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A Novel Saccharide Structure, Xyl 1→3 Gal 1→ (SO_3^-)3,4 Fuc→, Is Present in Acrosome Reaction-Inducing Substance of the Starfish, *Asterias amurensis*

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The jelly coat of echinoderm eggs contains a glycoconjugate, acrosome reaction-inducing substance (ARIS), that is essential for triggering the acrosome reaction in homologous spermatozoa. In the starfish, *Asterias amurensis*, ARIS is a sulfated glycoprotein of an apparent molecular size of $>10^7$. Since its biological activity is dependent mostly on its sugar moiety, oligosaccharides liberated by hydrolysis with 10 mM H_2SO_4 for 60 min at 100°C from pronase digests of ARIS (P-ARIS) were chemically analyzed. The main oligosaccharide purified by high-performance anion-exchange chromatography was determined to be Xyl1→3Gal1→ (SO_3^-)3,4Fuc by compositional analysis and FAB mass spectrometry. This structure indicates that ARIS possesses a novel saccharide chain having sulfated fucose as an internal residue.

Fertilization consists of serial interactions between the sperm and egg. One of the earliest direct interactions between the two gametes consists of the acrosome reaction and/or sperm binding, which occurs when the sperm encounters extracellular investments of the egg, such as the jelly coat. Although the acrosome reaction is an essential step for sperm to fuse with the egg plasma membrane, little is known about substances

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Abbreviations used are: Fuc, fucose; Xyl, xylose.

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responsible for the induction of acrosome reaction. Inducer of the acrosome reaction are present in the jelly coat of sea urchin (1) and starfish (2), and in the zona pellucida of mouse (3) and pig (4) eggs.

In starfish, acrosome reaction-inducing substance (ARIS) and a cofactor (Co-ARIS), which consists of steroid saponins (5), cooperatively induce the acrosome reaction in the sea water , whereas ARIS alone induces the acrosome reaction in sea water at an elevated pH or containing high levels of Ca^{2+} (6). ARIS is a sulfated glycoprotein of large molecular size (>10^7), whose activity remains after pronase digestion but disappears by periodate oxidation or solvolytic desulfation, demonstrating the importance of the sugar moiety for its activity (6).

Though sugar chains play important roles in fertilization of many animals (7), information on their structures is limited. Sugar chains of the porcine zona pellucida glycoprotein with sperm receptor activity have been determined recently and shown to be a neutral Asn-linked type (8,9). We report here a novel sugar chain which contains xylose and internal sulfated fucose as the partial carbohydrate structure of starfish ARIS.

MATERIALS and METHODS

Analytical Methods ----- Total sugar was measured by the method of Dubois (10) and reducing sugar by the method of Park and Johnson (11). The sugar composition was determined by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD; Dionex, CA) (12), after hydrolysis with 2 M trifluoroacetic acid for 2 h at 100°C. When necessary, the oligosaccharide was oxidized with 10 mM sodium periodate for 24 h at 4°C, or reduced with $NaBH_4$ (10 mg/ml in 50 mM NaOH) for 10 h at room temperature. Negative ion fast-atom bombardment mass spectra were obtained with a JEOL JMS-HX110 double-focusing mass spectrometer with triethanolamine as the matrix.

Preparation of Pronase Digest of ARIS (P-ARIS) ----- P-ARIS was prepared from the jelly coat of eggs in the starfish, *Asterias amurensis* (6). The egg jelly was solubilized in acidic sea water and dialyzed against deionized water. The non-diffusible fraction was digested with a pronase, Actinase E (Kaken Seiyaku, Tokyo), for 96 h at 37°C with further additions of pronase at every 24 h. The digest was applied to Sepharose CL-4B and the sugar-positive fraction at void volume was applied to DEAE-TOYOPEARL 650 M (Tosoh, Tokyo). P-ARIS fraction was eluted with an NaCl gradient, dialyzed and lyophilized.

Preparation of Oligosaccharides ----- P-ARIS was hydrolyzed with 10 mM H_2SO_4 for 1 h at 100°C. The hydrolyzate was applied to Bio-Gel P-2 using water for elution and the content of reducing sugar was monitored. The oligosaccharides were further separated

by HPAEC-PAD. The elution was carried out with 0.1 M sodium hydroxide at a flow rate of 1 ml/min with a two-stage linear gradient from 0.1 to 0.5 M sodium acetate for 10 min, and from 0.5 to 1 M for 5 min. For fractionation, the eluent was desalted by two sets of anion micro membrane suppressor (AMMS; Dionex, CA) with regenerating solution of 0.4 N HCl at a flow rate of 8.5 ml/min.

RESULTS and DISCUSSION

Analysis of P-ARIS and its Hydrolyzate

P-ARIS, the product derived from a pronase digestion of ARIS, was used for structural analysis. Table I shows the sugar composition of P-ARIS. P-ARIS was virtually free of mannose, suggesting that its sugar chains are not Asn-linked. However, the sugar chains were resistant to alkaline treatment which usually eliminates O-linked sugar chains from peptides. Therefore, P-ARIS was hydrolyzed with a mild acid (10 mM H_2SO_4 for 1 h at 100°C) to eliminate terminal fucoses and/or to obtain sugar chain fragments. The hydrolyzate was applied to Bio-Gel P-2 using water for elution (Fig. 1). Two fractions, A and B, of reducing sugar were found near the void position and several peaks at the small molecular size area. The molecular sizes of these oligosaccharides are difficult to estimate, since anionic molecules elute at a larger molecular size-position when water is used as the eluant. Fractions A and B were further analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Fig. 2). Fraction A was separated into 7 peaks and fraction B into 9 peaks. Peak B-6, which gave the largest response to PAD, was collected and subjected to further analysis.

