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ISOLATION OF A NOVEL GLYCOPROTEIN FROM THE EGGS OF RAINBOW TROUT: OCCURRENCE OF DISIALOSYL GROUPS ON ALL CARBOHYDRATE CHAINS

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SUMMARY The occurrence of disialosyl (α-N-glycolylneuraminyl-(2→8)-N-glycolylneuraminyl) groups has been shown in the carbohydrate chains of a glycoprotein newly isolated from the eggs of rainbow trout. Almost all sialic acid residues form disialosyl sequences at the terminal position of the oligosaccharide units consisting of galactose and N-acetylgalactosamine through which the units are linked to threonine residues of the peptide moiety. The sialic acid content of the glycoprotein was as high as 50 %. Such a glycoprotein has not been recognized as a constituent of fish eggs. Its unique sugar and amino acid composition is different from that of any class of the glycoproteins so far reported.

INTRODUCTION The occurrence of disialosyl groups in glycoproteins was first demonstrated in 1977 in a sample prepared from rat brain by Finne et al. (1). These authors also investigated the distribution of this sequence in different tissues and subcellular fractions and concluded that high proportion was found in the plasma membrane fraction of the young rat brain (2). But most sialic acid residues occupied terminal positions in the glycoproteins of mammalian tissues and the proportion of non-terminal to terminal sialic acid was 8.5 % in rat brain which showed a higher value than the other tissues examined.

In this paper we report the isolation of a new class of glycoprotein in which most sialic acid residues form disialosyl (α-N-glycolylneuraminyl-(2→8)-N-glycolylneuraminyl) sequences from the eggs of rainbow trout. This is the first demonstration of this sequence in a glycoprotein although it has been shown in a glycolipid (3).

EXPERIMENTAL The glycoprotein studied in this paper was isolated from the soluble fraction of ovulated eggs of rainbow trout (Salmo irideus). Throughout the isolation steps, the glycoprotein was accompanied by phosvitin, the major acidic egg protein. The two were separated from each other by gel filtration

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in the final step. Pig submaxillary mucins were prepared as described previously (4). The reduced oligosaccharides were prepared with $NaBH_4$ in 0.1 N NaOH and fractionated on a Sephadex G-25 column by the method of Spiro and Bhoyroo (5). Some oligosaccharides were further purified by paper electrophoresis.

Sialic acid was determined by the thiobarbituric acid reaction (6). Samples were hydrolyzed in 0.1 N trifluoroacetic acid at 80° until the maximum value was obtained. Neuraminidase from Clostridium perfringens (EC 3.2.1.18, Boehringer-Mannheim) was used at a concentration of 0.05 U per ml in 0.1 M acetate buffer (pH 5.0) at 37°. Sialic acid was identified by gas chromatography (7) and paper chromatography (8). Neutral sugars were analyzed as alditol trifluoroacetates by gas chromatography (9). Amino acids, hexosamines and hexosaminitols were analyzed with a Hitachi amino acid analyzer KLA-5.

The procedure of periodate oxidation and the assay of formaldehyde formation were as described previously (10).

Methylation analysis of the sialic acid derivatives by gas chromatography-mass spectrometry was according to Rauvala and Kärkkäinen (11). A Hitachi mass spectrometer M-60 was operated at 20 eV.

RESULTS The most striking feature of the trout egg glycoprotein was that sialic acid, identified as N-glycolylneuraminic acid, accounted for 50 % of its weight. Other constituents were neutral sugars (mainly galactose) 10 %, N-acetyl galactosamine 15 % and total amino acids 21 %. A molecular weight of 260,000 was obtained by sedimentation equilibrium. All carbohydrate chains were O-glycosidically linked to the peptide by N-acetylgalactosaminylthreonine bonds. The molar ratios of the sugar components of the intact glycoprotein and oligosaccharide fractions were given in Table 1. The oligosaccharide fractions I to IV represent all sugar components of the glycoprotein.

