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Purification and Characterization of Endo-β-*N*-Acetylglucosaminidase from Hen Oviduct

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Endo-β-*N*-acetylglucosaminidase from hen oviduct (Endo-HO) was purified to homogeneity by ammonium sulfate fractionation and then by column chromatographies on DEAE-Sephacel, hydroxyapatite, Octyl-Sepharose CL-4B, Co^{2+}-chelating Sepharose FF, and YMC-Pack Diol-200G. Partial purification of the enzyme was reported previously [Tarentino, A.L. and Maley, F. (1976) *J. Biol. Chem.* 251, 6537-6543]. The molecular weight was 54,000 by gel filtration and 52,000 by SDS-PAGE in the presence of 2-mercaptoethanol, indicating that Endo-HO is composed of a single polypeptide chain. The optimum pH was 6.5, and the K_m value was 25 μM when pyridylaminated $Man_6GlcNAc_2$ was used as a substrate. EDTA and metal cations tested, except Hg^{2+}, had no effects on Endo-HO activity. Substrate specificity results using pyridylaminated *N*-linked sugar chains revealed that Endo-HO hydrolyzed oligomannose-type sugar chains faster than complex- and hybrid-type chains, and that sugar chains containing the Manα1-2Manα1-3Manβ1-4GlcNAcβ1-GlcNAc structure were good substrates for the enzyme. These findings suggest that in cytosol the enzyme contributes to the production of a free oligosaccharide with one reducing end *N*-acetylglucosamine residue in cooperation with neutral α-mannosidase, an enzyme that specifically hydrolyzes oligosaccharides to Manα1-2Manα1-2Manα1-3(Manα1-6)Manβ1-4GlcNAc.

Key words: cytosol, endo-β-*N*-acetylglucosaminidase, hen oviduct, oligosaccharide.

The release of free sugar chains during glycoprotein biosynthesis has been reported for pig-liver (*1*), rat-spleen lymphocytes (*2*), hen oviduct (*3*), thyroid (*4*), Chinese hamster ovary cells (*5, 6*), and HepG2 cells (*7*). These free sugar chains, found in cytosol and the ER compartment, are anionic sugar chain-phosphates and neutral sugar chains possessing at their reducing end either di-*N*-acetylchitobiose residues (OS-GN2) or single *N*-acetylglucosamine residues (OS-GN1) (*4-8*). Among them, the major neutral sugar chain found in cytosol is M5B′ (*6, 7*), which seems to be derived from cytosolic OS-GN1 and OS-GN2 by two cytosolic enzymes, neutral α-mannosidase and endo-β-*N*-acetylglucosaminidase. The former has been purified and characterized for Japanese quail oviduct, bovine liver, and rat liver (*9-13*). From the results, it became apparent that OS-GN1 is a better substrate than OS-GN2 for neutral α-mannosidase (*9-13*) and that M5B′ is the end product of M9A′. On the other hand, the presence of cytosolic endo-β-*N*-acetylglucosaminidase in animal tissues has been reported (*14-22*); however, it has not been purified to homogeneity so far, though Tarentino and Maley reported a partial purification and characterization of the enzyme from hen oviduct (*17*). Here, we report the purification and characterization of endo-β-*N*-acetylglucosaminidase from hen oviduct.

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Abbreviations: Endo-D, endo-β-*N*-acetylglucosaminidase D (*Streptococcus pneumoniae*); Endo-H, endo-β-*N*-acetylglucosaminidase H (*Streptomyces griseus*); ER, endoplasmic reticulum; Fuc, L-fucose; Gal, D-galactose; Glc, D-glucose; GlcNAc, *N*-acetyl-D-glucosamine; Man, D-mannose; OS-GN1, *N*-linked sugar chains with a reducing end *N*-acetyl-D-glucosamine residue; OS-GN2, *N*-linked sugar chains with a reducing end di-*N*-acetylchitobiose residue; PA-, pyridylamino; PBS, phosphate-buffered saline; Sia, sialic acid. For abbreviations of sugar chains, see Table I.

