Structure of the Main Saccharide Chain in the Acrosome Reaction-inducing Substance of the Starfish, Asterias amurensis*

Souichi Koyota, K. M. Swarna Wimalasiri‡, and Motonori Hoshi§

From the Department of Life Science, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226, Japan

The structure of the main saccharide chain of the acrosome reaction-inducing substance in the egg jelly coat of the starfish, Asterias amurensis, is composed of the following pentasaccharide repeating units (Structure I).

$$\rightarrow 4, \beta-D-Xylp-(1 \rightarrow 3)-a-D-Galp-(1 \rightarrow 3)$$

$$-a-D-Fucp-(1 \rightarrow 3)-a-D-Fucp-(1 \rightarrow 4)-a-D-Fucp-(1 \rightarrow 4)$$

$$\uparrow \quad \uparrow \quad \uparrow$$

$$SO_3^- \quad SO_3^-$$

**Structure I**

The identities and linkage positions of constituent sugars were established using sugar, methylation, and sulfate analyses together with one- and two-dimensional nmr spectroscopy. The structure was supported by the data obtained for desulfation products and the Smith degradation of the polysaccharide.

The acrosome reaction is a prerequisite for fertilization in various animals including mammals. In starfish, three components of the egg jelly, namely a highly sulfated glycoprotein of an extremely large molecular size named acrosome reaction-inducing substance (ARIS), a steroid saponin named Co-ARIS, and a sperm-activating peptide (asterosap) are responsible for triggering the acrosome reaction (1). Three saponins consisting of a sulfated steroid and a pentasaccharide chain (Co-ARIS 1–3) were isolated from the egg jelly and structurally characterized (2). A mixture of glutamine-rich peptides consisting of 34 amino acid residues was isolated as asterosap, and the acrosome reaction in normal seawater. A Pronase digest of Co-ARIS (23) was reduced by about 50% by the Pronase digestion, which made it easier to handle in comparison with ARIS, P-ARIS was used as the starting material in this analysis. This study was carried out in an attempt to understand the structure-activity relationship of ARIS, and we now report the structural study of the main saccharide chain of P-ARIS.

**EXPERIMENTAL PROCEDURES**

**General Methods**—Water was purified with a Milli-Q reagent water system (Millipore Corp.). Sample concentrations were performed under diminished pressure at <40 °C or in a stream of nitrogen. For gas-liquid chromatography, a Shimadzu GC-7A instrument fitted with a flame ionization detector was used. Gas-liquid chromatography-mass spectrometry was performed on a Shimadzu GC-14A instrument coupled with a Shimadzu GCMS-QP2000A instrument. A Dionex HPAEC-PAD was used to determine sugar compositions. Alditol acetates and partially O-methylated alditol acetates were analyzed on a HP-5 capillary column using a temperature program (170 °C (2 min) → 250 °C (2 °C/min)). Gel filtrations were performed on a Bio-Gel P-2 column using deionized Milli-Q filtered water as the eluent unless otherwise specified. Fractions were monitored either by the Dubois method (7) or by the Park and Johnson method (8). Sulfate contents were determined according to Terho and Hartiala (9).

**Preparation of P-ARIS and Its Fragments**—P-ARIS was prepared by ethanol precipitation of the egg jelly solution of the starfish, Pronase digestion, and ion exchange chromatography on DEAE Toyopearl 650M described by Matsui et al. (5). A suspension of P-ARIS (30 mg) in water (10 ml) was sonicated using a Bransonic Ultrasonics apparatus model for 30 min and then filtered, and the product was freeze-dried. The sample was chromatographed on a column of DEAE Toyopearl 650M using water followed by a linear gradient of 0–1.0 M NaCl in water. The major fraction, eluted at ~0.9 M NaCl concentration, was dialyzed and freeze-dried to give purified Fragment 1 (12.6 mg, 42%).

**Sugar and Methylation Analysis**—The native and degraded polysaccharide samples were hydrolyzed with 2 M trifluoroacetic acid at 120 °C for 1 h. After evaporation to dryness, the products were analyzed as alditol acetates either by direct injection to the HPAEC-PAD or by gas-liquid chromatography (10). Methylation analysis was performed by a modified Hakomori procedure (11). Since the prolonged base and CH₃I treatments improved the stoichiometry of the partially O-methylated alditol acetates, the same procedure has been followed in all methylation analyses. The absolute configurations of the sugar residues were determined according to Gerwig et al. (12).

