity suggests the presence of a catabolic pathway for high mannose-type glycans in the cytosol together with the presence of endo-β-*N*-acetylglucosaminidase (*6*). (3) The enzyme is activated by Co^{2+}. These characteristics seem to be common to other reported neutral/cytosolic α-mannosidases (*4, 5, 7-17*).

The mechanism of activation by Co^{2+} has not yet been well elucidated. Mathur *et al.* (*12*) reported that Co^{2+}-stimulated aminopeptidase activity might be involved in the activation of monkey brain α-mannosidase. To further unveil the mechanism of activation by Co^{2+}, we purified neutral α-mannosidase from hen oviduct, which is a good source material for obtaining a large amount of the enzyme. In this paper we show the activation is due to the binding of Co^{2+}, and not to proteolytic cleavage by a Co^{2+}-activated protease.

MATERIALS AND METHODS

Materials—Hen oviducts were procured from a poulterer. pNP α-Man was purchased from Nacalai Tesque (Kyoto), M9A′ from BioCarb (Lund, Sweden) (for sugar chain abbreviations, see Table I), swainsonine from Wako Pure Chemicals (Osaka), and 1-deoxymannojirimycin from Genzyme (Boston). Yeast mannan and bovine serum albumin were obtained from Sigma (St. Louis, MO). Actinonin, Amastatin, Aprotinin, Arphamenin A, Arphamenin B, Bestatin, Chymostatin, Diprotin A, E-64, Leuhistin, Leu-

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Abbreviations: BSA, bovine serum albumin; CyDTA, *trans*-1,2-cyclohexanediamine tetraacetic acid; DTPA, diethylenetriaminepentaacetic acid; FPLC, fast protein liquid chromatography; GlcNAc, *N*-acetyl-D-glucosamine; Man, D-mannose; MES, 2-(*N*-morpholino)-ethanesulfonic acid; PA-, pyridylamino; PBS, phosphate-buffered saline; pNP α-Man, *p*-nitrophenyl α-D-mannopyranoside.

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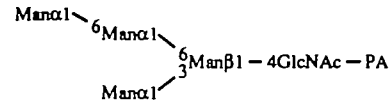
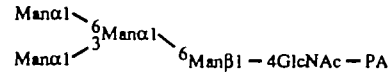
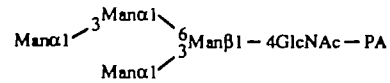
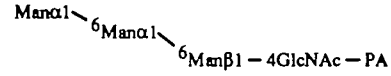
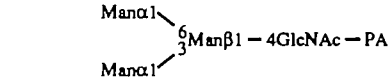
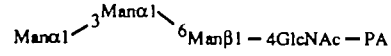
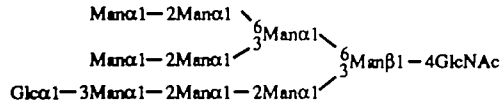
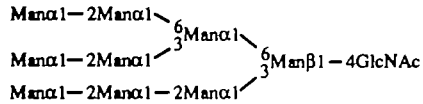
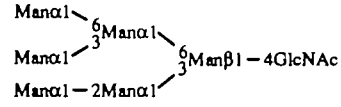
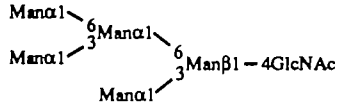
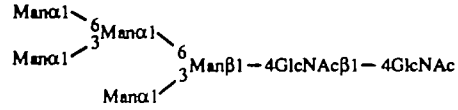
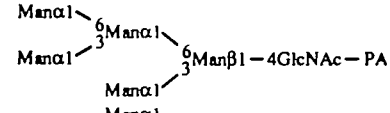
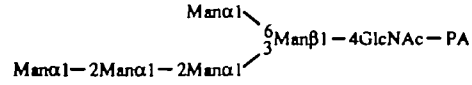
TABLE I. **Structures and abbreviations of sugar chains used.**

Abbreviation	Structure
M4A'-PA	
M4B'-PA	
M4C'-PA	
M3A'-PA	
M3B'-PA	
M3C'-PA	
GM9A'	
M9A'	
M6B'	
M5A'	
M5A	
M5A'-PA	
M5B'-PA	

peptin, Pepstatin A, and Phosphoramidon were from the Peptide Institute (Minoh). Endo-β-*N*-acetylglucosaminidase H (*Streptomyces griseus*) was purchased from Seikagaku Kogyo (Tokyo), and BCA protein assay reagent from Pierce (Rockford, IL). α1,2-Mannosidase (*Aspergillus oryzae*) was kindly donated by Dr. H. Yamaguchi (University of Osaka Prefecture). The columns and column materials used were TSKgel Amide-80 (0.46×7.5 cm) and TSKgel Sugar AXI (0.46×15 cm) from Tosoh (Tokyo), DEAE Sephacel, Octyl Sepharose CL-4B, Sephacryl S-300, Sepharose 4B, Sephadex G-25 superfine, and Mono Q HR 10/10 from Pharmacia (Uppsala, Sweden), YMC-Gel Sil S-5 from Yamamura Kagaku (Kyoto), and HCA A-7610 (0.76×10 cm) from Mitsui Toatsu (Tokyo). Mannan-Sepharose 4B was prepared as described previously (*18*).

