Free Sialooligosaccharides Found in the Unfertilized Eggs of a Freshwater Trout, _Plecoglossus altivelis_

A LARGE STORAGE POOL OF COMPLEX-TYPE BI-, TRI-, AND TETRAANTENNARY SIALOOLIGOSACCHARIDES*

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Unfertilized eggs of _Plecoglossus altivelis_ (a kind of freshwater trout, "Ayu" in Japanese) were found to contain a relatively large pool of three sialooligosaccharides. These free oligosaccharides were accumulated in the cytoplasm up to concentrations of about 35 ng per egg, which correspond to about 107 µg/g. Their structures were determined by a combination of chemical (composition and methylation analysis and Smith degradation) and instrumental (fast atom bombardment-mass spectrometry, secondary ion mass spectrometry, and 400-MHz 1H NMR) analyses. The structures established represent a typical type of bi-, tri-, and tetraantennary sialooligosaccharides all of which end with di-N-acetylchitobiose structure (-GlcNAcβ1-4GlcNAc) and α-2,3-linked NeuAc at reducing and nonreducing termini, respectively. These data show that these free sialooligosaccharides, which were originally protein-linked components, must be released during oogenesis. Although a specific functional requirement for their release is not known, the occurrence of such free sialooligosaccharides in normal animal cells or tissues has not been previously reported.

Inoue and Iwasaki (1) were the first who showed the presence of polysialoglycoproteins (PSGP, 1 M, 200,000) in rainbow trout eggs. PSGP represent a new class of glycoproteins which contain oligo(poly)sialyl groups (1). PSGP seems to be of general occurrence in all the salmonid fishes so far investigated (2). Whether PSGP plays a physiological role is at present an open question. However, we have already some important clues to answer this question: (a) PSGP is a major component of the cortical vesicles (alveoli) of the unfertilized eggs (3) and (b) 200-kDa PSGP was found to undergo proteolytic depolymerization to 9-kDa PSGP, the repeating unit of 200-kDa PSGP, upon fertilization or parthenogenetic activation (4, 6). Thus, we feel that it would be also challenging to study which macromolecules substitute for PSGP in other families of fishes, which are devoid of PSGP in their unfertilized eggs. We have thus undertaken a study to search for such macromolecules in the unfertilized eggs of _Plecoglossus altivelis_ (a freshwater trout, "Ayu" in Japanese) which is phylogenetically close to _Salmonidae_. We have found the presence of cortical alveolus-derived sialoglycoproteins, which can be regarded as functionally the same glycoprotein as PSGP by several criteria. During such studies we have also found that _P. altivelis_ eggs contain relatively large amounts of free sialooligosaccharides, which are accumulated in the cytoplasm. This was unexpected since free oligosaccharides are found only rarely in animal tissues and in general found in patients suffering from genetic lysosomal disorders of complex carbohydrate metabolism (6). The occurrence of free sialooligosaccharides in normal animal cells has not previously been reported, although it is known that, in sialidosis and I-cell disease, there is evidence for a defective catabolism of sialic acid-containing compounds, such as accumulation in fibroblasts and leucocytes and an excessive urinary excretion of sialooligosaccharides (7). The free sialooligosaccharides found in _P. altivelis_ eggs have been proven to be a family of typical complex-type oligosaccharide chains that are commonly found in glycoproteins. In this paper we report the results of the structural analysis of the free sialooligosaccharides and their intracellular localization. The present unprecedented findings would be of interest because they could provide some insight into the basic problem in the pathways and mechanisms by which carbohydrate is added to and removed from glycoproteins during intracellular transport.

