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Structure of a unique sulfated α-L-galactofucan from the tunicate *Clavelina*

Mauro S. G. Pavão, Paulo A. S. Mourão*,
Departamento de Bioquímica, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Caixa Postal 68041, Rio de Janeiro, RJ 21910 (Brazil)

and Barbara Mulloy
National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire, EN6 3QG (Great Britain)

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ABSTRACT

A purified sulfated α-L-galactofucan from *Clavelina* sp. has been studied before and after desulfation, using periodate oxidation, methylation analysis, and n.m.r. spectroscopy, and shown to be composed mainly of 3-sulfated 4-linked α-L-galactopyranosyl residues. There was also a small proportion of 3-sulfated 4-linked α-L-fucose residues.

INTRODUCTION

Sulfated polysaccharides are abundant[1] in the tunic of ascidians (Chordata, Tunicata). A high-molecular-weight fraction (F-1) composed mainly of galactose and sulfate is present in 5 species studied so far[2,3]. Structural studies[3] revealed large proportions of non-reducing L-galactopyranose end-units in the F-1 fractions from *Styela plicata* and *Ascidia nigra*, indicative of highly branched polymers, but the F-1 fraction from *Clavelina* sp. was largely unbranched.

We now report on the structure of the purified polysaccharide extract from whole *Clavelina*.

EXPERIMENTAL

Extraction, purification, and chemical analysis of the F-1 fraction from Clavelina *sp.* — The sulfated polysaccharides from *Clavelina* colonies were extracted by proteolysis and precipitation with ethanol, then purified by chromatography on DEAE-cellulose as described[4] for *S. plicata*. Further fractionation was effected by gel-filtration chromatography[2,4] on a column of Sepharose CL-4B. Fraction F-1, which contained the high-molecular-weight sulfated polysaccharide, was dialysed against distilled water and lyophilised. Total hexose was measured by the method of DuBois *et al.*[5]. After acid hydrolysis of the polysaccharide (4M trifluoroacetic acid, 6 h, 100°), total hexosamine

* Author for correspondence.

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was measured by a modified Elson–Morgan reaction[6] and sulfate by the $BaCl_2$–gelatin method[7]. The percentages of the various hexoses and hexosamines were determined by g.l.c. of the corresponding alditol acetates[8]. Optical rotations were measured with a Perkin–Elmer model 243-B polarimeter. Agarose and poly(acrylamide) gel electrophoresis of the sulfated polysaccharides was carried out as described[4].

Chemical modification of Fraction F-1. — (*a*) *Desulfation.* This reaction was performed as described for fraction F-1 from *S. plicata.*

(*b*) *Periodate oxidation.* A solution of fraction F-1 (5 mg) in 0.1M $NaIO_4$ (1 mL) was kept for 5 days at room temperature in the dark, then treated with a few drops of ethylene glycol, stored in the dark for 30 min, dialysed against distilled water for 24 h, and lyophilised. The product was reduced with 0.1M $NaBH_4$ in 0.05M NaOH for 24 h at room temperature. Excess of $NaBH_4$ was destroyed by the dropwise addition of glacial acetic acid at 0°, and the mixture was dialysed against distilled water for 24 h, then lyophilised. The resulting polymer was hydrolysed (6 M trifluoroacetic acid, 100°, 5 h), the products were reduced with borohydride, and the resulting alditols were treated conventionally with 1:1 acetic anhydride–pyridine. The products were analysed by g.l.c. on an AN-600 capillary column (30 m × 0.30 mm) (Thames Chromatography) with the temperature programme 140° for 10 min, then to 170° at 2°/min, and held at 170°. The carrier gas was hydrogen at 20 cm/s.

(*c*) *Methylation.* The native and desulfated fraction F-1 were each methylated as follows[9,10]. To a solution of the dry polysaccharide (4 mg) in methyl sulfoxide (1.28 mL) were added powdered NaOH (102 mg) and methyl iodide (90 μL). The mixture was stirred for 60 min at room temperature, cooled, mixed with M acetic acid (3.3 mL) and distilled water (1.28 mL), dialysed against distilled water, and lyophilised. This procedure was repeated three times.