Preparation of Sugar Chains—GM9A-PA was prepared from hen egg-yolk immunoglobulin, and M9A-PA from soybean agglutinin. M6B-PA, M5A-PA, and GNM5-PA were prepared from hen ovalbumin (*3*). GM9A′, M6B′, and M5A′ were prepared by digesting GM9A-PA, M6B-PA, and

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M5A-PA, respectively, with endo-β-*N*-acetylglucosaminidase H. M5A′-PA was prepared by pyridylamination of M5A′ (*19*). M5B′-PA was prepared by digesting M9A′ with neutral α-mannosidase of Japanese quail oviduct (*3*), followed by pyridylamination. M4A′-PA, M4B′-PA, M4C′-PA, M3A′-PA, M3B′-PA, and M3C′-PA were prepared as described previously (*3*).

Assay Method for α-Mannosidase—A mixture of 10 μl of the enzyme solution and 20 μl of 200 mM HEPES buffer, pH 7.0, containing 1.5 mM $CoCl_2$ was preincubated at 37°C for 45 min. Then, 20 μl of 10 mM pNP α-Man was added to the preincubated enzyme solution, and the mixture was further incubated at 37°C for 30 min. The enzymatic reaction was stopped by the addition of 950 μl of a 0.25 M Na_2CO_3 solution, and then the absorbance at 400 nm was measured. One unit of α-mannosidase activity was defined as the enzyme activity which released 1 μmol of *p*-nitrophenol per min at 37°C.

When a PA-sugar chain was used as a substrate, 10 μl of a solution containing 12.5 pmol PA-sugar chain was added to 15 μl of the preincubated enzyme solution and then the incubation was continued for 0.5–4 h. The enzymatic reaction was stopped by heating at 100°C for 3 min. The products were analyzed by size-fractionation HPLC on a TSKgel Amide-80 column. Prior to HPLC analysis, the hydrolysates of unlabeled sugar chains were pyridylaminated as described (*19*). PA-sugar chains were eluted and collected, and then their structures were analyzed using a TSKgel Sugar AXI column (*3*).

Purification of Neutral α-Mannosidase—All the procedures were carried out at 4°C. The amount of protein was determined using BCA protein assay reagent with bovine serum albumin as a standard.

Step 1. Preparation of a crude enzyme solution: Magnum sections (2 kg) of hen oviduct were washed several times with chilled PBS and then frozen with liquid nitrogen. The frozen magnum sections were powdered with a blender, suspended in 6 liters of 50 mM phosphate buffer, pH 7.5, and homogenized with a Polytron homogenizer (10,000 rpm, 1 min). The homogenate was centrifuged at 28,000× *g* for 30 min, and the supernatant was used as the crude enzyme solution.

Step 2. Ammonium sulfate precipitation: Ammonium sulfate was added to the crude enzyme solution to 30% saturation. The precipitates formed were collected by centrifugation, dissolved in 10 mM phosphate buffer, pH 6.3, and then dialyzed against the same buffer. The insoluble material was removed from the non-dialyzable fraction by centrifugation.

Step 3. DEAE Sephacel chromatography: The supernatant (770 ml) obtained in Step 2 was placed on a DEAE Sephacel column (5.5×30 cm) equilibrated with 10 mM phosphate buffer, pH 6.3, and the column was washed with 900 ml of the same buffer. Neutral α-mannosidase was eluted with a linear gradient of NaCl, from 0 to 0.4 M (6 liters). The fractions containing neutral α-mannosidase activity were pooled.

Step 4. Octyl Sepharose CL-4B chromatography: Disodium hydrogenphosphate was added to the pooled fraction from Step 3 to a concentration of 50 mM and then ammonium sulfate was added to the solution to a concentration of 1 M. The solution (1.1 liters) was applied to an Octyl Sepharose CL-4B column (2.5×20 cm) equilibrated with 50 mM phosphate buffer, pH 7.0, containing 1 M ammonium sulfate, and then the column was washed with 700 ml of the same buffer. Neutral α-mannosidase was eluted with 10 mM phosphate buffer, pH 7.0. The fractions containing α-mannosidase activity were pooled. Ammonium sulfate was added to the solution to 80% saturation. The precipitates were collected by centrifugation (28,000× *g*, 30 min), and then dissolved in 30 ml of 10 mM HEPES buffer, pH 7.3, containing 0.3 M NaCl.

Step 5. Sephacryl S-300 gel filtration: The enzyme solution (30 ml) was applied to a Sephacryl S-300 column (3.5×192 cm) equilibrated with 10 mM HEPES buffer, pH 7.3, containing 0.3 M NaCl. The fractions containing α-mannosidase activity were pooled.

Step 6. Mannan-Sepharose 4B chromatography: The pooled fraction obtained in Step 5 was dialyzed against 10 mM phosphate buffer, pH 7.0, and the non-dialyzable fraction was applied to a mannan-Sepharose 4B column (3.0× 21 cm) equilibrated with the phosphate buffer, pH 7.0. The column was washed with 400 ml of the same buffer, and then α-mannosidase was eluted with the same buffer containing 0.5 M NaCl. The fractions containing α-mannosidase activity were pooled.

Step 7. Hydroxyapatite chromatography: The pooled fraction obtained in Step 6 was dialyzed against 10 mM phosphate buffer, pH 7.0, and the non-dialyzable fraction was concentrated to 5 ml by ultrafiltration (Diaflo membrane PM-10, Amicon). Hydroxyapatite chromatography was performed with a fast protein liquid chromatography (FPLC) system (Pharmacia) on an HCA-column (0.76×10 cm). The column was equilibrated with 10 mM phosphate buffer, pH 7.0, at a flow rate of 1 ml/min. After injection of the enzyme solution, the column was washed with the same buffer for 20 min. α-Mannosidase was eluted with a linear gradient of phosphate, from 10 to 350 mM, in 30 min. The fractions containing α-mannosidase activity were pooled.