MATERIALS AND METHODS

Materials—Hen oviduct was purchased from a poultry farm. Pyridylamino derivatives of sugar chains (PA-sugar chains) were prepared as reported previously (*9, 10*). Columns used were DEAE-Sephacel, Octyl-Sepharose CL-4B, and chelating Sepharose Fast Flow from Pharmacia (Uppsala, Sweden), hydroxyapatite from Wako Pure Chemicals (Osaka), and YMC-Pack Diol-200G (8×500 mm) from YMC (Kyoto). A silver staining kit was purchased from Bio-Rad (Richmond, CA), a Diaflo membrane PM-10 from Amicon (Beverly, MA), and Ultracent-30 from Tosoh (Tokyo). Cosmosil 5C18-P was purchased from Nacalai Tesque (Kyoto) and Shodex NH2P-50 from Showa Denko (Tokyo). Endo-β-*N*-acetylglucosaminidase D (*Streptococcus pneumoniae*) and endo-β-*N*-acetylglucosaminidase H (*Streptomyces griseus*) were purchased from Seikagaku Kogyo (Tokyo). Ser-Asn-M5A was isolated from the pronase digest of Taka-amylase A.

Assay of Enzyme Activity—A reaction mixture containing 190 pmol of M6B-PA, 0.03 mg BSA, and an appropriate amount of enzyme solution in 50 μl of 65 mM sodium citrate buffer, pH 6.5, was incubated at 37°C for 20 min.

The enzymatic reaction was stopped by boiling for 2 min. The amount of PA-GlcNAc released in the reaction mixture was measured by reversed-phase HPLC. One unit was defined as the activity that released 1 nmol of PA-GlcNAc per min at 37°C.

The optimum pH of Endo-HO was measured using M6B-PA as a substrate under the conditions described above, except for the buffers. The buffers used were 0.1 M sodium citrate, pH 4-7 and 0.1 M Tris-HCl, pH 7-8.5.

Digestion of Ser-Asn-M5A and of Taka-amylase A, which has the sugar chain structure of M5A, as substrates was done under the conditions described above, and the sugar chains released (M5A′) were pyridylaminated (*24*). The amount of resultant M5A′-PA was quantified by size-fractionation HPLC.

High-Performance Liquid Chromatography—PA-sugar chains were analyzed by reversed-phase or size-fractionation HPLC. Reversed-phase HPLC was done using a Cosmosil 5C18-P column (4.6×50 mm) eluted with 0.1 M ammonium acetate buffer, pH 4.0, containing 0.05% 1-butanol, at a flow rate of 1.5 ml/min. The elution was monitored by measuring the fluorescence at 400 nm with excitation at 320 nm. Size-fractionation HPLC was done using a Shodex NH2P-50 column (4.6×50 mm). Two eluents were used: Eluent A was a mixture of acetic acid, water, and acetonitrile (3 : 800 : 200, v/v/v), adjusted to pH 7.0 with aqueous ammonia, and Eluent B a mixture of acetic acid, water, and acetonitrile (3 : 70 : 930, v/v/v), adjusted to pH 7.0 with aqueous ammonia. The column was equilibrated with 97% of Eluent B. After injection of a sample, the percentage of Eluent B was decreased linearly to 67 in 1 min, and then to 29 in 24 min. The flow rate was 0.6 ml/min and the elution was monitored by measuring the fluorescence at 380 nm with excitation at 310 nm.

Purification of Endo-HO—All purification procedures were carried out at 4°C. The amount of protein was determined by measuring the absorbance at 280 nm using BSA as a standard.

Step 1. Preparation of the crude extract: Magnum sections (2 kg) of hen oviduct were washed with chilled PBS and frozen with liquid nitrogen. Frozen magnum sections were powdered in a blender, suspended in 6 liters of 50 mM sodium phosphate buffer, pH 7.5, and homogenized in 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 a crude extract.

Step 2. Fractionation with ammonium sulfate: The precipitates formed from the crude extract with ammonium sulfate between 30 and 55% saturation were dissolved in 500 ml of 10 mM sodium phosphate buffer, pH 7.0, and dialyzed against the same buffer.