Each methylated polysaccharide was hydrolysed with 4M trifluoroacetic acid for 6 h at 100°, the products were reduced with $NaBH_4$ and then acetylated, and the resulting alditol acetates were analysed by g.l.c. as described above, and by g.l.c.–m.s. as described[3,11].

N.m.r. spectroscopy. — Spectra were recorded with a JEOL GSX500 spectrometer (500 MHz for ^1H, 125 MHz for ^{13}C). Each polysaccharide was repeatedly dissolved in D_2O and the solution was concentrated. The ^1H-n.m.r. spectra (internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate) were recorded at 80°, and the ^{13}C-n.m.r. spectra (internal 1,4-dioxane, 67.4 p.p.m.) at 60°. The COSY spectrum of desulfated fraction F-1 was obtained using the standard pulse sequence supplied.

RESULTS AND DISCUSSION

Purification and fractionation of the sulfated polysaccharides from Clavelina *sp.* — The polysaccharides were purified by ion-exchange chromatography on DEAE-cellulose (Fig. 1A). A single metachromatic peak that contained hexose was eluted with a high concentration of NaCl. The u.v.-absorbing materials emerged during washing of the column and at the beginning of the salt gradient, and a small proportion of neutral sugars also emerged in the washings.

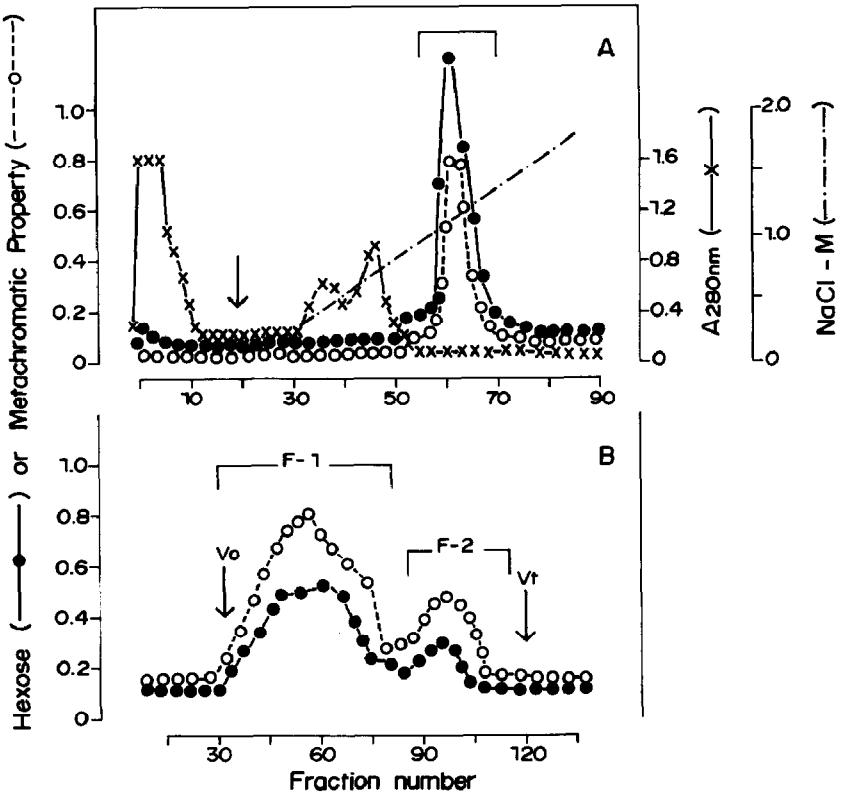

Fig. 1. Fractionation of the sulfated polysaccharides from *Clavelina* sp. *A*. Ion-exchange chromatography: the papain-extracted polysaccharides (~200 mg) were applied to a column (7 × 2 cm) of DEAE-cellulose which was washed with 0.1M sodium acetate buffer (100 mL, pH 5.0) and then a linear gradient with NaCl as described[3]. The fractions were checked for hexose (-●-), metachromasia (-o-), u.v. absorption (-x-), and NaCl concentration (---). The fractions 55–70 were combined, precipitated with 2 vols. of aqueous 95% ethanol, dialysed against distilled water, and lyophilised. *B*. The product (~40 mg) from *A* was eluted from a column (115 × 1.5 cm) of Sepharose CL-4B with 0.5M pyridine acetate buffer (pH 6.0) at 6 mL/h. Fractions (1.5 mL) were checked for hexose (-●-) and metachromasia (-o-). Fractions F-1 and F-2 were separately dialysed against distilled water and lyophilised.