Step 8. Mono Q chromatography: The pooled fraction obtained in Step 7 was dialyzed against 10 mM HEPES buffer, pH 7.5, and the non-dialyzable fraction was concentrated to 5 ml by ultrafiltration. Mono Q chromatography was performed with a FPLC system equipped with a Mono Q HR 10/10 column. The column was equilibrated with 10 mM HEPES buffer, pH 7.5, at a flow rate of 2 ml/min. After injection of the concentrated enzyme solution, the column was washed with the same buffer for 10 min. α-Mannosidase was eluted with a linear gradient of NaCl, from 0 to 0.6 M, in 54 min. The fractions containing α-mannosidase were pooled, and then stored at 4°C in the presence of 10% ethylene glycol.

Polyacrylamide Gel Electrophoresis (PAGE)—Gel electrophoresis was performed on a 5% polyacrylamide gel, pH 8.9, by the method of Davis (*21*). SDS-PAGE was performed on a 4–20% gradient polyacrylamide gel by the method of Laemmli (*22*). Proteins were stained with Coomassie Brilliant Blue R 250.

High-Performance Liquid Chromatography (HPLC)—A Beckman model 342 chromatograph equipped with a Hitachi 650-10 fluorescence spectrophotometer was used for the HPLC analysis of PA-sugar chains. The conditions for size-fractionation HPLC on TSKgel Amide-80 (*20*) and TSKgel Sugar AXI (*23*) were described previously.

Determination of the Co^{2+} Content by Atomic Absorption Spectrometry—The content of cobalt ions in Co^{2+}-activated

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neutral α-mannosidase was determined as follows. The purified enzyme was incubated in the presence of 1 mM Co^{2+}, and then unbound excess Co^{2+} was removed with a Sephadex G-25 superfine column. As a control, BSA was treated in the same way. Cobalt in the protein was determined using a Shimadzu atomic absorption spectrophotometer AA-6400G equipped with a Shimadzu graphite furnace atomizer GFA-6500. Atomized cobalt was detected at 240.7 nm using a cobalt hollow cathode lamp and the background interference was corrected by the D2 lamp method.

RESULTS

Purification and Characterization of Neutral α-Mannosidase—The results of the purification of neutral α-mannosidase are summarized in Table II, and the final chromatogram obtained with the Mono Q column is shown in Fig. 1. The purified α-mannosidase gave a single band on native PAGE (Fig. 2A). pNP α-Man hydrolyzing activity was detected at the position of the protein band and the enzyme was activated by incubation with Co^{2+} (Fig. 2B). The molecular mass of the neutral α-mannosidase was 480 kDa on Sephacryl S-300 gel filtration, and a 110-kDa band was detected on SDS-PAGE in the presence of 2-mercaptoethanol (Fig. 2C). The optimal pH of α-mannosidase activity was 6.8, and the K_m for M5A′ was 0.44 mM (data not shown). The Co^{2+}-activated enzyme was completely inhibited by Pb^{2+}, Zn^{2+}, and Cu^{2+} (0.5 mM), and swainsonine (0.01 mM), but 1-deoxymannojirimycin exhibited 19% inhibition at a concentration of 0.01 mM and 74% at 0.5 mM.

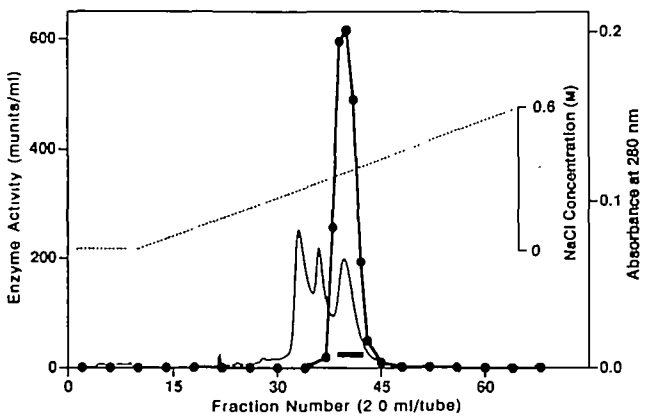

Fig. 1. **Final step of neutral α-mannosidase purification by Mono Q chromatography.** The fraction obtained on hydroxyapatite chromatography was applied to a Mono Q HR column (1.0×10 cm) equilibrated with 10 mM HEPES buffer, pH 7.5. The enzyme was eluted with a linear NaCl concentration gradient (·····), and the protein was monitored by measuring the absorbance at 280 nm (—). The fraction indicated by the bar was collected. α-Mannosidase activity was measured with pNP α-Man after preincubation at 37°C for 15 min in the presence of 1 mM Co^{2+} (●).

Substrate Specificity of Neutral α-Mannosidase—The rates of hydrolysis of high mannose-type sugar chains with Co^{2+}-activated α-mannosidase are summarized in Table III. M5A′, with a single GlcNAc residue at the reducing end, was hydrolyzed four times faster than M5A with two GlcNAc residues. The hydrolysates of M5A′ were pyridylaminated and then analyzed by size-fractionation HPLC (Fig. 3A) and on a Sugar AXI column (Fig. 3B): peaks F1 and F2 obtained on size-fractionation HPLC were collected, and separately analyzed by Sugar AXI column chromatography. Peak F1, exhibiting the molecular size of Man_4-GlcNAc-PA on size-fractionation HPLC, was eluted at the position of M4C′-PA on the Sugar AXI column, and peak F2, with the molecular size of Man_3GlcNAc-PA, was eluted at the position of M3B′-PA (Fig. 3B). Prolonged hydrolysis of M5A′ gave no product smaller than Man_3GlcNAc-PA. The activated neutral α-mannosidase was thus proved to hydrolyze M5A′ to M3B′ *via* M4C′. The hydrolysates of M9A′ were analyzed similarly (Fig. 4). Fraction A, the end product obtained on prolonged hydrolysis, was eluted at the

