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Substrate Specificity of Bovine Liver Cytosolic Neutral α-Mannosidase Activated by Co^{2+}

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A cytosolic neutral α-mannosidase was purified from bovine liver. Its molecular weight was found to be 500,000 on gel filtration. The activity of the enzyme toward Manα1-6(Manα1-3)Manα1-6(Manα1-3)Manβ1-4GlcNAc-PA was increased 26-fold by preincubation with 1 mM Co^{2+}. Manα1-6(Manα1-3)Manα1-6(Manα1-3)Manβ1-4GlcNAc was hydrolyzed by the enzyme to Manα1-3Manα1-6(Manα1-3)Manβ1-4GlcNAc, which was further hydrolyzed to Manα1-6(Manα1-3)Manβ1-4GlcNAc. The rate of hydrolysis was 15-fold greater than that of Manα1-6(Manα1-3)Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc. This substrate specificity suggested that the enzyme could be involved in the degradation of oligomannose-type sugar chains with one GlcNAc residue released from glycoproteins by endo-β-*N*-acetylglucosaminidase, and supported a pathway for glycoprotein catabolism *via* oligomannosyl glycans with one GlcNAc residue proposed on the basis of an earlier study on a cytosolic neutral α-mannosidase from Japanese quail oviduct [Oku, H. and Hase, S. (1991) *J. Biochem.* 110, 982-989].

Key words: α-mannosidase, cytosol, oligomannosyl glycan, substrate specificity.

Cytosolic neutral α-mannosidases activated by Co^{2+} in mammalian tissues have been proposed to be involved in glycoprotein catabolism like lysosomal enzymes. α-Mannosidase purified from Japanese quail oviduct displayed a remarkable substrate specificity in that it hydrolyzed oligomannose-type sugar chains with one GlcNAc residue more than ten times faster than the corresponding sugar chains with two GlcNAc residues (*1, 2*). This finding suggested a further physiological role for the enzyme—that is, there appeared to be a metabolic pathway for glycoproteins in the cytosol, in which α-mannosidase is involved in the degradation of oligomannose-type sugar chains with one GlcNAc residue produced from glycoproteins or glycopeptides by endo-β-*N*-acetylglucosaminidase (*1, 2*). Such enzymes, playing the same role, are thought to be widely distributed in the cytosol of animal cells, and to degrade oligomannosyl glycans with one GlcNAc residue in a similar manner. Thus, if cytosolic neutral α-mannosidases in mammalian tissues can be shown to possess a similar substrate specificity to that of the Japanese quail enzyme, the existence of the proposed pathway will be more likely. This paper describes the purification and characterization of a bovine liver cytosolic α-mannosidase activated by Co^{2+} in an attempt to confirm the pathway for glycoprotein catabolism *via* oligomannosyl glycans with one GlcNAc residue in the cytosol.

MATERIALS AND METHODS

Materials—Bovine liver was purchased from Nippon Ham (Tokyo). *p*-Nitrophenyl α-D-mannoside was from Wako Pure Chemicals (Osaka), M5A and M9A from Dionex (Sunnyvale), and M9A′ from BioCarb (Lund, Sweden) (for the sugar chain structures, see Table I). M5A-PA, M9A-PA, M5A′, M5A′-PA, M4C′-PA, M3A′-PA, M3B′-PA, and M3C′-PA were prepared as described previously (*2*).

BCA protein assay reagent was obtained from Pierce (Rockford), a silver stain kit from Bio-Rad (Richmond), and Ultracent-30 from Tosoh (Tokyo). Diaflo membranes, YM-30 and YM-100, and an H1P 100-43 hollow fiber cartridge were from Amicon (Beverly). DEAE-Sephacel, Butyl-Sepharose 4B, Sepharose 4B, Sephacryl S-300, and Mono Q HR 5/5 column were purchased from Pharmacia (Uppsala), an HCA column A-7610 (0.76×10 cm) from Mitsui Toatsu Chem. (Tokyo), a TSK gel sugar AXI column (0.46×15 cm) from Tosoh, and an Asahipak NH2-P column (0.46×5 cm) from Asahi Kasei (Tokyo).