Fractionation of the purified sulfated polysaccharide on Sepharose CL-4B (Fig. 1B) gave fractions of high (F-1) and low (F-2) molecular weight. On electrophoresis (Fig. 2), fraction F-1 gave a single wide band on agarose gel, which could not be distinguished from the original, unfractionated material (Fig. 2A). This fraction did not enter the polyacrylamide gel due to its high molecular weight (Fig. 2B). Fraction F-2 entered the polyacrylamide gel and had an average molecular weight of 18 000.

Composition of fractions F-1 and F-2. — Fraction F-1 was composed of galactose, fucose, and sulfate (Table I), and had $[\alpha]_D$ −104° (water) consistent with α-L-galactopyranose residues[2]. Fraction F-2 was more heterogeneous, had a high content of hexosamine, and had $[\alpha]_D$ −32° (water).

Ion-exchange chromatography of the original polysaccharide eliminated a neu-

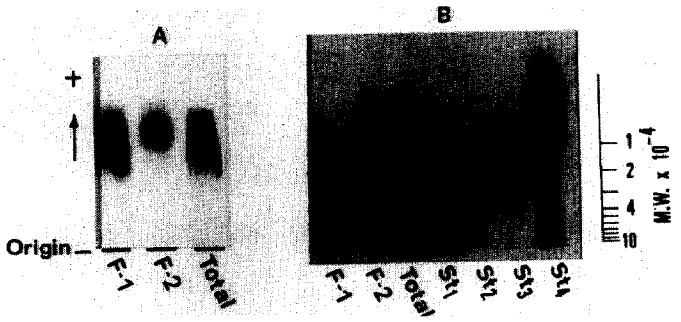

Fig. 2. Electrophoresis of fractions F-1 and F-2. *A*. Fractions F-1 and F-2 (~ 25 μg) were applied to a 0.5% agarose gel and run for 1 h at 110 V in 0.05M 1,3-diaminopropane–acetate buffer (pH 9.0). The polysaccharides in the gel were fixed with aqueous 0.1% cetyltrimethylammonium bromide. After 8 h, the gel was dried and stained with 0.1% Toluidine Blue in acetic acid–ethanol–water (0.1:5:5). *B*. Fractions F-1 and F-2 (~ 50 μg) were applied to 6% polyacrylamide gel in 0.02M sodium barbital buffer (pH 8.6) and run for 30 min at 100 V. The glycans in the gel were stained with 0.1% Toluidine Blue in aqueous 1% acetic acid. The molecular weight markers were dextran sulfate (St_1, 500 000), chondroitin 6-sulfate (St_2, 40 000), dermatan sulfate (St_3, 19 000), and dextran sulfate (St_4, 10 000).

TABLE I

Composition of fractions F-1 and F-2

Fraction	*Molar ratio*						$[\alpha]_D^{20}$
	Gal	*Glc*	*Man*	*Fuc*	*GlcN*	*Sulfate/total sugar*	
F-1	0.76	<0.01	<0.01	0.24	<0.01	0.68	−104°
F-2	0.20	0.16	0.05	0.18	0.41	0.59	− 32°

tral polysaccharide that migrated together with fraction F-1 in our earlier study[2,3]. At the same time, the glucose component (~20%), reported earlier, also disappeared. For fraction F-1 from the solitarian ascidian *S. plicata*, the analyses before and after ion-exchange chromatography were similar (Table I in ref. 3). For this organism, the tunic can be separated completely from other tissues, and neutral polysaccharides were not detected in fraction F-1. Since the polysaccharides from *Clavelina* and other colonial ascidians are extracted from the whole animal (the tunic cannot be separated from the viscera), the neutral polysaccharide may be derived from other tissues.