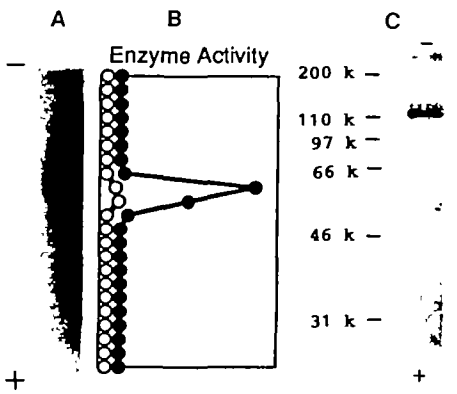

Fig. 2. **Polyacrylamide gel electrophoresis of the purified neutral α-mannosidase.** A: Native PAGE of the enzyme. B: Enzyme activity measured using pNP α-Man as a substrate in the presence (●) or absence (○) of Co^{2+}. C: SDS-PAGE of the enzyme in the presence of 2-mercaptoethanol.

TABLE II. **Summary of purification of neutral α-mannosidase from hen oviduct.**

	Protein[a] (mg)	Total activity[b] (units)	Recovery (%)	Specific activity (units/mg)	Purification (-fold)
Crude enzyme	190,000.00	350.0	100.0	0.0018	1
$(NH_4)_2SO_4$ ppt. (0-30%)	4,900.00	170.0	50.0	0.035	20
DEAE Sephacel	1,600.00	87.0	25.0	0.055	31
Octyl Sepharose CL-4B	370.00	37.0	11.0	0.10	56
Sephacryl S-300 HR	39.00	19.0	5.4	0.49	270
Mannan-Sepharose	12.00	14.0	4.1	1.2	650
Hydroxyapatite	1.90	7.8	2.1	4.1	2,300
Mono Q	0.51	5.7	1.6	11	6,200

[a]The amount of protein was measured using BCA protein assay reagent with bovine serum albumin as a standard. [b]pNP α-Man was used as a substrate.

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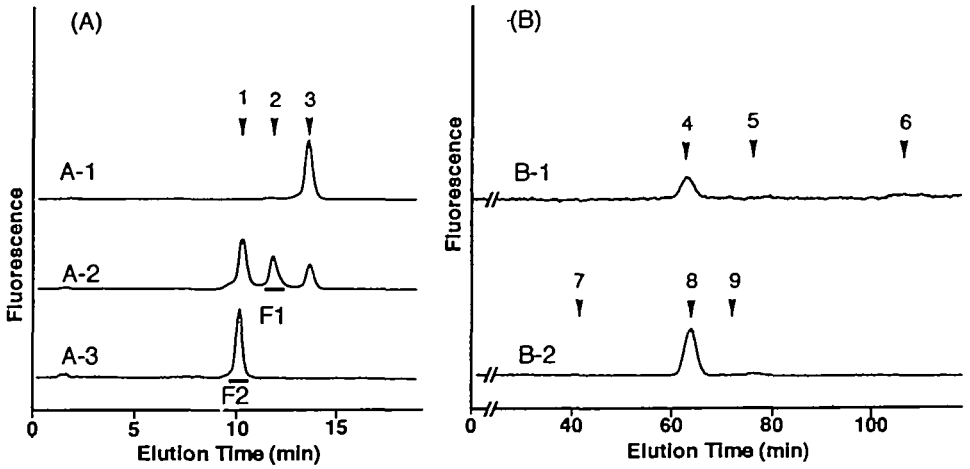
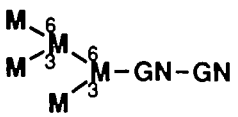
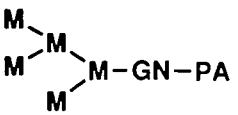
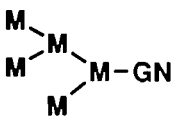
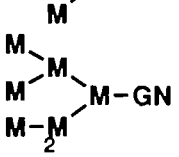
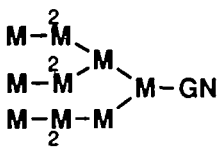
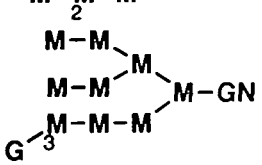


Fig. 3. **Analysis of digests of M5A′ with the activated neutral α-mannosidase.** (A) M5A′ was digested with the activated enzyme, followed by pyridylamination. The products were analyzed on TSKgel Amide-80. A-1, 0 min hydrolysate; A-2, 30 min hydrolysate; A-3, 180 min hydrolysate. (B) Fractions F1 and F2 were further analyzed (B-1 and B-2, respectively) by Sugar AXI chromatography. Arrowheads indicate the elution positions of standard PA-sugar chains: 1 and 9, M3A′-PA; 2, M4A′-PA; 3, M5A′-PA; 4, M4C′-PA; 5, M4B′-PA; 6, M4A′-PA; 7, M3C′-PA; 8, M3B′-PA.

TABLE III. **Relative rates of hydrolysis of high mannose-type oligosaccharides digested with neutral α-mannosidase.** The rate of hydrolysis of M5A was taken as 1.0. M indicates a mannose residue, GN an *N*-acetylglucosamine residue, and G a glucose residue. The numbers, 2, 3, and 6, at the chemical bonds indicate the linkage positions.