α-Mannosidase Assay—α-Mannosidase activity was measured as follows. A mixture of 40 μl of the enzyme solution and 40 μl of 0.2 M MES buffer, pH 6.5, containing 2 mM $CoCl_2$ and 0.5 mg/ml bovine serum albumin was preincubated at 37°C for 45 min to activate the enzyme. To the solution, 20 μl of 10 mM *p*-nitrophenyl α-mannoside was added and then the mixture was further incubated at 37°C for an appropriate period (5-15 min). The enzymatic reaction was stopped by adding 400 μl of a 2.5% sodium carbonate solution. *p*-Nitrophenol liberated was measured as the absorbance at 400 nm. One unit of α-mannosidase activity was defined as the amount of the enzyme which released 1 μmol of *p*-nitrophenol per min at 37°C.

Protein Assay—Protein was assayed using the BCA

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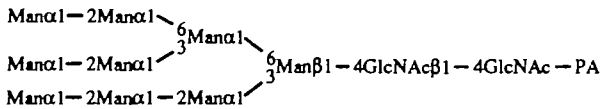
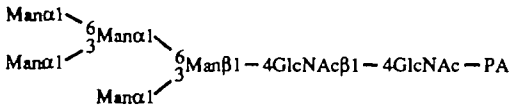
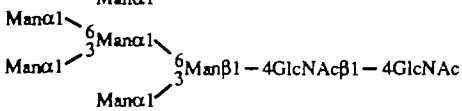
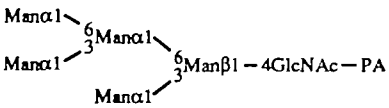
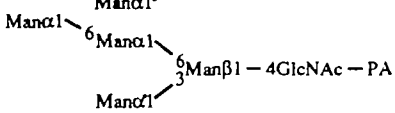
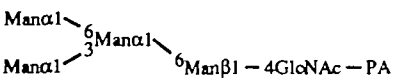
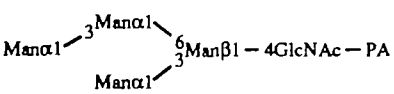
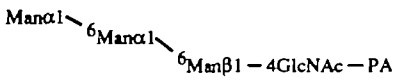
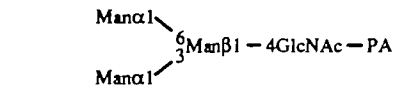
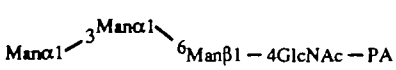
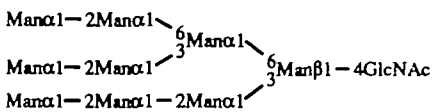
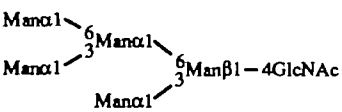
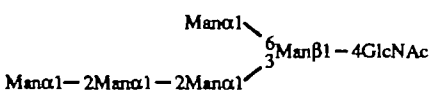
Abbreviations: EDTA, ethylenediaminetetraacetate; GlcNAc, *N*-acetyl-D-glucosamine; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Man, D-mannose; MES, 2-(*N*-morpholino)-ethanesulfonic acid; PA-, pyridylamino; PAGE, polyacrylamide gel electrophoresis. For sugar chain abbreviations, see Table I.

TABLE I. **Sugar chain structures and abbreviations.**

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

protein assay reagent with bovine serum albumin as a standard.

Purification of Cytosolic Neutral α-Mannosidase—Step 1. Preparation of a crude enzyme solution: Bovine liver tissue (5.4 kg) was homogenized in 16 liters of 50 mM HEPES buffer, pH 7.4, containing 0.25 M sucrose with a Polytron homogenizer. The homogenate was centrifuged at 28,000×*g* for 30 min, and the resultant supernatant was used as a crude enzyme solution.