MATERIALS AND METHODS

RESULTS

Isolation of Free Sialooligosaccharides

The crude glycoprotein material (3.28 g) obtained from the aqueous phase after phenol treatment of the extract of the unfertilized eggs (562 g in wet weight) of _P. altivelis_ was subjected to ion-exchange chromatography on DEAE-Sephadex A-25. The elution profiles monitored by the phenolsulfuric acid method and the resorcinol method are shown in Fig. 1. Three discrete free sialooligosaccharide fractions (denoted as A, B, and C) were found. The "Materials and Methods" are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
FIG. 1. Isolation of free sialooligosaccharides by anion exchange chromatography on DEAE-Sephadex A-25 of the crude extract obtained from unfertilized eggs (526 g) of *P. altivelis*. Column size, 2.6 × 70 cm. The column was first washed with 400 ml of equilibration buffer, 0.01 M Tris-HCl (pH 8.0), then eluted with a linear gradient (−−−) of NaCl from 0 M (2 liters) to 0.7 M (2 liters). 15-m1 fractions were collected, monitored by the resorcinol method, and pooled as indicated with the bars. SGT, sialoglycoproteins. Elution profile obtained by the phenol-sulfuric acid method is included in the inset.

TABLE I

| Sample | Man | Gal | GlcNAc | GlcNAcol | NeuAc |
|--------|-----|-----|--------|----------|-------|
| A-1    | 3.0 | 1.9 | 3.8    | 0.9      | 2.2   |
| A-1-ol | 3.0 | 2.0 | 2.8    | 0.9      | 2.2   |
| Asialo A-1 | 3.0 | 2.0 | 3.9    |          |       |
| A-2    | 3.0 | 2.2 | 5.2    | 0.9      | 2.7   |
| A-2-ol | 3.0 | 3.0 | 3.8    | 0.9      | 2.7   |
| Asialo A-2 | 3.0 | 3.0 | 5.0    |          |       |
| A-3    | 3.0 | 4.2 | 6.2    | 0.9      | 4.4   |
| A-3-ol | 3.0 | 4.1 | 5.1    | 0.9      | 4.4   |
| Asialo A-3 | 3.0 | 3.7 | 5.8    |          |       |

void of amino acid residues), labeled A-1, A-2, and A-3 were eluted ahead of sialoligocarbohydrate. The yields of the isolated fractions were 38.2 mg (A-1), 16.6 mg (A-2), and 4.7 mg (A-3). The actual amounts may even be higher, because at the initial steps of the isolation the egg extract was diazyl against water to remove phenol so that small amounts of these oligosaccharides possibly leaked out of the cellophane bag. Each of the purified material of A-1, A-2, and A-3 appeared as a single spot in the thin-layer system (Silica Gel 60 plate; 1-propanol/concentrated NH₄OH/H₂O, 6:1:2.5 by volume; detection, resorcinol spray). At present we have not succeeded in separating A-2A and A-2B even after high-performance liquid chromatography analysis.

**Structural Elucidation of Sialooligosaccharides**

**Carbohydrate Composition**—Results of carbohydrate analysis are given in Table I for A-1, A-2, and A-3 together with those for the corresponding oligosaccharide alditols obtained by reduction with sodium borohydride. Table I shows that each oligosaccharide yielded 1 mol each of GlcNAcol at the expense of GlcNAc when treated with NaBH₄, and therefore represents the presence of the GlcNAc residue at the reducing end of the oligosaccharide.

The carbohydrate compositions of A-1, A-2, and A-3 are typical for complex oligosaccharides of the di-, tri-, and tetraantennary types with 2, 3, and 4 NeuAc residues/3 mannose residues, respectively.

**FAB-MS and SIMS Spectra**—The SIMS spectrum of asialo A-1 is reproduced in Fig. 2A. The spectrum showed a prominent pseudo-molecular ion (M + Na)⁺ at m/z 1663, which is consistent with molecular weight of 1640 estimated from the data based on composition analysis for asialo A-1 (Gal/MAN/GlcNAc = 2:3:4). There are also significant peaks at m/z 1484, 1442, 1118, 931, and 899. These observed masses are in agreement with the indicated formula shown in Fig. 2A, and all of these data are consistent with the assignment of the monosaccharide sequence to the asialo A-1:

Gal-GlcNAc-Man

Gal-GlcNAc-GlcNAc

Demonstrating the presence of a GlcNAc-GlcNAc unit at the reducing end instead of a single GlcNAc residue.