	Substrate	Relative hydrolysis rate
M5A		1.0
M5A′-PA		1.6
M5A′		3.9
M6B′		2.7
M9A′		2.2
GM9A′		1.6

position of $Man_5GlcNAc$-PA on size-fractionation HPLC and at the position of M5B′-PA on a Sugar AXI column (Fig. 4B). The structure of fraction A was further confirmed by digestion with α1,2-mannosidase, followed by analysis of

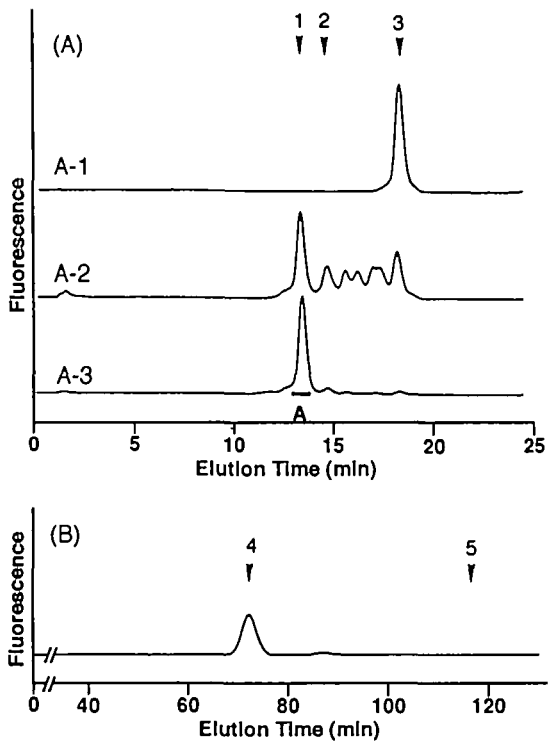


Fig. 4. **HPLC-analysis of digests of M9A′ with the activated neutral α-mannosidase.** M9A′ was digested with the activated enzyme, followed by pyridylamination. (A) The products were analyzed on a TSKgel Amide-80 column. A-1, 0 min hydrolysate; A-2, 1 h hydrolysate; A-3, 5 h hydrolysate. (B) Fraction A was collected and chromatographed on a Sugar AXI column. Arrowheads indicate the elution positions of standard PA-sugar chains: 1 and 5, M5A′-PA; 2, M6B′-PA; 3, M9A′-PA; 4, M5B′-PA.

the products by size-fractionation HPLC and Sugar AXI column chromatography. The product was eluted at the position of M3B′-PA (data not shown). These results showed that Fraction A was M5B′.

Activation of Neutral α-Mannosidase—Activation by Co^{2+} was preeminent among that by the metal cations tested; the rate of hydrolysis of M5A′-PA increased 24-fold on incubation with Co^{2+} (Table IV), while that of pNP α-Man rose 10-fold. In the presence of 1 mM Co^{2+} at 37°C,

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the maximum activity was obtained after incubation for more than 15 min (Fig. 5). Higher concentrations of Co^{2+} increased the activation rate, but did not change the maximum activation level (Fig. 5). EDTA (8.3 mM) inhibited the activation, but protease inhibitors (Actinonin, Amastatin, Aprotinin, Arphamenin A, Arphamenin B, Bestatin, Chymostatin, Diprotin A, E-64, Leuhistin, Leupeptin, Pepstatin A, or Phosphoramidon) did not inhibit the activation at a concentration of 10 μM (data not shown). The activated enzyme was analyzed by SDS-PAGE under reducing conditions, but the band pattern was unchanged (data not shown). The optimal pH for activation was 7.1 (Fig. 6), which was almost the same as that of the enzyme activity. A higher pH (7.5 or 8.0) made the rate of activation greater, but the maximum activation level was lowered (data not shown).

Effects of Chelating Reagents on the Activated α-Mannosidase—The activated enzyme was stable in the presence of 1 mM Co^{2+} at 37°C, however, the addition of EDTA reduced the activity according to the first-order rate (Fig. 7). This activity reduction was not dependent on the concentration of EDTA, and occurred in similar manner with stronger chelating reagents (CyDTA and DTPA).

Reactivation of the EDTA-Treated Activated α-Mannosidase—The Co^{2+}-activated enzyme lost 85% of its activity on incubation with 6.25 mM EDTA for 4 h (Fig. 7). The addition of Co^{2+} to the EDTA-treated enzyme immediately restored the activity (Fig. 8). Similar reactivation was observed for the enzyme inactivated with EDTA for 8 h (data not shown).

TABLE IV. **Activation of neutral α-mannosidase by metal cations.** Neutral α-mannosidase was preincubated with a metal cation at a concentration of 1 mM for 45 min at 37°C, and then the substrate, M5A′-PA, was added to the solution. The reaction mixture was further incubated, and the products were analyzed by size-fractionation HPLC as described under "MATERIALS AND METHODS."

Metal cation	Relative hydrolysis rate
Control	1
Co^{2+}	24
Mn^{2+}	4.8
Ca^{2+}	2.2
Ba^{2+}	2.0
Mg^{2+}	1.9
Sr^{2+}	1.5
Fe^{2+}	1.4
Ni^{2+}	1.3
Fe^{3+}	1.2
Zn^{2+}	0.0
Cu^{2+}	0.0
Hg^{2+}	0.0