Step 2. Ammonium sulfate precipitation: The precipitate with ammonium sulfate at 30% saturation was dissolved in 4 liters of 50 mM phosphate buffer, pH 7.4, and then dialyzed against 10 mM phosphate buffer, pH 6.8.

Step 3. DEAE-Sephacel chromatography: One-fifth of the above dialysate (800 ml) was applied to a DEAE-Sephacel column (4.6×50 cm) equilibrated with 10 mM phosphate buffer, pH 6.8, and the column was washed with 2.7 liters of the same buffer. Cytosolic α-mannosidase was

eluted with a linear gradient of NaCl, from 0 to 0.4 M (6 liters). The α-mannosidase fraction was concentrated to 2.3 liters by ultrafiltration on the H1P 100-43 hollow fiber cartridge. Sodium phosphate and ammonium sulfate were added to the solution to 50 mM and 1 M, respectively.

Step 4. Butyl-Sepharose 4B chromatography: The α-mannosidase fraction from Step 3 was applied to a Butyl-Sepharose 4B column (4.6×26 cm) equilibrated with 50 mM phosphate buffer, pH 7.0, containing 1 M ammonium sulfate, and the column was washed with 720 ml of the buffer. Cytosolic α-mannosidase was eluted with a linear gradient of ammonium sulfate, from 1 to 0 M, and phosphate, from 50 to 10 mM (5 liters). The α-mannosidase fraction was pooled and dialyzed against 10 mM phosphate buffer, pH 7.0. The dialysate was concentrated to 76 ml by ultrafiltration on a YM-30 Diaflo membrane, and then Triton X-100 was added to a concentration of 0.05% (w/v).

Step 5. Sephacryl S-300 gel filtration: One-half of the cytosolic α-mannosidase fraction obtained in Step 4 was applied to a Sephacryl S-300 column (3.2×190 cm) equilibrated with 10 mM HEPES buffer, pH 7.3, containing 0.3 M NaCl and 0.05% (w/v) Triton X-100, and then eluted with the same buffer. The cytosolic α-mannosidase fraction was pooled, and then dialyzed against 10 mM phosphate buffer, pH 7.0, containing 0.05% (w/v) Triton X-100.

Step 6. Mannan-Sepharose 4B chromatography: The dialysate from Step 5 (165 ml) was applied to a Mannan-Sepharose 4B column (2.6×13 cm), equilibrated with 10 mM phosphate buffer, pH 7.0, containing 0.05% (w/v) Triton X-100, and the column was washed with 600 ml of the same buffer. Cytosolic α-mannosidase was then eluted with a linear gradient of NaCl, from 0 to 0.4 M (1 liter). The cytosolic α-mannosidase fractions were pooled, and then dialyzed against 10 mM phosphate buffer, pH 7.0, containing 0.05% (w/v) Triton X-100. The dialysate was concentrated to 8.8 ml with a YM-100 Diaflo membrane.

Step 7. Hydroxyapatite chromatography: Hydroxyapatite chromatography was carried out on an HCA column A-7610 (0.76×10 cm) using a fast protein liquid chromatography system (Pharmacia). The column was equilibrated with 10 mM phosphate buffer, pH 7.0, containing 0.05% (w/v) Triton X-100. After injecting the cytosolic α-mannosidase fraction obtained in Step 6, the column was washed with 16 ml of the same buffer, and then the concentration of the phosphate buffer was increased to 180 mM in 30 min at the flow rate of 1 ml/min. The cytosolic α-mannosidase fraction was dialyzed against 10 mM HEPES buffer, pH 7.5, containing 0.05% (w/v) Triton X-100.

Step 8. Mono Q chromatography: Anion-exchange chromatography was performed on a Mono Q HR 5/5 column (0.5×5 cm). The column was equilibrated with 10 mM HEPES buffer, pH 7.5, containing 0.05% (w/v) Triton X-100, at the flow rate of 0.5 ml/min. The dialysate (14.4 ml) obtained in Step 7 was injected onto the column, which was washed with 5.5 ml of the same buffer, and then the concentration of NaCl was linearly increased from 0 to 0.6 M in 84 min. The cytosolic α-mannosidase fraction was pooled, and kept in the presence of 10% (w/v) ethylene glycol at 4°C.

