J. Biochem. 125, 70-74 (1999)

Detection of $Man_5GlcNAc$ and Related Free Oligomannosides in the Cytosol Fraction of Hen Oviduct[1]

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Received September 8, 1998; accepted September 28, 1998

The presence of free oligomannosides in cytosol has been demonstrated by metabolic radiolabeling, and Manα1-2Manα1-2Manα1-3(Manα1-6)Manβ1-4GlcNAc was detected as the main free oligomannoside in Chinese hamster ovary cells [Kmiécik, D., Herman, V., Stroop, C.J.M., Michalski, J.C., Mir, A.M., Labiau, O., Verbert, A., and Cacan, R. (1995) *Glycobiology*, 5, 483-494]. In the present paper, the isomeric structures and amounts of oligomannosides in the cytosol fraction of hen oviduct were analyzed by pyridylamination and exoglycosidase digestion. Hen oviduct was used since our group has already characterized the cytosolic neutral α-mannosidase and endo-β-*N*-acetylglucosaminidase obtained from the same source. The amounts of $Man_2GlcNAc$, $Man_3GlcNAc$, $Man_4GlcNAc$, and $Man_5GlcNAc$ were 0.6, 0.6, 0.5, and 0.8 nmol/g tissue, respectively, but $Man_6GlcNAc$-$Man_9GlcNAc$ were not detected. The isomeric structures of the $Man_3GlcNAc$-$Man_5GlcNAc$ found were (Manα1-2)$_{0-2}$Manα1-3(Manα1-6)Manβ1-4GlcNAc, which were compatible with the substrate specificities of cytosolic endo-β-*N*-acetylglucosaminidase and neutral α-mannosidase, indicating that these enzymes participate in the formation of the oligomannosides present in the cytosol.

Key words: cytosol, free oligosaccharides, hen oviduct, oligomannoside.

We recently purified endo-β-*N*-acetylglucosaminidase (*1*) and neutral α-mannosidase (*2*) from hen oviduct and analyzed their substrate specificities. These enzymes are thought to participate in the catabolism of high-mannose type sugar chains and probably produce oligomannosides in the cytosol, because (i) oligomannosides have been found in the cytosol of a number of cultured cells using metabolic radiolabeling of sugar chains (*3-11*), and (ii) Manα1-2Manα1-2Manα1-3(Manα1-6)Manβ1-4GlcNAc (M5B′) is reported to be a major oligomannoside in the cytosol (*9, 11*). The release of free oligosaccharides is associated with glycoprotein synthesis (*7-11*). However, the oligomannosides in the cytosol of hen oviduct have not yet been analyzed. To clarify the contribution of these glycosidases to oligomannoside degradation, in the work reported here we analyzed the molar ratios of free oligomannosides present in the cytosol of hen oviduct by pyridylamination and disclosed the presence of M5B′, the endo product of M9A by digestion with these two enzymes (*1, 2*), together with some smaller oligomannosides.

[1] This study was supported in part by the Research for the Future Program of the Japan Society for the Promotion of Science.

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Abbreviations: GN1, an oligosaccharide terminating with a single GlcNAc residue; GN2, an oligosaccharide terminating with a di-*N*-acetylchitobiose; M2′-M9′, $Man_2GlcNAc$-$Man_9GlcNAc$; PA-, pyridylamino-; PBS, phosphate-buffered saline. Abbreviations for oligosaccharides, see Table I.

MATERIALS AND METHODS

Materials—TSK-Gel HW-40F, a TSK-Gel amide-80 column (4.6×75 mm), and a TSK-Gel sugar AXI column (4.6×150 mm) were purchased from Tosoh (Tokyo), a YMC-Gel Sil S-5 column (4.6×75 mm) from Yamamura Kagaku (Kyoto), Bio-Gel P-2 from Bio-Rad (Hercules, CA), and Dowex 50W-X2 (50-100 mesh) from Dow Chemicals (Midland, MI). α1,2-Mannosidase (*Aspergillus oryzae*) (*12*) was a gift from Dr. H. Yamaguchi (University of Osaka Prefecture). α-Mannosidase (jack bean), β-*N*-acetylhexosaminidase (jack bean), endo-β-*N*-acetylglucosaminidase H (*Streptomyces griseus*), and β-mannosidase (*Acatina fulica*) were obtained from Seikagaku Kogyo (Tokyo). Phenol reagent was obtained from Wako (Osaka), and Dulbecco's modified Eagle medium (D-MEM) and fetal calf serum from Gibco (Gaithersburg, MD).