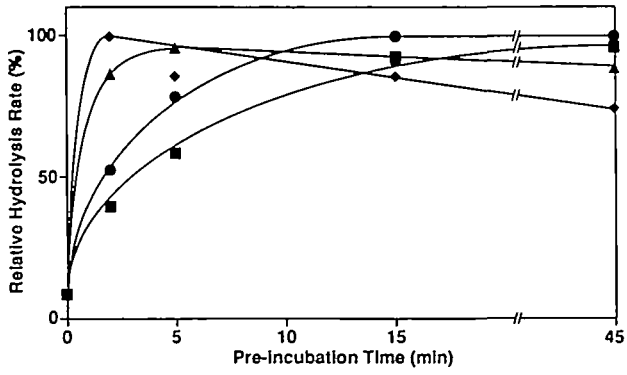

Fig. 5. **Effect of the Co^{2+} concentration on activation of neutral α-mannosidase.** Neutral α-mannosidase (0.3 milliunit in 5 μl) was mixed with 10 μl of 200 mM HEPES buffer, pH 7.0, containing $CoCl_2$ at 0.5 mM (■), 1 mM (●), 2 mM (▲), or 5 mM (◆), and then the mixture was incubated at 37°C. An EDTA solution (5 μl of 25 mM EDTA in 200 mM HEPES buffer, pH 7.0) was added to terminate the activation. pNP α-Man was then added, and the mixture was incubated for 5 min at 37°C. The hydrolysis rates relative to that obtained when the enzyme was activated with 1 mM Co^{2+} for 15 min are shown.

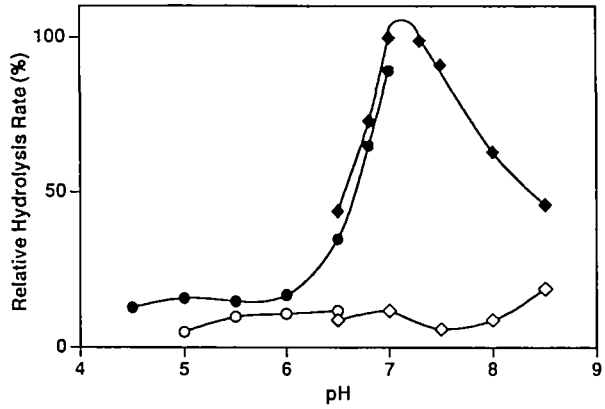

Fig. 6. **Effect of pH on activation of neutral α-mannosidase.** The enzyme (0.3 milliunit) was activated in 15 μl of 50 mM MES buffer, pH 4.5-6.8 (●, ○), or 50 mM HEPES buffer, pH 6.5-8.4 (◆, ◇) in the presence (●, ◆) or absence (○, ◇) of 1 mM Co^{2+} at 37°C for 3 min. Then, 15 μl of 400 mM HEPES buffer, pH 7.0, and 15 μl of 10 mM pNP α-Man were added to the preincubation mixture, followed by incubation for 5 min at 37°C.

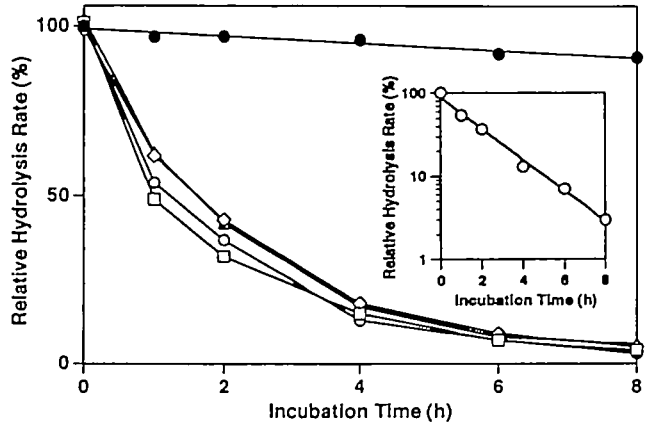

Fig. 7. **Effects of chelating reagents on the activated neutral α-mannosidase.** A chelating reagent (5 μl in 200 mM HEPES buffer, pH 7.0) was added to the neutral α-mannosidase solution (0.3 milliunit) which had been activated for 15 min at 37°C in 15 μl of 130 mM HEPES buffer, pH 7.0, containing 10 μg BSA and 1.0 mM Co^{2+}. The chelating reagents used were 6.25 mM EDTA (○), CyDTA (△), and DTPA (◇), and 62.5 mM EDTA (□). No chelating reagent was added in the control (●). Then, 10 μl of 10 mM pNP α-Man was added, and the mixture was incubated at 37°C for 5 min. The residual enzyme activity is expressed as a percentage of that of the activated enzyme. The inset shows a plot of the relative hydrolysis rates for 6.25 mM EDTA.

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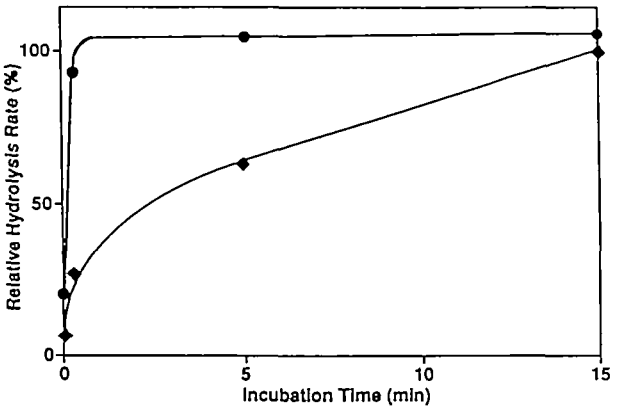

Fig. 8. **Reactivation of the EDTA-inactivated enzyme with Co^{2+}.** To the enzyme activated as described in the legend to Fig. 7 was added 5 μl of 200 mM HEPES buffer, pH 7.0, containing 25 mM EDTA, and then the mixture was incubated at 37°C for 4 h. Then, 5 μl of 200 mM HEPES buffer, pH 7.0, containing 27 mM Co^{2+} and 10 ml of 10 mM pNP α-Man was added, and then the mixture was incubated at 37°C for 5 min (●). As a control experiment, to the purified neutral α-mannosidase (0.3 milliunit in 5 μl) was added 10 μl of 200 mM HEPES buffer, pH 7.0, containing 12.5 mM EDTA and 10 μg of BSA, and the mixture was incubated for 4 h. Then, 10 μl of 200 mM HEPES buffer, pH 7.0, containing 15 mM Co^{2+} and 10 μl of 10 mM pNP α-Man was added, and the mixture was incubated at 37°C for 5 min (◆).