Polyacrylamide Gel Electrophoresis (PAGE)—Electrophoresis was carried out on a 5% polyacrylamide gel, pH 8.9, by the method of Davis (*3*). The gel was stained with the silver stain kit.

Molecular Weight Measurement of the Purified Enzyme—The molecular weight of the enzyme was estimated by gel filtration. Gel filtration on a Sephacryl S-300 column was performed as described above. Thyroglobulin (MW 669,000), ferritin (MW 440,000), catalase (MW 232,000), and aldolase (MW 158,000) were used as standards.

pH Dependence of the Enzyme Activity—A mixture of 15 μl of the enzyme solution (0.03 unit/ml) and 5 μl of 0.2 M MES buffer, pH 6.5, containing 4 mM $CoCl_2$ and 0.5 mg/ml bovine serum albumin was preincubated at 37°C for 45 min. To the solution, 10 μl of 10 mM *p*-nitrophenyl α-mannoside and 20 μl of 0.2 M MES buffer (pH 4.0-7.0) or 0.2 M HEPES buffer (pH 6.5-9.0) were added, and then the enzyme activity was measured as described above.

Measurement of the Rates of Hydrolysis for Oligomannose-Type Sugar Chains—A mixture of 10 μl of the enzyme solution (1 milliunit) and 10 μl of 0.2 M MES buffer, pH 6.5, containing 2 mM $CoCl_2$ and 0.5 mg/ml bovine serum albumin was preincubated at 37°C for 45 min. To this solution, 10 μl of a substrate solution (1 nmol of sugar chain) was added and then the mixture was incubated at 37°C. The enzymatic reaction was stopped by heating at 100°C for 3 min at the stage where more than 75% of the substrate remained intact. In the cases of the fluorogenic substrates, the products in the digests were directly analyzed by size-fractionation HPLC. For the substrates exhibiting no fluorescence, pyridylamination (*4*) of the digests were carried out prior to HPLC analysis.

HPLC—Size-fractionation HPLC of the α-mannosidase digest of oligomannose-type sugar chains was carried out on an Asahipak NH2-P column (0.46×5.0 cm). Two eluents, A and B, were used: Eluent A was a mixture of acetic acid, water and acetonitrile (3 : 800 : 200, v/v/v), titrated to pH 7.0 with 6 M ammonium hydroxide; and Eluent B was a mixture of acetic acid, water, and acetonitrile (3 : 70 : 930, v/v/v), whose pH was adjusted to 7.0 with 12% ammonia water. The elution was monitored by measuring the fluorescence at 380 nm (excitation at 310 nm). Isocratic elution was performed with 65, 71, or 75% of Eluent B. Gradient elution was carried out as follows: The column was equilibrated with 95% of Eluent B. After injection of a sample, the percentage of Eluent B was decreased linearly to 67% in 1 min, and then decreased linearly to 46% in 19 min. The flow rate was 0.6 ml/min.

Anion-exchange HPLC was carried out on a TSKgel sugar AXI column (0.46×15 cm) as described previously (*5*). Elution was performed with a mixture of 0.7 M boric acid-KOH buffer, pH 9.0, and acetonitrile (9 : 1, v/v) at the flow rate of 0.2 ml/min at 65°C. The elution was monitored in the same way as for size-fractionation HPLC.