Fig. 1 shows the rate of hydrolytic release of N-glycolylneuraminic acid from the intact glycoprotein and one of the oligosaccharide fractions. Similar profiles were obtained for the other oligosaccharide fractions. It is noted that the release of N-glycolylneuraminic acid reached the maximum value after 4 h and 2 h from the intact trout egg glycoprotein and its oligosaccharide fraction, respectively. Under similar conditions, the maximum release of N-glycolylneuraminic acid was observed after 1 h and 40 min from intact pig submaxillary mucin and an oligosaccharide fraction derived from it, respectively. Neuraminidase from Clostridium perfringens released all sialic acid residues from the intact glycoprotein in 1 h under the conditions described. Methanolysis in methanolic 0.05 N HCl at 80° for 1 h gave β-methyl ketoside methyl ester of N-glycolylneuraminic acid in a yield of more than 80 % as determined by gas chromatography (7).

Table 1. Composition of the trout egg glycoprotein and its oligosaccharide fractions obtained by alkaline borohydride treatment.

	Intact glycoprotein	Oligosaccharide fraction[a] I	II	III	IV	III-2-2
N-Acetylgalactosaminitol		1.0[b]	1.0	1.0	1.0	1.0
N-Acetylgalactosamine	1.0	1.8	1.6	0.6	0.3	nil
Galactose	0.8	2.1	2.5	1.5	1.0	1.1
Fucose	0.08	0.12	0.19	0.05	0.01	nil
N-Glycolylneuraminic acid	2.3	4.3	3.2	1.9	2.0	1.8

a. Oligosaccharide fractions I to IV were eluted from a Sephadex G-25 column in this order. Fraction III-2-2 was obtained by paper electrophoresis of fraction III.

b. Numbers represent molar ratios relative to N-acetylgalactosaminitol and N-acetylgalactosamine for the oligosaccharides and the intact glycoprotein, respectively.

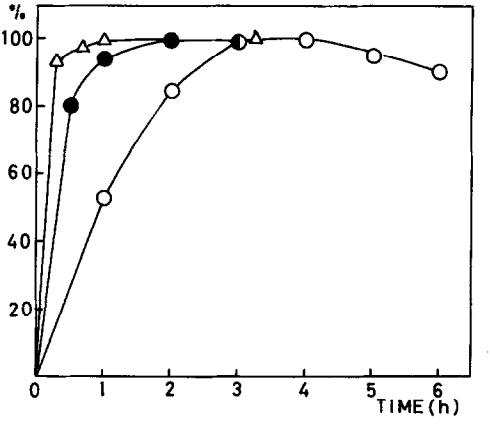

Fig. 1. Rate of release of N-glycolylneuraminic acid from the trout egg glycoprotein and an oligosaccharide derived from it. Ordinate: per cent of the maximum value obtained by acid hydrolysis. o , acid hydrolysis of the intact glycoprotein; ● , acid hydrolysis of the oligosaccharide; Δ , enzymatic hydrolysis of the intact glycoprotein.

The results of periodate oxidation (Table 2) show that while half the sialic acid residues of the glycoprotein occupy terminal positions and produce formaldehyde, the other half are 8-O-substituted and do not produce formaldehyde (3). This was further confirmed by the effect of periodate oxidation on the reduced oligosaccharide. Of the 2 moles (obs. 1.7 moles) of formaldehyde formed,

Table 2. Formaldehyde formed by periodate oxidation.

	mole/mole sialic acid
N-Glycolylneuraminic acid	1.0
Intact trout egg glycoprotein	0.5
	moles/mole N-acetylgalactosaminitol
Oligosaccharide III-2-2	1.7

1 mole was originated from N-acetylgalactosaminitol and the other 1 mole was produced by one of the two sialic acid residues: one sialic acid residue was resistant to periodate oxidation.