**nmr Spectroscopy**—All 1H and 13C nmr spectra of solutions in D₂O were recorded either at 20 °C or at 70 °C using either a Varian UNITY plus 400-MHz or a JEOL GX 270-MHz instrument. Chemical shifts are reported in ppm using sodium 3-trimethylsilyl propionate-d₅ (δ 0.00 ppm) for 1H as the external reference and acetone (δ 31.07 ppm) for 13C as the internal reference.

**Desulfation of Fragment 1**—A solution of Fragment 1 was dialyzed against 0.1 M pyridinium acetate buffer (pH 5.4) for 2 days. The resulting pyridinium salt (12 mg) was treated with 10% methanol in dimethyl

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§ To whom correspondence should be addressed. Tel.: 81-45-924-5720; Fax: 81-45-924-5777.

The abbreviations used are: ARIS, acrosome reaction-inducing substance; P-ARIS, Pronase digest of ARIS; DS, desulfated fraction; NOE, nuclear Overhauser effect; HPAEC-PAD, high-performance anion exchange chromatograph with pulsed amperometric detector.

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sulfoxide (Me$_3$SO) for 12 h at 50 °C (13), concentrated to dryness, and passed through a column of Bio-Gel P-2. The fraction that eluted in the void volume (DS-1) was analyzed for its composition of sugars and their linkages. Another sample of the pyridinium salt (12 mg) was treated with 10% methanol in Me$_2$SO for 5 h at 80 °C (14) and treated in a similar manner as above to obtain DS-2.

The Smith Degradation of Fragment 1—The Smith degradation (15) was applied to P-ARIS. Namely, a dried sample of Fragment 1 (3 mg) was dissolved in 1 ml of 0.1 M ammonium acetate buffer containing 9.0 M urea at pH 4.0. NaIO$_4$ (0.5 ml of a 1% solution) and isopropyl alcohol (6 µl) were added to the Fragment 1 solution and stirred in the dark at room temperature for 2 h. The reaction mixture was stored in the dark for 7 days at 4 °C. The reaction was quenched by the addition of ethylene glycol, and the mixture was dialyzed against water. The non-dialyzable fraction was freeze-dried and reduced with NaBH$_4$ (1 ml of a 1% solution) for 6 h at room temperature. The product was dialyzed against water and then hydrolyzed with 0.1 N trifluoroacetic acid (0.5 ml) at 100 °C for 30 min. After evaporation to dryness, the mixture was chromatographed on a column of Bio-Gel P-2 using 0.1 M NaCl as the eluent. The tri-, di-, and monosaccharide fractions obtained were desalted by passing through Dowex 50W, and the samples were hydrolyzed with 2 N trifluoroacetic acid at 120 °C for 1 h and then analyzed for their sugar compositions using a Dionex HPAEC-PAD.

### Determination of Acrosome Reaction-inducing Activity—

The acrosome-reaction-inducing capacities were scored with the starfish spermatozoon for ARIS, P-ARIS, Fragment 1, DS-1, and DS-2. The sperm suspension was prepared by diluting 5 ml of 2-fold artificial seawater and passed through a column of Sepharose CL-4B, and 55 ml of high calcium solution, 55 ml of 2-fold artificial seawater, and 5 ml of 5% glutaraldehyde in artificial seawater. The samples were stained with erythrosine and observed using a Nomarski microscope. A total of 200 spermatozoa were randomly scored, and the intensity ratio of 1:1:1:1:1 that were assigned for the presence of fucose, xylose, and galactose in a molar ratio of 3:1:1 (Table I). The absolute configurations, determined by gas-liquid chromatography of the trimethylsilylated (+)-2-butyl glycosides (12), were L for fucosyl and D for xylosyl and galactosyl residues. Methylation analysis (11) under reported conditions was not satisfactory due probably to the steric hindrance caused by the O-sulfation. Therefore, the base and methyl iodide treatments were prolonged by 24 and 5 h, respectively, to obtain a satisfactory stoichiometry of the analysis. The methylation analysis of Fragment 1 revealed the presence of 4-linked xylopyranosyl, 4-linked fucopyranosyl, 3,4-linked fucopyranosyl, and 3-linked galactopyranosyl residues in a molar ratio of 1:1:2:1 (Table II). The presence of 3,4-linked fucopyranosyl residues suggested a branching or substitution.