Step 3. DEAE-Sephacel chromatography: The non-dialyzable fraction (770 ml) was placed on a DEAE-Sephacel column (4.6×45 cm) equilibrated with 10 mM sodium phosphate buffer, pH 7.0, and the column was washed with the same buffer until unadsorbed proteins were removed. The enzyme activity was eluted with 30 mM sodium phosphate buffer, pH 7.0. Ammonium sulfate was added to the pooled fraction until 60% saturation. The precipitates formed were collected, dissolved in 10 mM sodium phosphate buffer, pH 7.0, and dialyzed against the same buffer.

Step 4. Hydroxyapatite chromatography: The pooled fraction obtained in step 3 was applied to a hydroxyapatite column (2.6×17 cm) equilibrated with 10 mM sodium phosphate buffer, pH 7.0, and the column was washed with the same buffer. The enzyme was eluted with a gradient of sodium phosphate from 10 to 100 mM (total elution volume, 800 ml). Ammonium sulfate was added to the pooled fraction to 1.4 M.

Step 5. Octyl-Sepharose chromatography: The solution obtained in step 4 was applied to an Octyl-Sepharose CL-4B column (2.0×8.0 cm) equilibrated with 10 mM sodium phosphate buffer, pH 7.0, containing 1.4 M ammonium sulfate, and the column was washed with the same buffer. The enzyme was eluted with a linear gradient of ammonium sulfate from 1.4 to 0 M (total elution volume, 1,000 ml). The fraction with the enzyme activity was pooled and concentrated to 7.4 ml by ultrafiltration (Diaflo membrane PM-10, Amicon).

Step 6. Co^{2+}-Chelating Sepharose chromatography: The solution obtained in step 5 was applied to a Co^{2+}-chelating Sepharose Fast Flow column (1.0×3.0 cm) equilibrated with 50 mM sodium phosphate buffer, pH 7.0, containing 0.5 M NaCl. The column was washed with 15 ml of the same buffer and then with 15 ml of 50 mM sodium phosphate buffer, pH 7.0, containing 2 M ammonium chloride. The enzyme activity was eluted with 50 mM sodium phosphate buffer, pH 7.0, containing 0.5 M NaCl and 5 mM EDTA.

Step 7: The pooled fraction obtained in step 6 was concentrated to 30 μl by ultrafiltration (Ultracent-30, Tosoh) and applied to a YMC-Pack Diol-200G column (8.0×500 mm) equilibrated with 0.1 M potassium phosphate buffer, pH 7.0, containing 0.2 M NaCl at a flow rate of 0.7 ml/min. The main peak eluted at around 23 min had the enzyme activity and was used as the purified enzyme.

SDS-Polyacrylamide Gel Electrophoresis (PAGE)—SDS-PAGE was carried out by the method of Laemmli (*25*) using a 10% acrylamide gel under reducing conditions with 2-mercaptoethanol. Proteins were stained with a Bio-Rad silver staining kit.

RESULTS AND DISCUSSION

Purification and Characterization of Endo-HO—The results of the purification of endo-HO are summarized in Table II. The enzyme was purified 56,000-fold with a yield of 2.8%. The purified enzyme gave a single band on SDS-PAGE (Fig. 2). Gel filtration on YMC-Pack Diol-200G (Fig. 1E) indicated that the molecular weight of Endo-HO was 54,000, which is consistent with the value of 56,000 reported by Tarentino and Maley (*17*). These values also agree with the value of 52,000 obtained by SDS-PAGE (Fig. 2), indicating a single polypeptide chain. The optimum pH of Endo-HO was 6.5 (Fig. 3) for M6B-PA; Tarentino and Maley reported 5.5 for dansylated glycopeptide (*17*). The purified enzyme was unstable and lost its activity within two to three days at 4°C. However, the inactivation could be prevented by the addition of BSA at a concentration of 1 mg/ml. EDTA and metal cations had no effect on the activity of Endo-HO, with the exception of Hg^{2+}, which was an inhibitor (data not shown). The K_m value for M6B-PA was 25 μM. The enzyme seems to be located in cytosol judging from the fact that most of the enzyme activity was found in the soluble fraction obtained by cell fractionation of hen oviduct (data not shown).