Structure of fraction F-1. — (*a*) *Periodate oxidation and methylation.* The molar ratios of the products formed from native and desulfated fraction F-1 after periodate oxidation, borohydride reduction, and acid hydrolysis are shown in Table II. The native fraction F-1 resisted periodate oxidation, but large proportions of threitol and butane-1,2,3-triol were produced from desulfated fraction F-1. These products were derived from galactose and fucose, respectively, indicating that HO-2,3 were exposed on desulfation (Table II).

Methylation of fraction F-1 yielded 2,6-di-*O*-methylgalactose (74%) and 2-*O*-

TABLE II

Molar ratios of the products obtained from native and desulfated fraction F-1 after periodate oxidation, borohydride reduction, and strong acid hydrolysis

Product[a] *(as alditol acetate)*	T[b]	*Native F-1*	*Desulfated F-1*
Butane-1,2,3-triol	0.99	<1	26[c]
Threitol	5.02	<1	38
Fucose	6.79	12	12
Galactose	10.68	88	24

[a] The identity of each peak was established by mass spectrometry. [b] Retention time on an AN-600 capillary column relative to that of acetylated glycerol. [c] Since glycerol and butane-1,2,3-triol have similar retention times on this column, it is possible that small proportions of glycerol are also formed.

TABLE III

Molar ratios of the methylated sugars obtained from native and desulfated fraction F-1

Methylated sugar[a] *(as alditol acetate)*	T[b]	*Native*	*Desulfated*
2,3-Me_2-Fuc	1.08	nd[c]	16
2,3,4,6-Me_4-Gal	1.11	nd	6
2-Me-Fuc	1.22	26	1
2,3,6-Me_3-Gal	1.57	nd	71
2,6-Me_2-Gal	1.98	74	6

[a] The identity of each peak was established by mass spectrometry. [b] Retention time on an AN-600 capillary column relative to that of 2,3,4,6-tetra-*O*-methylglucitol. [c] Not detected.

methylfucose (26%) (Table III). This ratio suggested the presence of large amounts of galactose, either 4-linked and 3-sulfated or 3-linked and 4-sulfated. The formation of 2-*O*-methylfucose indicated the presence of intra-chain residues of sulfated fucose.

Methylation of desulfated fraction F-1 yielded 2,3,6-tri-*O*-methylgalactose and 2,3-di-*O*-methylfucose (Table III), indicating that the polysaccharide contained a core of 4-linked and 3-sulfated α-L-galactopyranose residues. The fucose was present as 4-linked 3-sulfated residues, thereby confirming previous data[3]. The small proportion of 2,3,4,6-tetra-*O*-methylgalactose formed may have originated from non-reducing galactose end-groups present originally or exposed during desulfation. The formation of 2,6-di-*O*-methylgalactose indicated that a few sulfate residues survived.

(*b*) ^{1}*H-N.m.r. data.* The ^{1}H-n.m.r. spectra of native and desulfated fraction F-1 are shown in Fig. 3. The spectrum of F-1 was assigned by analogy with that of a polysaccharide with a similar backbone, derived[12] from *S. plicata*, and that of the desulfated F-1, using a 2D-COSY spectrum (data not shown). The chemical shifts of the ^{1}H resonances of desulfated F-1 accorded with those of methyl α-D-galactopyranoside[13] (Table IV). The resonances in the spectrum of native fraction F-1 were somewhat upfield of their