Preparation of Standard PA-Oligosaccharides—M1′-PA was prepared from α-mannosidase digests of M3B′-PA. M3B-PA was prepared from ovomucoid of Japanese quail (*13*), and agalactoBi-PA from riboflavin-binding protein of hen egg yolk (*14*). Other PA-oligosaccharides were prepared as reported previously (*15, 16*).

Cell Fractionation of Hen Oviduct—Connective tissues of the magnum section of hen oviduct were removed and the rest was cut into pieces with scissors. A part of the material (10 g) was homogenized using a Potter-Elvehjem homogenizer (2-3 strokes at 550 rpm) in 100 ml of 50 mM Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose and 5 mM $MgCl_2$, and the homogenate was centrifuged at 150×*g* for 10 min. The supernatant was fractionated to a nucleous fraction

(Fraction N), mitochondrial fraction (Fraction M), microsomal fraction (Fraction E), and cytosolic fraction (Fraction S) by centrifugation at 700 × *g* for 10 min, 20,000 × *g* for 15 min, and 148,000 × *g* for 60 min. All procedures were carried out below 4°C.

Preparation of Oligosaccharides from Fraction S—Fraction S was heated at 100°C for 5 min and ethanol was then added up to a concentration of 60%. After removal of the precipitates by centrifugation at 15,000 × *g* for 20 min, the supernatant was concentrated and applied onto a Bio-Gel P-2 column (2.5 × 140 cm) equilibrated with 20 mM ammonium acetate. The fraction eluted between the void volume and just before the elution position of sucrose was collected, concentrated, and placed onto a Dowex 50W-X2 (H^+ form) column, and the pass-through fraction was used for the analysis of oligomannosides.

Preparation of PA-Oligosaccharides—Pyridylamination of the oligomannosides was carried out according to the reported methods (*15*). Most excess reagents were removed using a Palstation 1000 system (Takara Biomedicals, Kyoto). The residue was futher purified by gel filtration on a TSK-Gel HW40-F column equilibrated with 0.01 M ammonium acetate buffer, pH 6.0.

High-Performance Liquid Chromatography (HPLC)—A Beckman 344M liquid chromatograph and a Hitachi F-3000 fluorescence spectrophotometer were used. Size-fractionation HPLC was performed on a TSK-Gel amide-80 column with a guard column of YMC-Gel Sil-5 to protect the amide-80 column. The column was equilibrated with acetonitrile:water (85:15, v/v) containing 3% (v/v) acetic acid, pH 7.3, adjusted with triethylamine. The flow rate was 1.0 ml/min. PA-oligosaccharides were detected by fluorescence using an excitation wavelength of 310 nm and an emission wavelength of 380 nm. Reversed-phase HPLC was performed on a Cosmosil 5C18-P column under isocratic conditions using Eluent a or b: Eluent a was 0.1 M ammonium acetate buffer, pH 4.5, containing 0.05 or 0.125% (v/v) 1-butanol; Eluent b was 0.1 M ammonium acetate buffer, pH 6.0, containing 0.05% (v/v) 1-butanol. The flow rate was 1.5 ml/min. PA-oligosaccharides were detected by fluorescence using an excitation wavelength of 320 nm and an emission wavelength of 400 nm. Anion-exchange HPLC was performed on a TSK-Gel sugar AXI column using 0.8 M borate-KOH buffer, pH 9.0, containing 10% (v/v) acetonitrile (*16*). The flow rate was 0.3 ml/min. PA-oligosaccharides were detected by fluorescence using as an excitation wavelength of 310 nm and an emission wavelength of 380 nm.

