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Analysis of monosaccharides, fatty constituents and rare *O*-acetylated sialic acids from gonads of the starfish *Asterias rubens*

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Abstract

A previous study (Bergwerff et al., Biochimie 74 (1992) 25–37) reported that sialic acids present in *Asterias rubens* gonads were essentially composed of 8-methyl-*N*-glycolylneuraminic acid (Neu5Gc8Me), a large part of it being acetylated in position 9. Using GC/MS of heptafluorobutyrate derivatives (Zanetta et al., Glycobiology 11 (2001) 663–676) on the chloroform/methanol soluble and insoluble fractions, we showed that most sialic acids were found in the latter and demonstrated that all sialic acids were derived from *N*-glycolylneuraminic acid, most of them being 8-methylated, but that the majority were also acetylated in position 4 or 7 (or both positions). GC/MS analyses of the constituents liberated using acid-catalysed methanolysis verified that major glycoprotein-bound glycans were N-linked and of the gluco-oligomannosidic type. Major fatty acids were poly-unsaturated (especially C20:4) and long-chain bases were C22:1 phytosphingosine and C22:2 6-hydroxysphingenine. Major monosaccharides found in the chloroform/methanol extract (quinovose and fucose) were derived from steroidal saponins.

Keywords: Asterias; *N*-acetylneuraminic acid; *N*-glycolylneuraminic acid; *N*-glycolyl-8-methyl-neuraminic acid; *O*-acetylated sialic acids; N-linked glycans; Unsaturated lipids

1. Introduction

In recent years, increasing interest was drawn to the diversity of sialic acids, since these monosaccharides could play important biological roles (for reviews see Refs. [1–4]). For example, 9-*O*-acetyl *N*-acetylneuraminic acid is a specific ligand for the agglutinin of *influenza* virus C [5], whereas 4-*O*-acetylated *N*-acetylneuraminic acid is a specific ligand for the agglutinin of murine hepatitis S virus [6]. Based on previous work [1,2], sialic acids present an extreme diversity and more than 40 different compounds were identified differing in the presence in position 5 of an amino group (neuraminic acid derivatives) or an hydroxyl group (3-deoxy-D-glycero-D-galacto-nonulosonic acid (Kdn)), different acylations of the NH_2 group at position 5 (acetyl, glycolyl) and various substituents of the different hydroxyl groups (acetyl, lactyl, methyl, sulphate, phosphate, etc.). Complex monosaccharides structurally identical with sialic acids were identified in bacteria (legionaminic acid and derivatives), which may represent important epitopes [7,8]. Sialic acids show sometimes a clear species specificity. For example, *N*-glycolylneuraminic acid (Neu5Gc) (but not Kdn) is absent from man, although it was found in all other mammalian species so far analysed. The starfish *Asterias rubens* presents the

Abbreviations: aag, alkyl-acyl-glycerol; C22:1phyt, 1,3,4-trihydroxy-2-amino-docosene; C22:2sphe6oh, 1,3,6-trihydroxy-2-amino-4,x-ene-docosene; CM, chloroform methanol-soluble; CMI, chloroform methanol-insoluble; EI, electron impact; FAME, fatty acid methyl-ester; HFB, heptafluorobutyrate; HFBAA, heptafluorobutyric acid anhydride; GC, gas chromatography; Kdn, 3-deoxy-D-glycero-D-galacto-nonulosonic acid; LCB, long-chain base; MS, mass spectrometry; the nomenclature used for sialic acids is according to Schauer and Kamerling (1997): Neu5Ac = *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; Neu5Gc8Me, 8-methyl-*N*-glycolylneuraminic acid; Neu4Ac5Gc8Me, 4-*O*-acetyl-8-methyl-*N*-glycolylneuraminic acid; Neu7Ac5Gc8Me, 7-*O*-acetyl-8-methyl-*N*-glycolylneuraminic acid; Neu4,7$Ac_2$5Gc8Me, 4,7-di-*O*-acetyl-8-methyl-*N*-glycolylneuraminic acid; Qui, quinovose (6-deoxyglucose).

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peculiarity of producing 8-*O*-methyl-5-glycolylneuraminic acid (Neu5Gc8Me) as the major compound [9,10], thus providing a good model for studying the sialate 8-methyltransferase [11]. Using a new method, which allows the determination of the sialic acid diversity without prior purification of the monosaccharides [12], we demonstrate that the major sialic acids of the gonad of *A. rubens* are mono-*O*-acetylated derivatives (in position 4 and 7) of Neu5Gc8Me. Therefore, this material also constitutes a good model for studying the sialate-*O*-acetyltransferases involved in their biosynthesis.