RESULTS AND DISCUSSION

Purification of Cytosolic α-Mannosidase Activated by Co^{2+}—Cytosolic α-mannosidase was purified from bovine liver as described under "MATERIALS AND METHODS" (Fig. 1). Chromatographies on Sephacryl S-300, Mannan Sepharose 4B, hydroxyapatite, and Mono Q columns were carried out with 0.05% Triton X-100, as the enzyme seemed to be too hydrophobic to aggregate with other proteins. The smaller α-mannosidase fraction obtained on Sephacryl S-300 chromatography was collected as cytosolic neutral α-mannosidase considering that the molecular

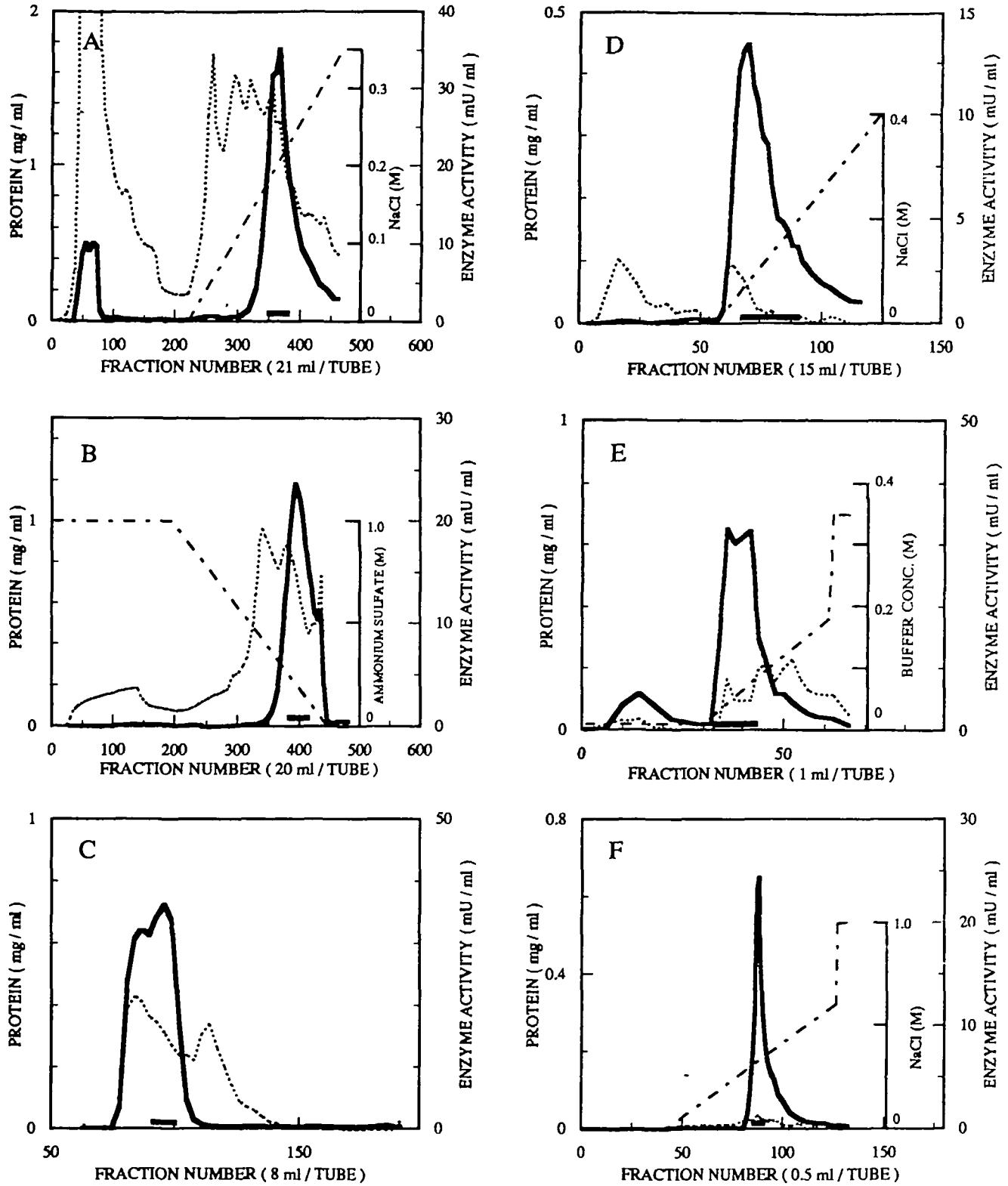


Fig. 1. **Purification of cytosolic α-mannosidase from bovine liver.** Chromatographies were carried out as described under "MATERIALS AND METHODS." (·····) protein; (——) enzyme activity. The fractions indicated by bars were collected for further purification. (A) DEAE-Sephacel chromatography of the precipitate on 30% saturation with ammonium sulfate. (B) Butyl-Sepharose 4B chromatography of the α-mannosidase fraction obtained on DEAE-Sephacel chromatography. (C) Sephacryl S-300 gel filtration of the α-mannosidase fraction obtained on Butyl-Sepharose 4B chromatography. (D) Mannan-Sepharose 4B chromatography of the α-mannosidase fraction obtained on Sephacryl S-300 gel filtration. (E) Hydroxyapatite chromatography of the α-mannosidase fraction obtained on Mannan-Sepharose 4B chromatography. (F) Mono Q chromatography of the α-mannosidase fraction obtained on hydroxyapatite chromatography.

weight of the Japanese quail oviduct enzyme is 330,000 (*1*) (Fig. 1C). The α-mannosidase fraction obtained on Mono Q chromatography gave a major broad band and a band at the gel top, which was probably due to aggregation, on PAGE (Fig. 2). However, the rather broad band was coincided with the α-mannosidase activities for *p*-nitrophenyl α-mannoside and M5A′-PA, and the other bands did not contain α-mannosidase activity. We thus considered that the enzyme preparation was