Exoglycosidase Digestions—PA-oligosaccharides (10-500 pmol) were digested at 37°C for 15 h with an exoglycosidase. Digestions were performed as follows: with α-mannosidase (50 mU) in 10 μl of 0.05 M sodium acetate buffer, pH 4.5; with α1,2-mannosidase (1 mU) in 20 μl of 0.1 M acetate buffer, pH 5.1; with β-mannosidase (1.25 mU) in 10 μl of 0.05 M citrate-phosphate buffer, pH 4.5; with β-galactosidase (5 mU) in 30 μl of 0.07 M citrate-phosphate buffer, pH 5.5; and with β-*N*-acetylhexosaminidase (50 mU) in 10 μl of 0.05 M ammonium acetate buffer, pH 5.0. Enzymatic reactions were terminated by heating at 100°C for 3 min, and a portion of the digest was analyzed by HPLC.

Protein Assay—The protein concentration in each subcellular fraction was assayed by the Lowry method (*17*) with bovine serum albumin as a standard.

Marker Enzyme Assays of Subcellular Fractions—Lysosomal acid phosphatase was assayed by the procedure of Lowry *et al.* (*18*), microsomal glucose-6-phosphatase by the procedure of Swanson (*19*), and cytosolic lactate dehydrogenase by the procedure of Wroblewski and Grace (*20*).

RESULTS AND DISCUSSION

Subcellular Fractionation of Hen Oviduct—The distributions of marker enzymes in the preparations from hen oviduct are shown in Fig. 1. The cytosol fraction, Fraction S, was used in this study. The lysosomal enzyme fractionated mainly into Fraction E, but a part of the enzyme was dispersed into Fraction S. However, degradation of free oligosaccharides in the cytosol fraction caused by lysosomal glycosidase contamination was thought to be negligible because the isolated cytosol fractions were immediately heated at 100°C for 5 min and the proteins were then removed by ethanol precipitation.

Analysis of GN1-Type Oligomannosides in the Cytosol Fraction—Oligomannosides in Fraction S were pyridylaminated and separated by size-fractionation HPLC into six fractions—designated Fractions 2, 3, 4, 5, 6, and 9—corresponding to the elution positions of the authentic standard PA-oligomannosides M2′, M3′, M4′, M5′, M6′-8′, and M9′ (Fig. 2). PA-oligomannosides in Fractions 3, 4, 5, and 9 were further separated by reversed-phase HPLC (Fig. 3). No peak appeared at the elution position of M9A′-PA in the chromatogram of Fraction 9. The detection limit of the analysis was about 10 pmol/g tissue. The peaks corresponding to the standard PA-oligosaccharides were collected as Fractions 3a, 4a, and 5a, and their sugar chain structures were identified as M3B′-PA, M4D′-PA, and

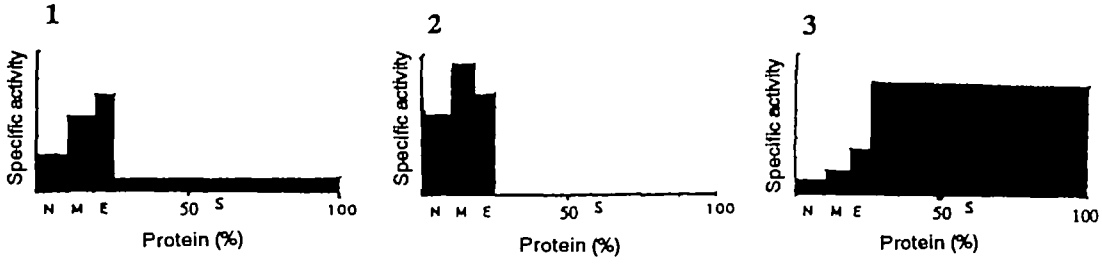


Fig. 1. **Distributions of marker enzymes in fractions obtained from hen oviduct.** Cells were homogenized and fractionated into fractions N, M, E, and S as described in "MATERIALS AND METHODS." 1, acid phosphatase; 2, glucose-6-phosphatase; 3, lactate dehydrogenase.