The 1H nmr spectrum of Fragment 1 recorded at 20 °C in D$_2$O solution showed five anomeric signals at δ (ppm) 5.28 (1H, d, J = 3.5 Hz), 5.09 (1H, d, J = 3.4 Hz), 5.03 (1H, d, J = 3.8 Hz), 5.00 (1H, d, J = 4.0 Hz), and 4.63 (1H, d, J = 6.3 Hz) in an intensity ratio of 1:1:1:1:1 that were assigned for α-galactopyranosyl, α-fucopyranosyl, α-fucopyranosyl, α-fucopyranosyl, and β-xylopyranosyl residues, respectively (Fig. 1).

The 1H nmr integral was in good agreement with the sugar composition data. Two unresolved signals appearing at δ (ppm) 1.23 (6H, dd, J = 6.7 Hz) and 1.32 (3H, d, J = 6.7 Hz) were attributed to C-6 methyl groups. The 13C nmr spectrum showed anomeric carbon signals at δ (ppm) 104.1, 100.1, 99.2, and 98.6 that could be assigned to α-fucopyranosyl, α-galactopyranosyl, α-fucopyranosyl, and α-fucopyranosyl residues, respectively (Fig. 1). Furthermore, this observation suggested that the signals at δ (ppm) 4.76 and 4.70 in the 1H nmr spectrum of Fragment 1 recorded at 70 °C are non-anomeric (Fig. 2). Since Fragment 1 showed the presence of sulfate as a possible substituent, these two signals were assigned to the ring protons shifted downfield due to the O-sulfation of the fucosyl residues. This observation was supported by the presence of 2 mol of 3,4-linked fucosyl residues in the methylation analysis of Fragment 1. Furthermore, this observation confirmed that the presence of 3,4-linked fucopyranosyl residues was not due to a branching of the main saccharide chain. All the information that we obtained suggested that Fragment 1 had a repeating branched structure of five sugar residues. The isolation of a trisaccharide, Xylp-(1→3)-Galp-(1→3)-SO$_3$-Fucp, after mild acid hydrolysis of P-ARIS (in our laboratory, Okinaga et al. (6)) was a positive clue of a five-sugar repeating unit in Fragment 1.

### Desulfation Studies of Fragment 1—

Fragment 1 was subjected to desulfation to determine the position of O-sulfation and the structure-activity relationship. Two desulfation products (DS-1 and DS-2) were obtained by the treatment of 10%
methanol in dimethyl sulfoxide for 12 h at 50 °C (13) and 80 °C (14), respectively. DS-1 showed the removal of 1 mol of sulfate by the disappearance of a proton signal at δ 4.70 (ppm) in its 1H nmr spectrum (Fig. 2). This was corroborated by the methylation analysis in which an approximate mole of 3,4-linked fucosyl residue was replaced by 3-linked fucosyl residue (Table II). However, in the case of DS-2, total desulfation was achieved as indicated by the disappearance of both non-anomeric proton signals from the anomeric region in its 1H nmr spectrum (Fig. 2). Moreover, complete replacement of 3,4-linked fucosyl residues by 3-linked fucosyl residues in the methylation analysis of DS-2 was also observed. All these observations indicated possible 4-O-sulfation of two of the fucosyl residues present in Fragment 1.

The biological activity of the sample was determined by means of the acrosome reaction-inducing capacity in high-calcium seawater. The acrosome reaction induced by egg jelly, ARIS, P-ARIS, Fragment 1, and DS-1 under the conditions used was found to be 77, 64, 70, 63, and 7%, respectively (Fig. 3). The near-base-line level of activity of DS-1 suggests that the removal of 1 mol of sulfate/repeating unit destroys the biological activity of Fragment 1 and, thus, displays the importance of that particular sulfate group for activity.