usable for studies on its substrate specificity. The results of the enzyme purification are summarized in Table II. Although the yield of the purified enzyme was low, the existence of several enzymes hydrolyzing *p*-nitrophenyl α-D-mannoside in the crude enzyme preparation (Fig 1, A, C, and E) and the relative instability of the enzyme resulting in its inactivation during the purification must be taken into consideration. However, measures to increase the yield were not taken, since the amount of enzyme obtained was sufficient to examine its enzymatic properties.

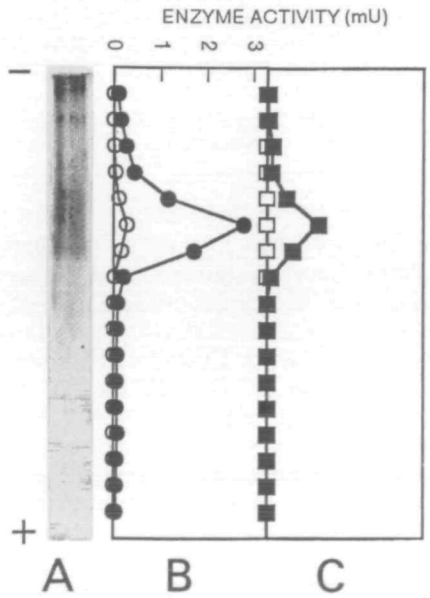


Fig 2. **Polyacrylamide gel electrophoresis of the purified α-mannosidase.** The purified enzyme was applied on two lanes, which were separated after electrophoresis One lane was stained with a silver stain kit, and the other was sliced into 3.5-mm-wide sections and the enzyme activity was extracted from each section with 150 μl of 100 mM MES buffer, pH 6.0, at 37°C for 2.5 h The activity was measured as described under "MATERIALS AND METHODS" A· Stained with a silver stain kit. B. Activity for *p*-nitrophenyl α-mannoside with (●) or without (○) addition of 1 mM $CoCl_2$ C: Activity for M5A′-PA with (■) or without (□) addition of 1 mM $CoCl_2$.

Characterization of the Purified α-Mannosidase—The molecular weight of the purified enzyme was found to be 500,000 on Sephacryl S-300 gel filtration, and the optimal pH was 6.0-6.5 when *p*-nitrophenyl α-D-mannoside was used as the substrate. The enzyme was activated on incubation with 1 mM Co^{2+} or Mn^{2+}, but inactivated with Ca^{2+}, Zn^{2+}, and 5 mM EDTA (Table III). The activity increased by preincubation with Co^{2+} fell after treatment with EDTA, indicating that Co^{2+} is closely related to the change in activity. It is noteworthy that the bovine liver enzyme was inactivated by Fe^{2+}, whereas the activity of the Japanese quail enzyme increased 13-fold with the addition of this ion (*1*).