TABLE I. **Structures and abbreviations of sugar chains, and relative rates of hydrolysis.** Hydrolysis was done using PA-derivatives.

Abbreviation	Structure	Relative rate of hydrolysis *
GM9A	Manα1-2Manα1-6 Manα1-2Manα1-3 Manα1-6 Glcα1-3 Manα1-2Manα1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc	0.63
M9A	Manα1-2Manα1-6 Manα1-2Manα1-3 Manα1-6 Manα1-2Manα1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc	0.51
M8A	Manα1-2Manα1-6 Manα1-3 Manα1-6 Manα1-2Manα1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc	0.69
M8C	Manα1-6 Manα1-2Manα1-3 Manα1-6 Manα1-2Manα1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc	0.68
M7A	Manα1-2Manα1-6 Manα1-3 Manα1-6 Manα1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc	0.86
M7B	Manα1-6 Manα1-3 Manα1-6 Manα1-2Manα1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc	0.84
M7C	Manα1-2Manα1-6 Manα1-2Manα1-3 Manα1-6 Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc	0.17
M6B	Manα1-6 Manα1-3 Manα1 Manα1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc	1.0
M5A	Manα1-6 Manα1-3 Manα1-6 Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc	0.25
GM5B	Manα1-6 Glcα1-3 Manα1-2Manα1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc	0.97
M5B	Manα1-6 Manα1-2Manα1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc	0.69
M3A	Manα1-6 Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc	0.13

* The value obtained with M6B was taken as 1.00.

Comparison of the Enzyme Activities of Endo-HO with Those of Endo-D and Endo-H—The substrate specificities of Endo-D and Endo-H from microorganisms (*26-29*) were compared with those of Endo-HO using a PA-sugar chain, glycopeptide, and glycoprotein as substrates (Table III). Endo-HO hydrolyzed M5A-PA as fast as the glycopeptide, whereas Endo-D and Endo-H hydrolyzed M5A-PA much more slowly than the glycopeptide, indicating that the pyranose form of the GlcNAc at the reducing end is not essential to Endo-HO.

Substrate Specificity of Endo-HO—The substrate specificity of Endo-HO was studied using PA-sugar chains. As reported (*17*), Endo-HO hydrolyzed oligomannose-type sugar chains faster than the complex (Com1, Com2, Com3) and hybrid types (Hyb1, Hyb2, Hyb3) (Table I). Tarentino and Maley found that the enzyme hydrolyzed M3F, and that it hydrolyzed M5A faster than M3A, and M3A faster than M6B (*17*). In our case, Endo-HO did not hydrolyze M3F-

TABLE I. (Continued)

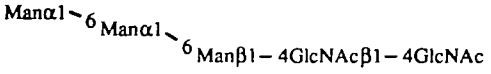
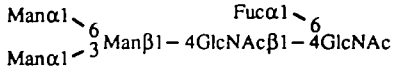
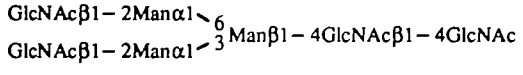
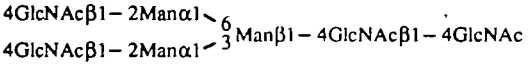
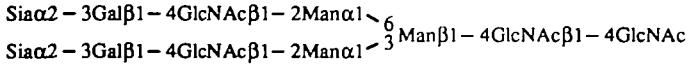
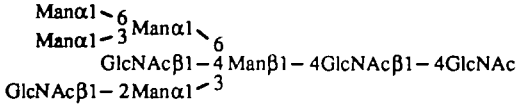
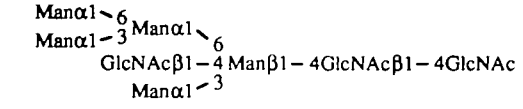
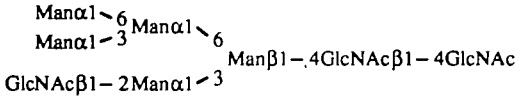
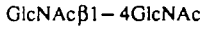
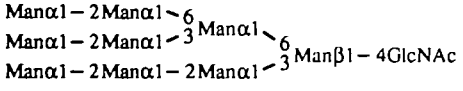
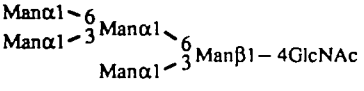
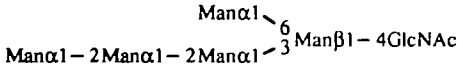
Abbreviation	Structure	Relative rate of hydrolysis *
M3B		< 0.01
M3F		< 0.01
Com1		0.05
Com2		0.03
Com3		< 0.01
Hyb1		0.08
Hyb2		0.03
Hyb3		0.19
GN_2		< 0.01
M9A'		
M5A'		
M5B'		