2. Materials and methods

2.1. Materials

A. rubens (2–3 years old) was collected in the North-Friesian part of the North Sea in early spring. The gonads were dissected, lyophilised and stored at –80 °C. The powder was extracted using chloroform/methanol (CM) mixtures [13] and the CM soluble (CM) and insoluble (CMI) fractions were separated by centrifugation (4000 rpm at room temperature). The two samples were evaporated using a rotary evaporator (at room temperature) and the remaining water was eliminated by lyophilisation. Samples were shipped to Villeneuve d'Ascq as dried material.

2.2. Analyses of the sialic acid diversity

The samples were weighted (about 1 mg of each) in reaction vials [14] and submitted first to a mild acid hydrolysis (1 ml, 2 M acetic acid during 105 min at 80 °C) and dried using a rotary evaporator. The samples were suspended adding 500-µl anhydrous methanol and 500 µl of a diazomethane solution in diethyl-ether [12]. The samples were left overnight in the closed vials. Before analysis, the samples were dried under a stream of nitrogen and supplemented with 400 µl dried acetonitrile and 50 µl of heptafluorobutyric acid anhydride (HFBAA; Fluka, 99% purity). After cooling at room temperature, the samples were evaporated under a stream of nitrogen and taken up in 400 µl of dried acetonitrile. 1–2 µl were injected onto the needle of the Ross injector of the GC/MS apparatus.

For alkaline treatment of sialic acids, the dried material obtained after mild acid hydrolysis (see above) was suspended in 500 µl of 0.1 M ammonia and incubated for 15 min at room temperature, neutralised with 0.1 M formic acid and evaporated to dryness at room temperature in a rotary evaporator. The dried residue was methyl-esterified and derivatised with HFBAA as above.

For sialic acid purification, 300 mg of the CMI fraction was submitted to mild acid hydrolysis under the conditions described above. After centrifugation, the pellet was submitted again to mild acid hydrolysis. Resorcinol staining [15] indicated that the supernatant obtained after this second hydrolysis contained less than 5% of the sialic acids liberated during the first step. The combined supernatants were evaporated to dryness with a rotary evaporator and passed through a DE52 column (Whatman; 20 × 1 cm) equilibrated in water. The column was washed with 40 ml water and eluted successively using 25 ml 0.1 M KCl, 0.4 M KCl and 1 M KCl in water and the eluates were evaporated to dryness. All sialic acids were found in the 0.4 M KCl fraction. An aliquot was analysed for sialic acids after direct methyl-esterification with diazomethane followed by acylation with HFBAA.

2.3. Monosaccharide analyses

Once the analyses of sialic acids were performed, the samples were dried under a stream of nitrogen and supplemented with 1 ml of methanolysis reagent (0.5 M gaseous HCl dissolved in anhydrous methanol [16]) and heated for 20 h at 80 °C. After drying under a stream of nitrogen, the samples were supplemented with dried acetonitrile and HFBAA as above and heated for 15 min at 150 °C. Before analysis the samples were evaporated under nitrogen and taken up in 400-µl dried acetonitrile. Again 1–2 µl were injected on the needle of the Ross injector.

2.4. GC/MS analyses

For GC/MS analysis, the GC separation was performed on a Carlo Erba GC8000 gas chromatograph equipped with a 25 m × 0.32 mm CP-Sil5 CB Low bleed/MS capillary column, 0.25 µm film phase (Chrompack France. Les Ulis. France). The temperature of the Ross injector was 260 °C and the samples were analysed using the following temperature program: 90 °C for 3 min, 90–260 °C at 5 °C/min and 260 °C for 20 min. The column was coupled to a Finnigan Automass II mass spectrometer (mass detection limit 1000) or, for masses larger than 1000, to a Nermag 10-10H mass spectrometer (mass detection limit 2000). The analyses were performed routinely in the electron impact mode (ionisation energy 70 eV; source temperature 150 °C). In order to preserve the filament of the ionisation source, the GC/MS records were performed 5 min after the injection of the sample. The quantitation of the different constituents was performed on the total ion count (TIC) of the MS detector using the Xcalibur software (Finnigan Corp.). For ascertaining the mass of the different derivatives, the MS analyses were also performed in the chemical ionisation mode in the presence of ammonia (ionisation energy 150 eV, source temperature of 100 °C). The detection was performed for positive ions. For quantitative data, the relative molar response factors on the major peaks were those reported elsewhere [14].

3. Results and discussion

3.1. Bulk analysis of CM soluble and insoluble fraction

Although these data were obtained in the second step of the analysis, they were found to be very informative. Indeed,