Similarly, the FAB-MS spectrum of asialo A-2 (Fig. 2B) exhibits an intense ion at m/z 2029 which is supposed to be (M + Na)⁺ but ions in this mass range are actually observed as (M + Na + 1)⁺ due to isotope effect. This pseudo-molecular ion is in good agreement with the molecular weight for asialo A-2 (Mr = 2055) estimated on the basis of the composition analysis (Gal/MAN/GlcNAc = 3:3:5), but the positive ion FAB-MS spectrum otherwise provides little structural information because it showed predominantly pseudomolecular ion and no fragment ions (Fig. 2B). Nevertheless, the FAB-MS data and the results of sugar analysis suggest that A-2 has a triantennary sialooligosaccharide structure having a di-N-acetyltchitobiosyl unit at the reducing end.

**400-MHz 1H NMR Spectra**—The 400-MHz 1H NMR spectrum of A-1, as shown in Fig. 3A, indicated the presence of α-2,3-linked NeuAc as is evident from the chemical shift values, 1.78 and 2.76 ppm, assignable to H-3α and H-3β, respectively, and both of the 2 NeuAc residues in A-1 were found to be α-2,3-linked by methylation analysis (see below). By detailed comparison of diagnostic proton resonances of A-1 with those reported for some oligosaccharide chains of biantennary type (22, 23), the structure of A-1 is proposed to be identical with that included in Fig. 3A. A-1 exhibits resonance peaks at 2.03 and 2.07 ppm in a 5:1 ratio. The first peak is attributed to the five N-acetyl methyl signals of the 2 NeuAc residues and 3
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**FIG. 2.** A, positive ion SIMS spectrum of asialo A-1. The structure and masses of the proposed fragment ions are also shown. B, positive mode FAB-MS spectrum of triantennary sialooligosaccharides (asialo A-2). The peaks at *m/z* 1663 (panel A) and *m/z* 2029 (panel B) correspond to (M + Na)<sup>+</sup> and (M + Na + I)<sup>+</sup> ions, respectively.

GlcNAc residues (GlcNAc-1, GlcNAc-5, and GlcNAc-5'), and the other peak (92.07 ppm) can be assigned to the N-acetyl-methyl proton of the GlcNAc-2 residue (cf. Refs. 24 and 25). The anomeric proton resonances appearing at 4.53–5.18 ppm could be interpreted as indicated in Fig. 3A on the basis of a set of reference data reported in the literature (22, 23, 26). No signal reminiscent of H-1 of the "bisecting GlcNAc" residue attached in β-1→4-linkage to the β-linked Man residue is seen in Fig. 3A. These results led us again to suggest for A-1 a biantennary structure with the di-N-acetylchitobiosyl group at the reducing end.

The <sup>1</sup>H NMR spectrum of A-2 is shown in Fig. 3B and exhibits a higher degree of complexity. This is due to the presence of an additional antenna. The signals were assigned by procedures similar to those employed for A-1, using the previously assigned chemical shifts of triantennary oligosaccharides for comparison (cf. Refs. 23 and 26). Two types of branching structures, A-2A and A-2B shown in Scheme I, can be considered for the "triantennary" oligosaccharide A-2. These two types of triantennary oligosaccharides are known to differ in the chemical shifts of anomeric protons of Man-4' and H-2 protons of Man-3 (26). The presence of a pair of
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**FIG. 3.** 400 MHz $^1$H NMR spectra of (A) A-1 and (B) A-2 in D$_2$O at 60 °C. The 4.0–5.2 ppm region of the spectra at 27 °C is also shown in each panel. In B, the 4.0–5.2 ppm region of the spectrum at 60 °C of the preparation A-2 obtained from another lot of the unfertilized eggs of *P. altivelis* is shown to demonstrate the difference in the relative amounts of A-2A and A-2B in the two preparations of A-2. The finally established structures and the assignments of the selected proton resonances are given.
signals at 6.48 ppm (Man-4' H-1) for A-2A and 6.46 ppm (Man-4' H-1) for A-2B is indicative of the presence of two types of structures in A-2, and the relative intensity of the corresponding peaks indicates that A-2 is composed of A-2A and A-2B with the ratio of about 1:1, which is further confirmed by the analysis of Smith degradation products (see below). (Lower proportion of A-2B, i.e. A-2A:A-2B = 7:3, was detected in another preparation of A-2 from a different lot of P. altivelis eggs (see Fig. 3B, inset; 6.50 ppm (Man-4' H-1) for A-2A and 6.57 ppm (Man-4' H-1) for A-2B).) All 3 NeuAc residues in A-2 were again interpreted to be α-2-3- linked from the data based on the chemical shifts of H-3′α (32.76 ppm) and H-3′β (31.87 ppm) (see below for supporting evidence from methylation analysis). Signal assignments for the selected proton resonances in the spectrum of A-2, based on their order of elution from the column. The composition analysis of these two fractions showed the presence of Man, GlcNAc, and XylNAc, identified by gas-liquid chromatography analysis of the oligosaccharide fraction isolated by Bio-Gel P-2 chromatography (data not shown).