TABLE II. **Summary of purification of endo-β-*N*-acetylglucosaminidase from hen oviduct.**

Purification step	Protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Recovery (%)	Purification (-fold)
Crude extract	220,000	970	0.0043	100	1
$(NH_4)_2SO_4$	76,000	690	0.0092	72	2
DEAE-Sephacel	10,000	390	0.037	40	9
Hydroxyapatite	130	350	2.7	36	630
Octyl-Sepharose CL-4B	7.1	280	39	29	9,000
Co^{2+}-chelating Sepharose FF	n.d.[a]	110	n.d.[a]	11	n.d.[a]
YMC-Pack Diol-200G	0.11	27	240	2.8	560,000

[a]n.d., not determined.

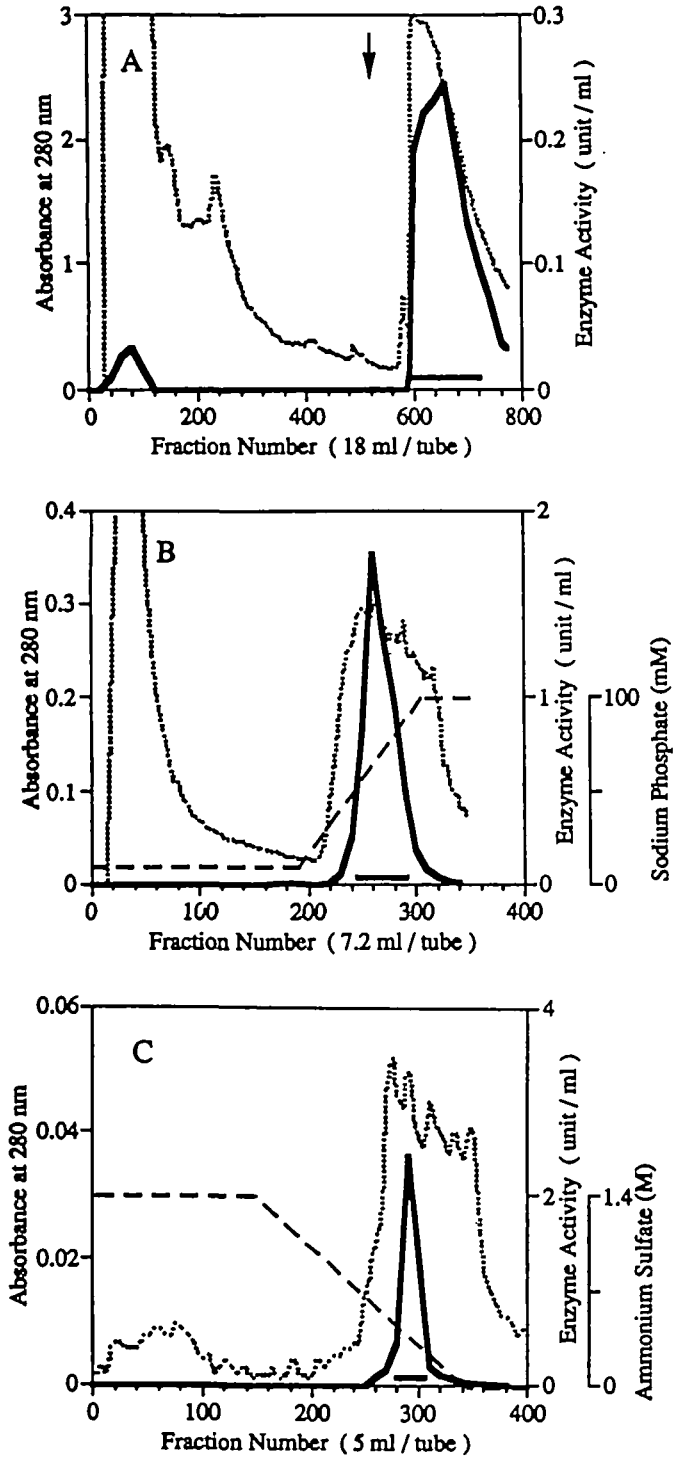


Fig. 1. **Purification of endo-β-*N*-acetylglucosaminidase from hen oviduct.** A: DEAE-Sephacel chromatography of ammonium sulfate precipitates. The column was washed with 30 mM sodium phosphate buffer, pH 7.0, from the position of the arrow. B: Hydroxyapatite chromatography of the pooled fraction indicated by the bar in A. The enzyme was eluted with a linear gradient of 10-100 mM sodium phosphate buffer, pH 7.0 (- - -). C: Octyl-Sepharose CL-4B chromatography of the pooled fraction in B. The enzyme was eluted with a linear gradient of 1.4-0 M ammonium sulfate (- - -). D: Co^{2+}-chelating Sepharose Fast Flow chromatography of the pooled fraction in C. The column was washed with 50 mM sodium phosphate buffer, pH 7.0, containing 2 M NH_4Cl as indicated by arrow a. The eluent was changed to 50 mM sodium phosphate buffer, pH 7.0, containing 5 mM EDTA and 0.5 M NaCl at the position indicated by arrow b. E: gel filtration with YMC-Pack Diol-200G of the pooled fraction in D. The arrows indicate the elution positions of standard