Table I *Sugar Compositions of P-ARIS and B-6*

	Xyl	Gal	Fuc	GalNAc	GlcNAc
P-ARIS[a]	2.7	3.1	8.8	0.64	1.00
B-6[b]					
intact	1.02	1.00	1.00	—[c]	—
reduced	1.09	0.98	0.18	—	—
oxidized	0.15	0.84	0.01	—	—

[a]Results normalized to GlcNAc = 1; [b]Results normalized to intact Fuc = 1; [c] not detected.

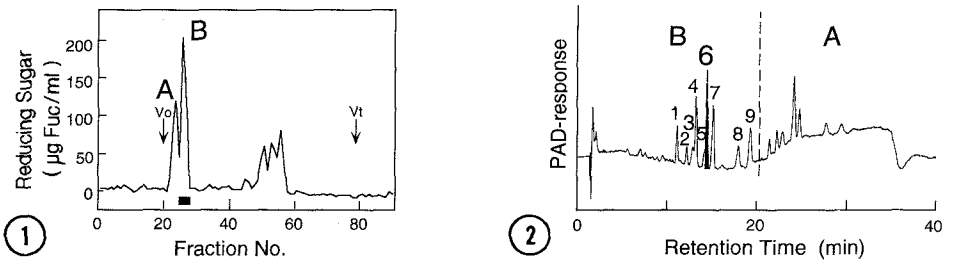

Figure 1. *Gel-filtration of P-ARIS hydrolyzate.* The hydrolyzate was fractionated by Bio-Gel P-2 using water for elution. An aliqout of each fraction was measured for the content of reducing sugar. Peak B contained the oligosaccharide, Xyl-Gal-(SO_3^-)Fuc, described in this report.

Figure 2. *HPAEC-PAD of fractions A and B from gel-filtration.* Fractions A and B were simultaneously injected into HPAEC. The chromatogram shows the result of analysis of A and B. B-6, which gave the largest response to PAD, was collected and further analyzed.

Fractionation of B-6

Peak B-6 was collected after on-line desalting by two sets of anion micro membrane suppressors (AMMS) and evaporated with a rotary evaporator. HCl was used instead of H_2SO_4 as the regenerating solution, since leakage of H_2SO_4 at the AMMS degraded oligosaccharides during evaporation. This method allowed us to collect 5 μg of B-6 during one operation. When larger amounts of oligosaccharides were fractionated, they were degraded during the evaporation. This is probably due to the acidity after desalting, owing to acetate ion, chloride ion leaked from AMMS and the sulfate group of B-6, as described below.

Structural Analysis of B-6

The sugar composition of B-6 was Fuc, Gal and Xyl in an equimolar ratio (Table I). Reduction of B-6 with sodium borohydride resulted in a loss of Fuc, which indicates that B-6 has a Fuc at its reducing terminal and that B-6 is a trisaccharide. The sugar sequence of B-6 was determined by negative ion fast-atom bombardment mass spectrometry (negative FAB-MS, Fig. 3). The ions at m/z 97 and 80 indicate the existence of a sulfate group (13). The fragmentation suggests the sequence of B-6 as Xyl → Gal → (SO_3^-)Fuc. The information about linkages was obtained by periodate oxidation (Table I). Degradation of Xyl and Fuc by oxidation indicates that Xyl is linked to 3-OH of Gal, and that Gal and sulfate group are attached to 3-OH and 4-OH of Fuc. The definitive positions of galactose and sulfate linked to Fuc remain to be determined.

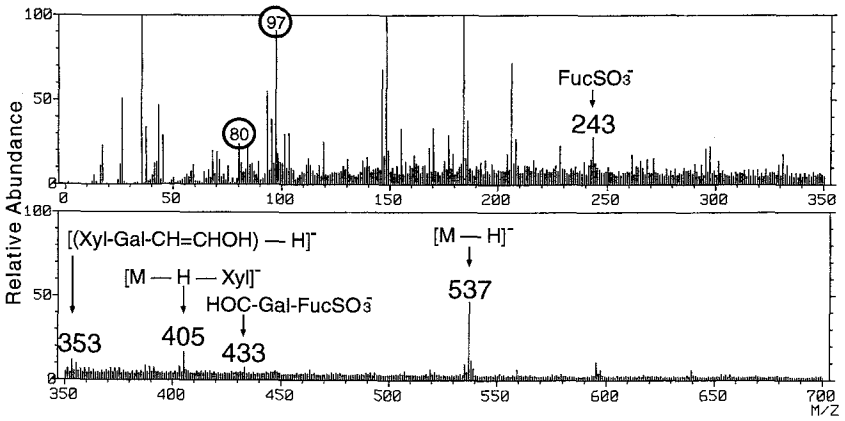

Figure 3. *Negative ion FAB-MS of B-6.* The ions at *m/z* 97 and 80 confirm the presence of a sulfate group. The ions corresponding to the sugar sequence are indicated by arrows.

The partial structure of the sugar chains of P-ARIS is unusual in two regards. First, P-ARIS contains internal fucose as indicated by the fact that B-6 has Fuc at its reducing terminal. Except for polyfucose, internal fucose has been rarely found in glycolipids (14,15) and glycoproteins (16). Second, P-ARIS contains xylose, which in animals is usually found at the sugar-protein linkage region of proteoglycan. It is noteworthy that there has been few reports of Xyl in animal glycoproteins (17,18,19).

HPAEC-PAD is a powerful method for analysis of mono- and oligosaccharides. Recently, it has been used for fractionation and structural analysis of neutral (20,21) and phosphorylated (22) oligosaccharides. In this report we have shown that HPAEC-PAD is also useful for analysis and for fractionation of sulfated oligosaccharides.

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