Confirmation for the presence of two isomeric forms in A-2, which was inferred from the 1H NMR data (see above), was provided by Smith degradation of asialo A-2-ol. The oligosaccharide fragments thus obtained were eluted in two distinct sugar-containing peaks from Bio-Gel P-2 (1.4 × 105 cm). These peaks were designated A-2S1 and A-2S2, respectively, based on their order of elution from the column. The composition analysis of these two fractions showed the presence of Man, GlcNAc, and XylNAc in the molar ratios of 2:3:1 and 1:1:1, suggesting the former (A-2S1) to have the structure GlcNAc(1→4)[GlcNAc(1→2)Man(1→3)Man(1→4)GlcNAc(1→4)XylNAc] and the latter (A-2S2) to have the structure Man(1→4)GlcNAc(1→4)XylNAc, which were presumably derived from asialo A-2A-ol and asialo A-2B-ol, respectively. Asialo A-2-ol yielded A-2S1 and A-2S2 upon Smith degradation in the relative molar proportions 1:1, confirming the presence of approximately equal amounts of A-2A and A-2B isomers in fraction A-2, which was indicated by 1H NMR spectrum of A-2 (Fig. 3B). Owing to the shortage of material, Smith degradation could not be carried out on asialo A-3-ol.

**Methylation Analysis**—Confirmatory evidence for the intersugar linkages in A-1, A-2, and A-3 was obtained by methylation analysis of A-1, asialo A-1-ol, asialo A-2, and asialo A-3. The results are summarized in Table II. Methylation analysis indicated that all the Gal residues in A-1, A-2, and A-3 were sialylated and the NeuAc residues were invariably linked α-2,3 to the penultimate Gal residues. Neither 6-substituted nor unsubstituted Gal residue was detected for A-1, A-2, and A-3. All GlcNAc residues in A-1, A-2, and A-3 were substituted at positions C-1 and C-4. In A-1-ol, 3 of the GlcNAc residues were substituted at C-1 and C-4, and 1 GlcNAc residue was substituted at C-6. Although we were unable to separate a pair of isomers of partially methylated alditol acetates derived from 1,2,4- and 1,2,6-substituted Man residues, all the data of methylation analysis were consistent with the previously described procedure (17).
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A-1
NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1
6
3
Manβ1-4GlcNAcβ1-4GlcNAc

A-2
NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1
6
3
Manβ1-4GlcNAcβ1-4GlcNAc

Scheme I. The established structures of A-1, A-2, and A-3.

with the presence of tri- and tetraantennary structures in the intact A-2 and A-3, respectively.

Subcellular Localization of Sialooligosaccharides

The amounts of A-1, A-2, and A-3 present in the two subcellular fractions obtained from the unfertilized eggs (see above) are shown in Table III. It can be concluded that the free sialooligosaccharides were localized in the cytoplasmic fraction.