proteins; a, bovine serum albumin (M_r 66,000); b, ovalbumin (M_r 45,000); c, chymotrypsinogen A (M_r 25,000). The solid and the dotted lines in the chromatograms show the enzyme activity and the absorbance at 280 nm, respectively, except in E, where the solid line shows the absorbance at 280 nm and the broken line, the enzyme activity.

PA, but it did hydrolyze M6B-PA faster than M5A-PA, and M5A-PA faster than M3A-PA. These disparities might be due to structural differences in the aglycons of the substrates; Tarentino and Maley used ^{14}C-acetylated glycopeptides while we used PA-sugar chains.

The substrate specificities of mammalian neutral endo-β-*N*-acetylglucosaminidases have been reported for rat liver (*14, 21*), and for human skin fibroblasts (*15*) and human tissues (*22*). The enzymes were found in the cytosol fraction and hydrolyzed oligomannose-type sugar chains, but not M3F. These characteristics are consistent with those of Endo-HO. However, the enzyme from rat liver hydrolyzed M3A faster than M5A, and M5A faster than M6B (*14*). The enzyme from human saliva (*23*) is different from Endo-HO in that it does not hydrolyze oligomannose-type sugar chains but complex type.

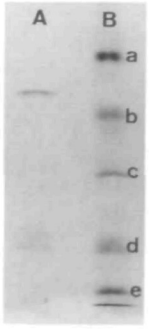


Fig. 2. **SDS-PAGE of Endo-HO.** Lane A: The protein was analyzed using a 10% gel under reducing conditions with 2-mercaptoethanol and stained with a Bio-Rad silver staining kit. Lane B: a, bovine serum albumin (M_r 66,000); b, ovalbumin (M_r 45,000); c, carbonic anhydrase (M_r 31,000); d, trypsin inhibitor (M_r 21,500); e, lysozyme (M_r 14,300).