Detection of Cobalt Ions in the Activated α-Mannosidase—The activated α-mannosidase had almost 4 cobalt ions per molecule of the enzyme, while the purified enzyme had about 2 (Table V). Incubation of the Co^{2+}-activated enzyme with EDTA for 4 h removed all the Co^{2+} ions, while the enzyme retained 15% of its activity.

TABLE V. **Cobalt content and activity of neutral α-mannosidase.** The cobalt content of the enzyme purified by gel filtration was measured after treatment with Co^{2+} or EDTA. BSA was treated in the same way as a control. The enzyme activity was measured using pNP α-Man as a substrate.

Sample	Molar ratio (cobalt/enzyme)	Relative activity[a]
Purified enzyme	1.5	11
Co^{2+}-activated enzyme	3.9	100
EDTA-treated enzyme[b]	Not detected	15
BSA	Not detected	–

[a]The activity of the Co^{2+}-activated enzyme was taken as 100%. [b]Co^{2+}-activated and inactivated with EDTA for 4 h.

DISCUSSION

Neutral α-mannosidase was purified from hen oviduct to homogeneity. Its molecular mass was determined to be 480 kDa. This value corresponds to those for neutral α-mannosidases obtained from other sources: human liver, 420 kDa (*15*); bovine liver, 500 kDa (*5*); quail oviduct, 330 kDa (*4*); monkey brain, 230 kDa (*12*); rat brain, 397 kDa (*13*); and rat liver, 372–490 kDa (*11*). SDS-PAGE of the hen neutral α-mannosidase gave a 110-kDa band in the presence of 2-mercaptoethanol (Fig. 2), suggesting a subunit structure of α_4.

The enzyme hydrolyzed certain α1-2, α1-3, and α1-6 linked mannose residues of M9A′, but not two mannose residues linked to the Man α1-3 branch of the trimannosyl core through an α1-2 linkage. The end product from M9A′ on digestion with the activated enzyme was M5B′. These substrate specificities were almost the same as those reported for Co^{2+}-activated neutral/cytosolic α-mannosidases from quail oviduct (*3*), bovine liver (*5*), rat liver (*8*), human liver (crude enzyme) (*10*), and rat liver (*9*). The activated hen neutral α-mannosidase hydrolyzed sugar chains with a single GlcNAc residue at their reducing end four times faster than corresponding sugar chains with two GlcNAc residues (Table III). This is similar to the results reported for quail and bovine neutral α-mannosidases (*3–5*). The enzyme activity was enhanced 24-fold on incubation with Co^{2+}, which is also comparable to the activation levels for the quail and bovine neutral α-mannosidases. These three characteristics, *i.e.* substrate specificity for mannose residues, substrate specificity for the reducing end structure, and activation by Co^{2+}, thus seem to be common to neutral α-mannosidases in addition to a pH optimum of around 7.

Faster activation was achieved at a higher concentration of Co^{2+} (Fig. 5) and at a higher pH (data not shown). The rate of inactivation was not affected by the concentration of a chelating reagent or by the reagent used, and obeyed first-order reaction kinetics (Fig. 7, inset), indicating that the inactivation was the result of a conformational change of the enzyme. The loss of activity by the enzyme from bovine liver resulting from EDTA addition (*5*) was faster than that by the enzyme from hen oviduct. The activated neutral α-mannosidase contained 4 Co^{2+} ions per molecule. The Co^{2+} ion already bound to the purified neutral α-mannosidase seems to be insufficient for its activation. Although Co^{2+} was lost during incubation with EDTA for 4 h, 15% of the enzyme activity was retained (Table V). These results suggest that the rates of Co^{2+} release by chelating reagents are faster than the rate of loss of enzyme activity. Immediate reactivation of the EDTA-treated enzyme was observed when Co^{2+} was added (Fig. 8), which is in contrast to the slower activation of the isolated enzyme (Fig. 5). Considering the increase in the Co^{2+} content of the enzyme from about 2 to 4 per molecule of the enzyme (α_4) on the activation (Table V), and the prompt reactivation of the EDTA-treated enzyme on the addition of Co^{2+} (Fig. 8), the activation of the neutral α-mannosidase appears to depend on the binding of Co^{2+} to specific sites of the enzyme. Our results are not compatible with those of Mathur *et al.* showing the contribution of aminopeptidase activity stimulated by Co^{2+} (*12*). The possibility of such activation due to a protease in the enzyme preparation was investigated. No significant change in the molecular size of hen neutral α-mannosidase after the activation was observed (data not shown), and the protease inhibitors tested did not show any inhibition of the activation by Co^{2+}.

The activated hen α-mannosidase was inhibited strongly by swainsonine and weakly by 1-deoxymannojirimycin. The enzymes from human liver and quail were inhibited by these inhibitors (*4, 10*), and that from rat liver by swainsonine but not 1-deoxymannojirimycin (*8, 11*), indicating that neutral α-mannosidases are sensitive to swainsonine. As the neutral α-mannosidase had specific characteristics found among cellular α-mannosidases, including Co^{2+} activation, and substrate specificity for both mannose and *N*-acetylglucosamine residues, the activated neutral α-

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mannosidase is thought to play a role such as the trimming of the mannose residues of oligosaccharides in the cytosol after the sugar chains have been hydrolyzed by cytosolic endo-β-*N*-acetylglucosaminidase.