De Gasperi *et al.* characterized the activity of bovine liver cytosolic α-mannosidase and its mode of action on oligomannose-type sugar chains with two GlcNAc residues using a partially purified enzyme preparation (*6*). They found that the activity was unaffected by added Co^{2+} and EDTA, showing that their enzyme was not identical with that purified in the present study.

Substrate Specificity of the Purified α-Mannosidase—In order to examine the substrate specificity of the enzyme, six oligomannose-type sugar chains were incubated with the purified α-mannosidase, and the products in the digests were analyzed by HPLC (Fig. 3). M5A′ was hydrolyzed much faster than M5A, M5A-PA, and M5A′-PA, and M9A′ faster than M9A-PA, indicating that the enzyme was specific to oligomannosyl glycans with one GlcNAc residue.

TABLE III. **Effects of metal cations and EDTA on the enzyme activity.** A mixture of 10 μl of the enzyme solution (0.1 unit/ml) and 10 μl of a 0 2 M MES or 0 2 M cacodylate buffer solution, pH 6 5, containing 2 mM metal cation or 10 mM EDTA was incubated at 37°C for 45 min. To the solution, 10 μl of an M5A′-PA solution (50 pmol) was added After incubation at 37°C for an appropriate period, the reaction mixture was heated at 100°C for 5 min to inactivate the enzyme The amount of product in the digest was determined by size-fractionation HPLC.

Ion or EDTA added	Relative rate of hydrolysis
Control	1[b]
Co^{2+}	26
Mn^{2+}	3 6
Ca^{2+}	1 1
Mg^{2+}	1.1
Fe^{2+}[a]	0.3
Zn^{2+}	0.0
Cu^{2+}[a]	0 0
EDTA	0.4
Co^{2+}/EDTA[c]	4.2

[a]Cacodylate buffer was used [b]The activity without addition of a metal cation was taken as unity. [c]After 45-min preincubation with Co^{2+}, EDTA was added, and then the enzyme activity was measured.

TABLE II. **Summary of cytosolic neutral α-mannosidase purification from bovine liver.**

Purification step	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification (-fold)
Crude enzyme	420,000	1,900	0.0044	100	1
$(NH_4)_2SO_4$	27,000	530	0.020	29	4.5
Precipitate DEAE-Sephacel	2,000	71	0.035	3 8	8.0
Butyl-Sepharose 4B	240	12	0.050	0.64	11
Sephacryl S-300	47	5 7	0.12	0 31	28
Mannan-Sepharose 4B	3.9	2 9	0.75	0.16	170
Hydroxyapatite	0.66	0.39	1 3	0 021	310
Mono Q	0.043	0.24	5.5	0 013	1,200

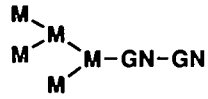
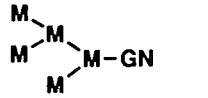
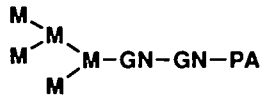
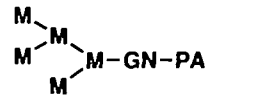
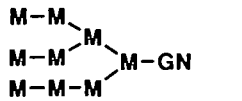
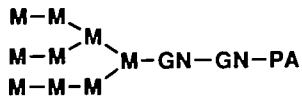
Substrate		Relative rate of hydrolysis
M5A		1.0
M5A'		15
M5A-PA		0.71
M5A'-PA		1.9
M9A'		4.3
M9A-PA		0.22

Fig. 3. **Hydrolysis rates for oligomannose-type sugar chains with the purified enzyme.** The relative rate of hydrolysis is expressed by taking the initial velocity of hydrolysis of M5A as unity.