DISCUSSION

The present study of the eggs of P. altivelis revealed the presence of free sialooligosaccharides. Three sialooligosaccharides have been isolated (A-1, A-2, and A-3). A-1 is by far the most abundant. A-3 was found in small amounts. From the data presented, the precise structures were established for A-1 and A-2 (Scheme I). Sufficient material was also obtained for structural analysis of A-3, and the results of 1H NMR spectral measurement (data not shown), composition analysis (Table I), and methylation analysis (Table II), together with reasoning by analogy to A-1 and A-2, all suggest A-3 to have the structure shown in Scheme I. A-1, A-2, and A-3 are the typical complex-type oligosaccharides and the proposed structures are consistent with the bi-, tri-, and tetraantennary structures from glycoproteins and with what is known about the biosynthesis of N-linked oligosaccharides (27).

Three points of special interest about the occurrence of free sialooligosaccharides need comment. First, this study revealed for the first time the presence of free oligosaccharides in normal animal cells such as mature oocytes. The occurrence of the complex-type oligosaccharides similar to A-1, A-2, and A-3 has only been demonstrated in patients with metabolic diseases such as sialidosis, who store in their cells and tissues and excrete in the urine excessive amounts of glycoprotein-derived sialic acid-containing free oligosaccharides (7).

The second point relates to the present findings of bi-, tri-, and tetraantennary sialooligosaccharides having di-N-acetylchitobiosyl structure, ---GlcNAcβ1-4GlcNAc, at their reducing termini. Such a terminal structure differs radically from the structure of various sialooligosaccharides stored or excreted by patients with genetic defect in the catabolism of sialic acid-containing oligosaccharides and sialoglycoproteins. Patients suffering from glycosidases due to the inborn deficiency of lysosomal exoglycosidases accumulate within the lysosomes of their tissues and excrete in the urine excessive quantities of sialooligosaccharides originating from glycoproteins. The structure of the reducing terminal portion of these oligosaccharides is invariably ---Manβ1-4GlcNAc instead of ---Manβ1-4GlcNAcβ1-4GlcNAc, suggesting that they are formed by the action of endo-β-GlcNAcases on the di-N-acetylchitobiosyl linkage of the patient sialoglycopeptides (6, 7). The limited numbers of the known examples against this general feature include sialooligosaccharides found in liver from GM1 gangliosidosis canine (28) and in the urine of a cat with mannosidosis (29). The third point relates to the finding that all antennae present in three sialooligosaccharides, A-1,
A-2, and A-3, are terminated by the structure NeuAcα2-3Galβ1-GlcNAcβ1-4. The exclusive expression of the NeuAcα2-3Gal linkage is tempting to speculate that these carbohydrate chains are synthesized in oocytes of certain stages of oogenesis if one considers the hypothesis that “the CMP-NeuAcGalβ1-4GlcNAc α2-3-sialyltransferase activity is specifically associated with fetal stages or stages of rapid controlled or uncontrolled cell divisions in general” (30). In this regard it is interesting to note that the asparagine-linked carbohydrate chains of human erythropoietin produced by recombinant Chinese hamster ovary cells (31), G protein of vesicular stomatitis virus grown in Chinese hamster ovary cells (32, 33), and recombinant γ-interferon produced in Chinese hamster ovary cells (34) all have been shown to contain only NeuAcα2-3Gal sequences.

From the presence of bi-, tri-, and tetraantennary sialooligosaccharides, it can be deduced that these free oligosaccharides are generated by cleavage of the bonds between the proximal GlcNAc and Asn residues in asparagine-linked glycoprotein(s). It should also be noted that the occurrence of free sialooligosaccharides is not confined to the eggs of P. altivelis but rather universal feature of at least five species including Tribolodon hakonensis. It may be rather likely that glycoprotein deglycosylation is a general phenomenon in all eukaryotic organisms and will be the subject of future studies which may open up an intriguing field in glycoconjugate research. The present findings and the arguments presented below have general implications concerning the mechanism to be followed in intracellular transport of glycoproteins via endo- and/or exocytosis. In our opinion the failure of various authors to detect cytoplasmic glycoprotein-derived free oligosaccharides is most likely due to preparative problems, primarily the almost inevitable loss of soluble material with small sizes.