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REFERENCES

1. Moremen, K.W., Trimble, R.B., and Herscovics, A. (1994) Glycosidases of the asparagine-linked oligosaccharide processing pathway. *Glycobiology* **4**, 113-125
2. Daniel, P.F., Winchester, B., and Warren, C.D. (1994) Mammalian α-mannosidases—multiple forms but a common purpose? *Glycobiology* **4**, 551-566
3. Oku, H. and Hase, S. (1991) Studies on the substrate specificity of neutral α-mannosidase purified from Japanese quail oviduct by using sugar chains from glycoproteins. *J. Biochem.* **110**, 982-989
4. Oku, H., Hase, S., and Ikenaka, T. (1991) Purification and characterization of neutral α-mannosidase that is activated by Co^{2+} from Japanese quail oviduct. *J. Biochem.* **110**, 29-34
5. Kumano, M., Omichi, K., and Hase, S. (1996) Substrate specificity of bovine liver cytosolic neutral α-mannosidase activated by Co^{2+}. *J. Biochem.* **119**, 991-997
6. Nishigaki, M., Muramatsu, T., and Kobata, A. (1974) Endoglycosidases acting on carbohydrate moiety of glycoproteins. Demonstration in mammalian tissue. *Biochem. Biophys. Res. Commun.* **59**, 638-645
7. Tulsiani, D.R.P. and Touster, O. (1987) Substrate specificities of rat kidney lysosomal and cytosolic α-D-mannosidases and effects of swainsonine suggest a role of the cytosolic enzyme in glycoprotein catabolism. *J. Biol. Chem.* **262**, 6506-6514
8. Grard, T., Saint-Pol, A., Haeuw, J.F., Alonso, C., Wieruszeski, J.M., Strecker, G., and Michalski, J.C. (1994) Soluble forms of α-D-mannosidases from rat liver. Separation and characterization of two enzymic forms with different substrate specificities. *Eur. J. Biochem.* **223**, 99-106
9. Haeuw, J.F., Strecker, G., Wieruszeski, J.M., Montreuil, J., and Michalski, J.C. (1991) Substrate specificity of rat liver cytosolic α-D-mannosidase. Novel degradative pathway for oligomannosidic type glycans. *Eur. J. Biochem.* **202**, 1257-1268
10. Al Daher, S., De Gasperi, R., Daniel, P., Hirani, S., Warren, C., and Winchester, B. (1992) Substrate specificity of human liver neutral α-mannosidase. *Biochem. J.* **286**, 47-53
11. Shoup, V.A. and Touster, O. (1976) Purification and characterization of the α-D-mannosidase of rat liver cytosol. *J. Biol. Chem.* **251**, 3845-3852
12. Mathur, R., Panneerselvam, K., and Balasubramanian, A.S. (1988) Co^{2+}-mediated time- and temperature-dependent activation of neutral α-D-mannosidase from monkey brain. *Biochem. J.* **253**, 677-685
13. Zanetta, J.P., Meyer, A., Dontenwill, M., Basset, P., and Vincendon, G. (1982) Rat brain α-mannosidase: purification, properties, and interaction with its antibodies. *J. Neurochem.* **39**, 1601-1606
14. Snaith, S.M. (1977) Multiple α-mannosidase activities in mammalian tissues as shown by metal-ion activation. *Biochem. J.* **163**, 557-564
15. Grabowski, G.A., Ikonne, J.U., and Desnick, R.J. (1980) Comparative physical, kinetic and immunologic properties of the acidic and neutral α-D-mannosidase isozymes from human liver. *Enzyme* **25**, 13-25
16. De Gasperi, R., Al Daher, S., Winchester, B.G., and Warren, C.D. (1992) Substrate specificity of the bovine and feline neutral α-mannosidases. *Biochem. J.* **286**, 55-63
17. Grard, T., Herman, V., Saint-Pol, A., Kmiecik, D., Labiau, O., Mir, A.-M., Alonso, C., Verbert, A., Cacan, R., and Michalski, J.-C. (1996) Oligomannosides or oligosaccharide-lipids as potential substrates for rat liver cytosolic α-D-mannosidase. *Biochem. J.* **316**, 787-792
18. Hudgin, R.L., Pricer, W.E., Ashwell, G., Stockert, R.J., and Morell, A.G. (1974) The isolation and properties of a rabbit liver binding protein specific for asialoglycoproteins. *J. Biol. Chem.* **249**, 5536-5543
19. Kuraya, N. and Hase, S. (1992) Release of *O*-linked sugar chains from glycoproteins with anhydrous hydrazine and pyridylamination of the sugar chains with improved reaction conditions. *J. Biochem.* **112**, 122-126
20. Oku, H., Hase, S., and Ikenaka, T. (1990) Separation of oligomannose-type sugar chains having one to five mannose residues by high-performance liquid chromatography as their pyridylamino derivatives. *Anal. Biochem.* **185**, 331-334
21. Davis, B.J. (1964) Disk electrophoresis. II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121**, 404-427
22. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
23. Suzuki, J., Kondo, A., Kato, I., Hase, S., and Ikenaka, T. (1991) Analysis by high-performance anion-exchange chromatography of component sugars as their fluorescent pyridylamino derivatives. *Agric. Biol. Chem.* **55**, 283-284

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