The results obtained by gas chromatography and mass spectrometry of the methylation products also support the disialosyl sequence. Fig. 2 shows a gas chromatogram of the methylated neuraminic acids from a purified oligosaccharide fraction of the trout egg glycoprotein. Three neuraminic acid derivatives were observed. These products were identified by mass spectrometry (12): peak 1, 8-O-acetyl-4,7,9-tri-O-methyl-N-methyl derivative of the methyl glycoside methyl ester of N-acetylneuraminic acid; peak 2, 4,7,8,9-tetra-O-methyl-N-methyl derivative of the methyl glycoside methyl ester of N-glycolylneuraminic acid; peak 3, 8-O-acetyl-4,7,9-tri-O-methyl-N-methyl derivative of the methyl glycoside methyl ester of N-glycolylneuraminic acid. Peak 2 was originated from the terminal sialic acid residues. Peak 1 and peak 3 indicate the presence of 8-O-substituted neuraminic acid in the sugar chains. The possibility that the neuraminic acid residues are 8-O-substituted by other sugars than neuraminic acid was ruled out by the complete liberation of the neuraminic acid residues with neuraminidase. O-Acyl substituents, if any, would be base-labile and replaced by methyl groups in the methylation. Area ratio of peak 1 + peak 3 to peak 2 was close to unity for not only the oligosaccharide but also the intact glycoprotein, suggesting that the disialosyl sequence was predominant in the glycoprotein. It is noted that in contrast to the N-glycolyl group of the permethylated derivative, the N-glycolyl group of the 8-non-methylated neuraminic acid was unstable under the methanolytic conditions (0.5 N HCl, 80°, 16 h).

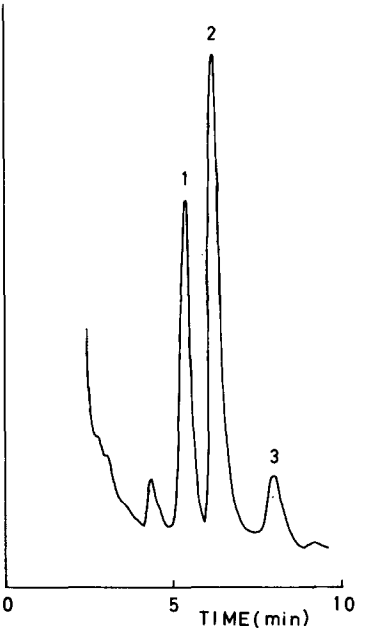

Fig. 2. Gas chromatogram of the methylated neuraminic acids from an oligosaccharide of the trout egg glycoprotein.
4 % OV-1, 220°. Detection by flame ionization.

More than 90 % of the N-glycolyl group was detached and N-acetylated in the subsequent step. Acetylation of the methanolysis products with hexadeuterioacetic anhydride gave the N-deuterioacetylneuraminic acid derivative (12). It has been reported that 4,7,9-tri-O-methyl-N-methyl derivative of the methyl glycoside methyl ester of N-acetylneuraminic acid was prepared by permethylation, methanolysis and re-N-acetylation of colominic acid (13).

DISCUSSION The present work showed the occurrence of a unique class of glycoprotein in which most sialic acid residues form disialosyl (α-N-glycolylneuraminyl (2→8)-N-glycolylneuraminyl sequences. This is the first report showing the occurrence of this sequence in glycoproteins. The glycoprotein isolated from the trout eggs in the present work has not been reported, although egg proteins of Salmonidae have been analyzed (14, 15). We have isolated a similar glycoprotein from the eggs of pacific salmon, Oncorhynchus keta. The newly isolated glycoproteins contain no phosphorus in contrast to phosvitins which also are

glycosylated in these fishes (14). These glycoproteins were found in the soluble fractions of the eggs and precipitated as lipovitellin-phosvitin complexes by lowering the ionic strength. The yield of the glycoprotein was 1 g from 1 kg wet trout eggs, corresponding to about one quarter of the phosvitin.

The biological function of sialic acid in glycoproteins has been elucidated in the limited cases (16). The occurrence of such a sialic acid-rich glycoproteins may be related to the occurrence of large amount of free sialic acids (both N-acetyl and N-glycolyl) in trout eggs (17).

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