The molecular mechanism by which the free bi-, tri-, and tetraantennary sialooligosaccharides are generated remains unknown. However, the present evidence implicates the action of an enzyme, peptide-Nα-(N-acetyl-β-glucosaminyl)asparagine amidase but not an endo-β-N-acetylgalactosaminidase on glycoprotein(s). Although PNGase activity was detected in plant seeds (35, 36) and in Flavobacterium meningosepticum (37), at present no report describes the presence of the corresponding enzyme (PNGase) in oocytes or more in general in other animal tissues or cells. It is likely that in the oocytes of P. altivelis, sialooligosaccharides are released from glycoproteins by PNGase during oogenesis, leading to their storage in cytoplasm in the mature egg cells. Our demonstration of large amounts of free sialooligosaccharides in cytoplasm of the unfertilized eggs suggests that these oligosaccharides may possibly be derived from vitellogenin, although their exact origin is at present not known. The amount of these sialooligosaccharides in 1 g of the mature eggs is about 5 × 10⁻⁴ mol, comparable with that of very abundant lipoprotein, vitellogenin. To our understanding vitellogenin is biosynthesized and glycosylated in the liver and then transported through the blood stream to oocytes (38). Following internalization, vitellogenin undergoes proteolytic processing during oogenesis (38, 39), and our findings would suggest that such a process of vitellogenin in oogenesis may be accompanied by the release of sialooligosaccharide chains by the direct action of a PNGase. However, as described above, the exclusive occurrence of NeuAcα2-3Gal intersugar linkages appears to argue against the possibility that these sugar chains are synthesized in the fish liver, but to support a possibility that the sugar chains are synthesized or at least sialylated in oocytes during oogenesis. To decide which possibility is correct must await practical verification. Here, it should be emphasized that the free sialooligosaccharides are by no means artifacts of isolation but arise naturally within the cell by a process as yet undetermined.

One must presume, therefore, that the cleavage of the asparagine-linked bi-, tri-, and tetraantennary sialooligosaccharide chains is not the beginning of a catabolic pathway; instead the action of putative PNGase on a progenitor glycoprotein and a deficiency of the degradative glycohydrolases in the cytoplasm of P. altivelis oocytes would cause the storage of these oligosaccharides. PNGase activity, responsible for the removal of carbohydrate chains from a certain glycoprotein as well as the storage pool of free oligosaccharides, may be needed in oocytes for fertilization or the subsequent early embryonic development until gastrulation. In conclusion, the present unusual findings may stimulate further investigations into the basic problem in glycoprotein research when one raises the questions such as: (i) How and why does removal of carbohydrate chains occur during oogenesis? (ii) Do other glycoproteins also undergo deglycosylation by a similar mechanism, at least during development? In this regard, a sensitive method of detection of PNGase activity recently developed by Plummer et al. (36) may be useful in search for PNGase-type enzymes in animal tissues or cells.

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Supplementary Material to
Free Sialooligosaccharides in Eggs of P. altivelis—Falki grown unfertilized eggs (66.7×0.8 mm in diameter of P. altivelis) were collected during October by courtesy of the Ranges, a Fresh-Water Trout, Solenodon Altivelis. Sialooligosaccharides were extracted by trichloroacetic acid (TCA) precipitation. The sialooligosaccharides were purified by a column of Sepharose 6B-50 columns (1.5×100 mm) pre-equilibrated with and eluted with 1% ethanol and analyzed for sialic acid and carbohydrate. The free sialooligosaccharides were further purified by preparative ultracentrifugation in the preparative ultracentrifugation method (9).}

Materials and Methods

Isolation of free sialooligosaccharides from P. altivelis eggs—Falki grown unfertilized eggs (66.7×0.8 mm in diameter of P. altivelis) were collected during October by courtesy of the Ranges, a Fresh-Water Trout, Solenodon Altivelis. Sialooligosaccharides were extracted by trichloroacetic acid (TCA) precipitation. The sialooligosaccharides were purified by a column of Sepharose 6B-50 columns (1.5×100 mm) pre-equilibrated with and eluted with 1% ethanol and analyzed for sialic acid and carbohydrate. The free sialooligosaccharides were further purified by preparative ultracentrifugation in the preparative ultracentrifugation method (9).