**Determination of the Sugar Sequence and the Location of the Activity-determining Sulfate Group**—The location of the activity-determining sulfate group was achieved with the aid of nmr spectrometry. Analyses of H, H-COSY, two-dimensional HO-HAHA, and one-dimensional HOHAHA difference spectra recorded at 20 °C in D₂O solutions led to the identification of almost all ring protons quite unambiguously (Table III). Comparative analyses of two-dimensional H-nuclear Overhauser effect spectroscopy spectra revealed the linkage positions and the exact sequence of some monosaccharide residues in the polymer chain (Table IV). Strong NOE contacts were obtained from H-1 of the xylosyl residue (E)² to H-3 of the galactosyl residue (A) and from galactosyl H-1 to H-3 of one of the sulfated fucosyl residues (B), supporting the previous determination of

² For use of capital letters, see Tables III and IV and Fig. 4.
the trisaccharide sequence by Okinaga et al. (6). The desulfation studies showed that it was the 4-O-sulfate of fucosyl residue B that showed a higher liability toward the desulfation. With the removal of the 4-O-sulfate of residue B, Fragment 1 showed a total loss of biological activity. These observations suggest the presence of a more potent sulfate group in the interior fucosyl residue.

H-1 of the fucosyl residue B showed a NOE contact to the H-3 of the second sulfated fucosyl residue (D) together with a somewhat stronger cross-peak with H-4 of the same residue (D), suggesting that the two sulfated fucosyl residues are next to each other in the polymer chain. Therefore the remaining unsulfated fucosyl residue (C) should be placed as the last residue in the pentasaccharide repeating unit. Even though the possible NOE contact between the H-1 of sulfated fucosyl residue D and the H-4 of the non-sulfated fucosyl residue was not observed, a strong NOE contact was obtained from H-1 of residue C to H-4 of the xylosyl residue (E), supporting the position of residue C in the pentasaccharide repeating unit.

To confirm the sequence of monosaccharides in the structure of Fragment 1, the Smith degradation was carried out. In the Smith degradation, vicinal diols are oxidized by periodate, resulting in an opening of C-C bonds. Therefore, the sugar residues susceptible to periodate oxidation in the pentasaccharide repeating unit should be the 4-linked xylosyl residue (E) and the 4-linked fucosyl residue (C). Thus the products by the Smith degradation of Fragment 1 should be a trisaccharide if the sequence E-A-B-D-C is correct or a disaccharide if the sequence E-A-B-C-D is correct. A dry sample of Fragment 1 was treated with sodium metaperiodate in ammonium acetate buffer containing urea at pH 4.0 in the dark at 4 °C. Urea was added to maximize the reaction, presumably by reducing hydrogen bonding. Indeed, in the absence of urea, the oxidation proceeded to a very limited extent. The product of Smith degradation was subjected to chromatography on a Bio-Gel P-2 column. A fraction corresponding to the elution volume of a trisaccharide contained galactosyl and fucosyl residues in an approximate ratio of 1:2, suggesting the trisaccharide A-B-D. A disaccharide fraction consisting only of fucosyl residues and a monosaccharide fraction galactose were also isolated, suggesting that the di- and monosaccharides resulted from further hydrolysis of the trisaccharide Gal-Fuc(SO3)2-Fuc(SO3)2. This observation confirmed the exact sequence of the monomer units in the repeating unit of Fragment 1. All the data on Fragment 1 indicate that the structure should be the one shown in Fig. 4.

CONCLUSIONS

The study of the acrosome reaction in the fertilization of the starfish, Asterias amurensis, has been a major research project in our laboratory. Previous work in our laboratory resulted in isolation of ARIS, Co-ARIS, and astersapos from starfish egg jelly as the components responsible for the induction of the acrosome reaction. ARIS has been thought to be most important among them for the induction of the acrosome reaction because only it alone is able to induce the acrosome reaction in high calcium or high pH seawater. This study has revealed that a saccharide polymer consisting of pentasaccharide repeating units as shown in Fig. 4 is responsible for the biological activity of ARIS.

In sea urchins the importance of fucose-rich glycoprotein for the activity has been observed, yet there has been a controversy about the crucial components, the sugar portion, or the protein portion. Suzuki and co-workers (16) suggested that a fucose-rich glycoprotein of the sea urchin egg jelly induces the acrosome reaction although it is 50% less active than egg jelly. They observed that the Pronase digestion of fucose-rich glycoprotein decreased the activity and that the activity was restored by the addition of a sperm-activating peptide after digestion. These results supported the conclusion by Ishihara and Dan (17) that a protein closely associated with the fucose sulfate polymer is responsible for the acrosome reaction-inducing activity. However SeGall and Lennerz (18) suggested that a fucose sulfate polymer is responsible for the induction of the acrosome reaction in the sea urchin and proposed that the observed species specificity in the activity of the sea urchin egg jelly is due to small differences in the structure of fucose sulfate polymer. Mulloy et al. (19) separated a fucose sulfate polymer from sea urchin egg jelly that was composed of tetrasaccharide repeating units in which the four residues were 1→3-linked α-L-fucopyranosyl units differing by specific patterns of sulfation at the O-2 and O-4 position. On the other hand, Keller and Vacquier (20) separated a fucose sulfate polymer from sea urchin egg jelly that had no significant activity. Instead, they separated a 138-kDa glycoprotein that contained the activity. Based on chemical analysis, they suggested that only the protein portion and N-linked oligosaccharides are responsible for the