M5B′-PA, respectively, by anion-exchange HPLC (Fig. 4A and Table II) and α1,2-mannosidase digestion of 5a and 4a (data not shown). These structures were further confirmed by the appearance of a $Man_1GlcNAc$-PA peak after the α-mannosidase digestion (Fig. 4B). $Man_1GlcNAc$-PA was also detected in the α-mannosidase digest of Fraction 2, but not in the digest of Fraction 6 (data not shown). These results indicated the presence of M2′, M3B′, M4D′, and M5B′ in Fraction S. Considering the substrate specificity of lysosomal α-mannosidases, these oligomannosides were not likely to be lysosomal contaminants, because lysosomal enzymes do not produce M5B′ from M8′ or M9A′ (*21-24*). Saint-Pol *et al.* (*25*) reported that M5B′ in the cytosol of HepG2 cells is translocated to a membrane-bound compartment, where the oligosaccharides are decomposed. However, this is contradicted by our finding of relatively high amounts of smaller oligomannosides (Table II). A high M5B′ content in cytosol seems to be common in animal cells, since M5B′ has been detected as a major component in the cytosol of Chinese hamster ovary (*9*) and HepG2 (*11*) cells by metabolic radiolabeling, and we have now confirmed M5B′ as the major oligomannoside in the cytosol of hen oviduct.

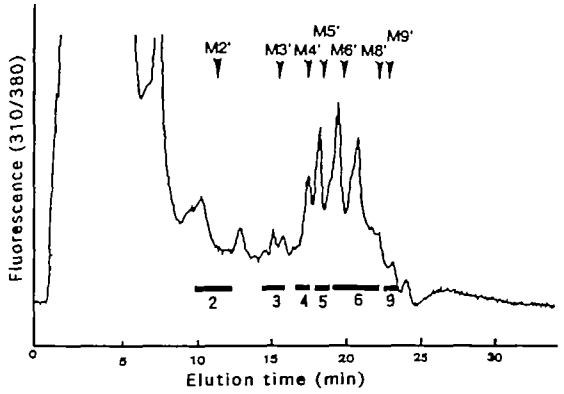


Fig. 2. **Size-fractionation HPLC of PA-oligosaccharides from Fraction S prepared from hen oviduct.** Fractions 2-6 and 9 were collected as indicated by the bars. The arrowheads show the elution positions of standard PA-oligosaccharides.

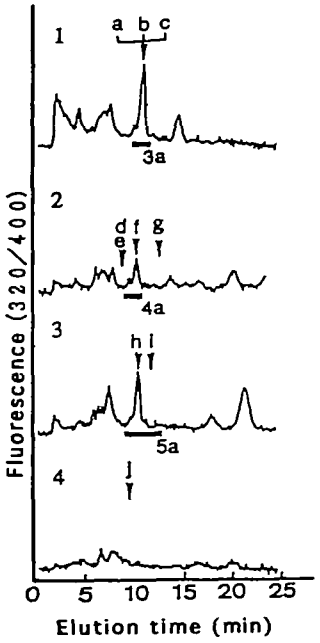


Fig. 3. **Reversed-phase HPLC of Fractions 3, 4, 5, and 9 isolated by size-fractionation HPLC (see Fig. 2).** 1, HPLC of Fraction 3; 2, Fraction 4; 3, Fraction 5; 4, Fraction 9. Fractions 3a, 4a, and 5a, corresponding to the elution positions of authentic PA-oligosaccharides, were collected as indicated by the bars. The arrowheads show the elution positions of standard PA-sugars: a, M3A′-PA; b, M3B′-PA; c, M3C′-PA; d, M4A′-PA; e, M4B′-PA; f, M4D′-PA; g, M4C′-PA; h, M5B′-PA; i, M5A′-PA; j, M9A′-PA.