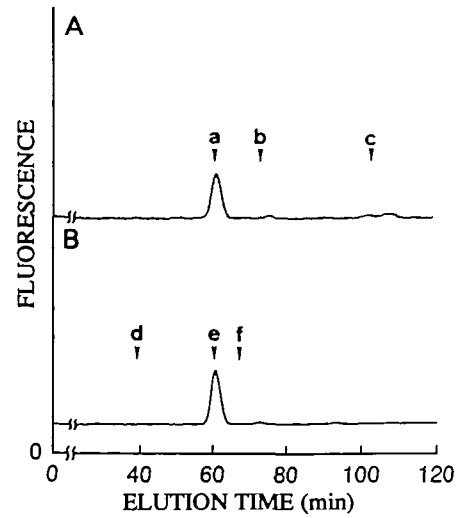


Fig. 4. **Anion-exchange HPLC of the digest of M5A′ with the enzyme.** M5A′ was digested with the enzyme, and then the digest was pyridylaminated. The M3′-PA and M4′-PA fractions were obtained by size-fractionation HPLC. Anion-exchange HPLC on a Sugar AXI column was carried out as described under "MATERIALS AND METHODS." A, M4′-PA fraction; B, M3′-PA fraction. Arrowheads indicate the elution positions of standard PA-sugar chains: a, M4C′-PA; b, M4B′-PA; c, M4A′-PA; d, M3C′-PA; e, M3B′-PA; f, M3A′-PA. For the structures, see Table I.

The course of hydrolysis of M5A′ by the purified α-mannosidase was examined. 1-h and 6-h digests were pyridylaminated, and then analyzed by size-fractionation HPLC. The peaks eluted at the positions of standard M4′-PA (a mixture of M4A′-PA, M4B′-PA, and M4C′-PA) and M3′-PA (a mixture of M3A′-PA, M3B′-PA, and M3C′-PA) were collected, and analyzed by anion-exchange HPLC on a TSKgel Sugar AXI column. M4C′-PA and M3B′-PA were detected (Fig. 4). Thus, M5A′ was hydrolyzed first to M4C′, and then to M3B′. Even though M5A′ was exhaustively digested for 6 h, no sugar smaller than M3′-PA was detected. Thus, M3B′ was an end product. The mode of action of the bovine liver α-mannosidase on M5A′ is similar to the actions of the enzymes from Japanese quail oviduct (*2*) and rat liver (*7*), but not to those from human liver (*8*) and rat kidney (*9*).

There have been several reports on the hydrolysis of oligomannosyl glycans with one or two GlcNAc residues by purified or crude preparations of cytosolic neutral α-mannosidases, as mentioned above (*1, 2, 6-10*), but no data exist on comparison of the rate of hydrolysis by a purified enzyme of a glycan having one GlcNAc residue with one possessing two such residues, except for in the case of the Japanese quail oviduct enzyme (*1, 2*). The substrate specificity of the bovine liver enzyme suggests that it is involved in the degradation of glycans with one GlcNAc residue in the cytosol, and supports the proposed glycoprotein catabolism pathway *via* oligomannosyl glycans with one GlcNAc residue (*2*), coupled with endo-β-*N*-acetylglucosaminidase, such as the one previously described as being cytosolic (*11*). M3B′ was an end product of hydrolysis of M5A′ by the bovine liver enzyme, and hydrolysis of M9A′ and M5A′ by the Japanese quail oviduct enzyme ceased at M5B′ and M3B′, respectively (*2*). The substrate specificities of cytosolic neutral α-mannosidases explain the occurrence of M5B′ in HepG2 and Chinese hamster ovary cell cytosol (*12, 13*), though the role of cytosolic oligomannosyl glycans in cells remains unknown. If it has a certain physiological role, cytosolic neutral α-mannosidase may be involved in another type of protein *N*-glycosylation regulation mechanism rather than in simple catabolic processing of glycoprotein oligomannose-type sugar chains. The bovine liver α-mannosidase is quite similar to the Japanese quail enzyme with respect to its substrate specificity, but dissimilar in molecular weight and the effect of Fe^{2+}. This may be due to the different species or organs.

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