## Table III

| Chemical shifts (ppm) in 1H nmr spectrum of Fragment 1 |
|--------------------------------------------------------|
| Measured at 400 MHz, 20°C in D2O. Chemical shifts are referenced to external sodium 3-trimethylsilyl propanoate-d4 at 0 ppm. |
| 3-Gal | 3-Fuc | 4-Fuc | 3-Fuc | 4-Xyl |
| (A) | (B) | (C) | (D) | (E) |
| H-1 | 5.32 | 5.12 | 5.06 | 5.03 | 4.66 |
| H-2 | 3.98 | 4.00 | 3.66 | 3.96 | 3.40 |
| H-3 | 4.39 | 4.21 | 3.92 | 3.98 | 4.08 |
| H-4 | 4.31 | 4.74 | 3.74 | 3.80 | 3.76 |
| H-5 | 3.82 | 4.52 | 4.40 | 4.70 | 3.44 |
| H-5’ | 3.64 | 3.44 | 3.40 | 3.76 | 3.44 |
| H-6 | 3.66 | 1.26 | 1.34 | 1.26 | 1.26 |
| H-6’ | 3.60 |

## Table IV

| Observed NOE contacts from anomeric protons at 400 MHz, 20°C, in D2O |
|-------------------------------------------------|
| Anomeric proton | NOE contacts |
|-----------------|--------------|
| 5.32(→3Gal, A) | 3.98(H-2, A), 4.21(H-3, B) |
| 5.12(→3Fuc, B) | 4.21(H-3, B), 4.00(H-4, D), 4.00(H-2, B), 4.08(H-3, D) |
| 5.06(→4Fuc, C) | 3.86(H-2, C), 3.97(H-4, E), 3.44(H-5, E), 3.76(H-3, E) |
| 5.03(→3Fuc, D) | 3.92(H-3, C), 4.00(H-5, C) |
| 4.66(→4Xyl, E) | 4.17(H-3, A), 3.40(H-2, E), 3.62(H-3, E), 3.76(H-4, E) |

![FIG. 4. Proposed structure for Fragment 1.](http://www.jbc.org/)
activity.

Previous work in our laboratory showed that, in starfish, ARIS and the Pronase digest of ARIS (P-ARIS) retain comparable activity to egg jelly, suggesting the importance of the sugar portion for the activity. In accordance with our conclusion, Florman and Wassarman (21) reported that O-linked oligosaccharides present in the glycoprotein ZP3 play a much more important role in the induction of the acrosome reaction in comparison with the protein portion of the molecule in the mouse.

This study was carried out in an attempt to understand the structure-activity relationship of ARIS, and it revealed that a saccharide polymer (Fragment 1) consisting of pentasaccharide repeating units is responsible for the biological activity of ARIS. Ushiyama et al. (22) estimated the molecular mass of the minimum functional unit(s) of ARIS by target analysis. The estimated average target size is in good agreement with the apparent molecular size of Fragment 1. It may be that a tertiary or higher order structure of this polysaccharide, determined by each sugar residue and sulfate group, is recognized by the receptor on the sperm head. The selective desulfation study (DS-1) shows that the removal of 1 mol of sulfate/repeating unit destroys the biological activity of Fragment 1. It suggests the importance of the sulfate group for the activity. However, the Fragment 1 oxidized by periodate did not show the activity although it retained the sulfate fucose residues (data not shown). Additionally, in our preliminary experiments, DS-1 and oxidized Fragment 1 lost the activity for specific binding to spermatozoa.

Since we have obtained an easily manageable active fragment of ARIS, future efforts will focus on the isolation and identification of sperm receptor for ARIS.

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