There have been several reports on the presence of oligomannosides in the cytosol, mostly based on the use of cultured cells including calf thyroid slices (*3, 7*), mouse splenocyte (*4, 5*), human fibroblast (*5*), Chinese hamster ovary (*6, 8, 9*), MDBK (*10*), and HepG2 cells (*11*) cells. Most of these studies were done using metabolic pulse-chase radiolabeling or short-term labeling of less than 1 h with [2-^{3}H]mannose or [2-^{3}H]glucose, which means the oligosaccharides detected were dependent on the chase time. In one study using 2-h labeling of Chinese hamster ovary cells, the major oligomannoside was reported to be M5B′, together with small amounts of M4′ and M6′ (*9*). However, the isomeric structures of oligomannosides other than M5B′ have not hitherto been reported. To detect oligomannosides in the cytosol on a molar basis and to analyze their isomeric structures, we used pyridylamina-

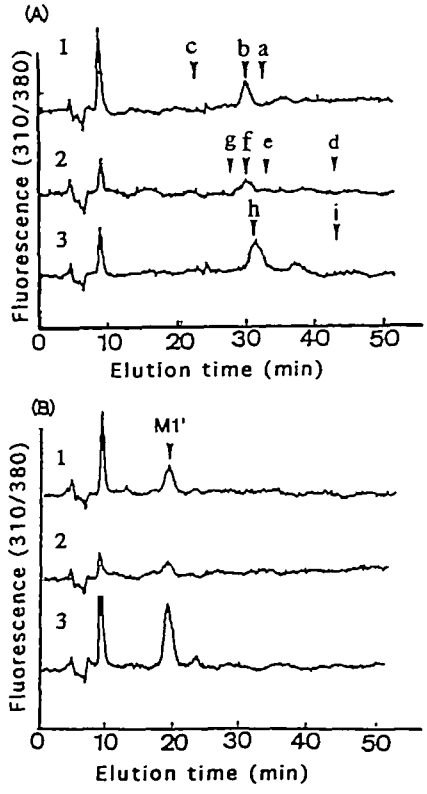


Fig. 4. **Anion exchange HPLC of fractions obtained in Fig. 3.** Fraction 3a, 4a, and 5a were analyzed directly (A) or after α-mannosidase digestion (B) on a sugar AXI column. 1, HPLC of Fraction 3a; 2, Fraction 4a; 3, Fraction 5a. The arrowheads show the positions of standard PA-sugars as in Fig. 3.

TABLE I. **Structures and abbreviations of oligosaccharides.**

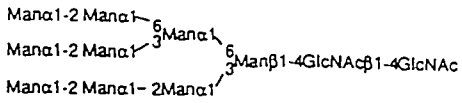
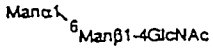
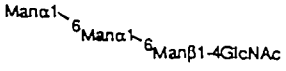
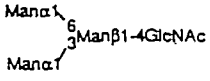
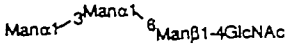
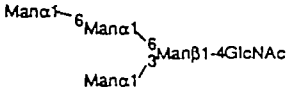
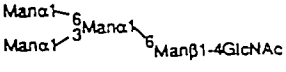
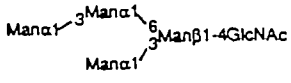
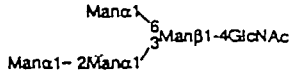
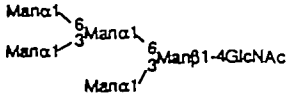
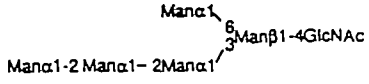
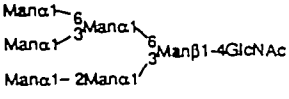
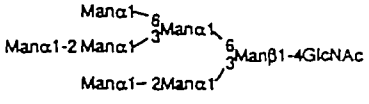
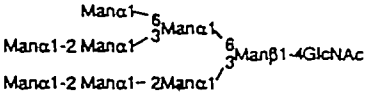
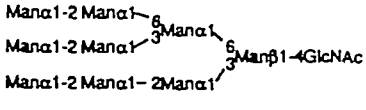
Abbreviation	Structure
M9A	
M2'	
M3A'	
M3B'	
M3C'	
M4A'	
M4B'	
M4C'	
M4D'	
M5A'	
M5B'	
M6B'	
M7'	
M8'	
M9A'	