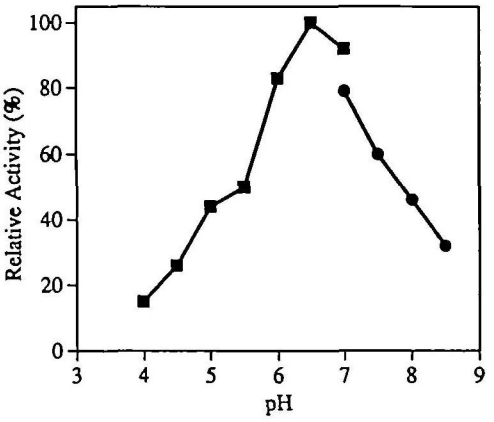


Fig. 3. **pH dependence of Endo-HO.** Enzyme activity was measured using M6B-PA as a substrate. The buffers used were sodium citrate (■) and Tris-HCl (●).

TABLE III. **Comparison of hydrolysis rates of Endo-HO with those of Endo-D and Endo-H.** M and GN are the abbreviations of Man and GlcNAc, respectively. Rate of hydrolysis of M5A-Asn-Ser was taken as unity.

Substrate		Relative rate of hydrolysis		
		Endo-HO	Endo-D	Endo-H
M5A-PA	M-GN-GN-PA	1.0	0.08	0.06
M5A-Asn(Ser)	M-GN-GN-Asn(Ser)	1.0	1.0	1.0
M5A-Protein (Taka-amylase A)	M-GN-GN-Asn-Protein	0.27	0.48	0.60

These substrate specificities of Endo-HO roughly resemble those of bacterial enzymes such as Endo-H, Endo-F, and Endo-D, which hydrolyze oligomannose- and hybrid-type sugar chains, but not complex-type chains. However, they differ slightly: Endo-H and Endo-F require Manα1-3Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc (*30, 31*) and Endo-D requires (Manα1-6)Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc in which the α1-3 branched mannose residue has no substitution at C2 or substitution with GlcNAc at C4 (*31*), while Endo-HO required Manα1-2Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc. This substrate specificity of Endo-HO seems to be suitable for the degradation of the oligosaccharides derived from the precursor, dolichol-pyrophosphate-oligosaccharide, in glycoprotein biosynthesis.

Judging from the presence of M5B′ in cytosol (*6, 7*), it is thought that Endo-HO acts on glycoconjugates or OS-GN2 in cytosol to provide OS-GN1. The following glycoconjugates are considered to be endogeneous substrates for Endo-HO: (i) OS-GN2 released during glycoprotein biosynthesis in the ER and then translocated to cytosol (*32*); (ii) cytosolically oriented lipid intermediates (*33*); (iii) OS-GN2 released from glycoproteins by a peptide:*N*-glycosidase (*34, 35*); (iv) *N*-linked sugar chains of membrane glycoproteins oriented to the cytosol (*36*). Among the oligomannose-type sugar chains tested, good substrates included the Manα1-2Manα1-3Manβ1-4GlcNAcβ structure (Table I). Sugar chains lacking a Manα1-2 residue(s) on the Manα1-3 branch, such as M3B, were poor substrates. The product of Endo-HO seems to be an endogeneous substrate for cytosolic α-mannosidase based on the fact that cytosolic α-mannosidase specifically hydrolyzes OS-GN1 rather than OS-GN2 (*9-13*). Recognition of the Manα1-2Manα1-3Manβ1-4GlcNAcβ structure by Endo-HO indicates that the enzyme is complementary to neutral α-mannosidase, which specifically hydrolyzes mannose residues linked to the Manα1-6 residue of M3B, and leaves mannose residues linked to the Manα1-3 branch intact. Taking these results together, Endo-HO and neutral α-mannosidase may cooperate in cytosol to produce M5B′. M5B′ was detected in hen oviduct cytosol in a preliminary experiment (data not shown).

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