TABLE II. **GN1-type oligomannosides found in Fraction S.**

Oligosaccharide	Hen oviduct Fraction S (nmol/10 g)
$Man_2GlcNAc$	5.6
M3B′	6.2
M4D′	5.1
M5B′	8.4
$Man_6GlcNAc$	n.d.[a]
$Man_7GlcNAc$	n.d.
$Man_8GlcNAc$	n.d.
M9A′	n.d.

[a]Not detected (less than 0.1 nmol/10 g).

tion. The isomeric structure of M4′ was found to be M4D′, and that of M3′ was M3B′.

From the isomeric structures M5B′, M4D′, and M3B′, we postulate that endo-β-*N*-acetylglucosaminidase participates in the supply of a GN1-type oligosaccharide and cytosolic α-mannosidase in its degradation, because the isomeric structures $(Man\alpha1\text{-}2)_{0-2}Man\alpha1\text{-}3(Man\alpha1\text{-}6)Man\beta1\text{-}4GlcNAc$ detected in the cytosol of hen oviduct are in accord with the substrate specificities of cytosolic α-mannosidase (*2*) and endo β-*N*-acetylglucosaminidase obtained from the same source (*1*). Namely, (i) cytosolic α-mannosidase hydrolyzed GN2-type oligosaccharides faster than GN1-type and formed M5B′ as an endo product in the digest of M9A′ (*2*), and (ii) endo β-*N*-acetylglucosaminidase hydrolyzed preferentially the oligomannosides containing the $Man\alpha1\text{-}2Man\alpha1\text{-}2Man\alpha1\text{-}3(Man\alpha1\text{-}6)Man\beta$ structure (*1*). A high content of M5B′ of several cells might be explained by the presence of both enzymes in the cytosol.

Oligomannosides in Fractions M and E were analyzed for comparison with those in the cytosol fraction. $Man_2GlcNAc$ and $Man_3GlcNAc$ were detected in Fraction M at roughly 73 and 22 pmol/g tissue, respectively. These oligomannosides are considered to be derived from lysosome. Little oligomannoside was detected in Fraction E, and the amount of $Man_1GlcNAc$ produced by α-mannosidase digestion was about 1/5th of that of Fraction M. These findings further confirm that there was little if any contamination of Fraction S by lysosomal oligomannosides.

Analysis of Total GN2-Type Oligomannosides in Cytosol Fractions—As GN2-type oligomannosides have been detected by radiolabeling in cultured cells (*3–11*), the total amounts of GN2- and GN1-type oligomannosides in the cytosol fraction were determined as follows. PA-oligosaccharides prepared from Fraction S were digested with α-mannosidase. The resulting end-products, $Man_1GlcNAc_2$-PA and $Man_1GlcNAc$-PA, were quantified as GN2-type and GN1-type oligomannosides, respectively, by reversed-phase HPLC after purification by size-fractionation HPLC. The amount of total GN2-type oligomannosides in Fraction S was 8.6 nmol/10 g hen oviduct. This was about 50% of that of the GN1-type. Metabolic radiolabeling showed that GN2-type oligomannosides were converted to GN1-type in less than 40 min (*9, 11*). The conversion appears to be performed by an endo-β-*N*-acetylglucosaminidase.

In conclusion, in this paper we have quantified the amounts of oligomannosides on a molar basis and analyzed the isomeric structures of M3′-M5′. The presence of oligomannosides in the cytosol of hen oviduct was thus

demonstrated, but their biological significance is still speculative (*9, 10, 26*).

The authors thank Dr. H. Yamaguchi for his generous gift of α1,2-mannosidase.

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