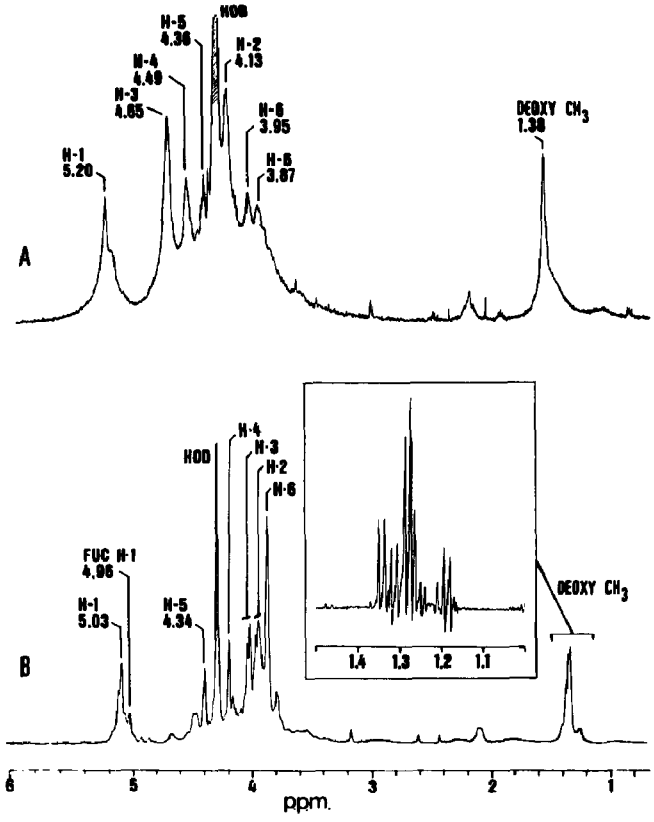

Fig. 3. ^{1}H-N.m.r. spectra (500 MHz) of fraction F-1 before (*A*) and after desulfation (*B*). The spectra were recorded for solutions in D_2O at 80°. The HOD peak is cross-hatched. The resolution of the expanded spectrum inset in *B* was enhanced by Lorentzian–Gaussian transformation.

TABLE IV

^{1}H chemical shifts (δ, p.p.m.) for native and desulfated fraction F-1

Compound	*Gal*							*Fuc*	
	H-1	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*	*H-6*	*H-1*	*H-6*
Native F-1	5.20	4.13	4.65	4.49	4.36	3.95	3.87		1.38
Desulfated F-1	5.03	3.89	3.95	4.13	4.34	3.81	3.81	4.96	1.18
									1.33
α-D-Gal*p*-OMe 3-SO_4[a]	4.90	3.98	4.48	4.32	3.94	3.75	3.76		
α-D-Gal*p*-OMe[a]	4.84	3.82	3.81	3.97	3.90	3.74	3.75		

[a] Data from Contreras *et al.*[13].

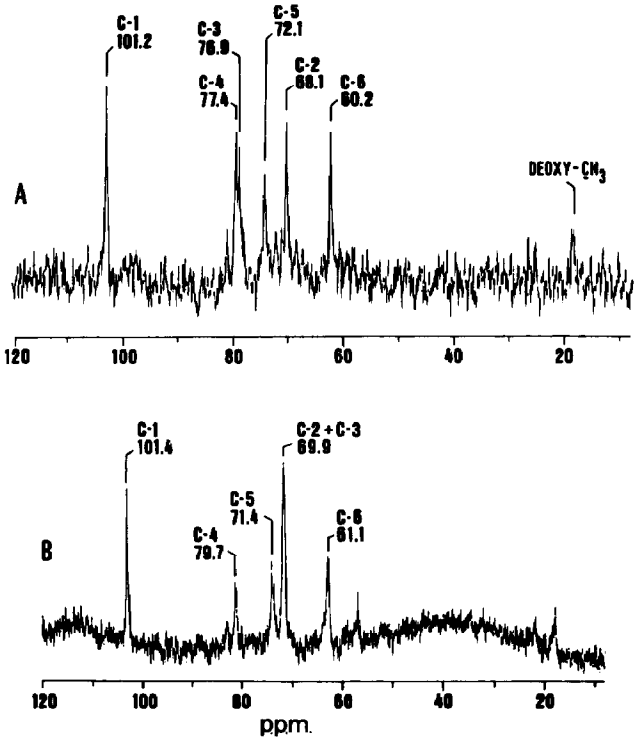

Fig. 4. ^{13}C-N.m.r. spectra (D_2O, 60°) of fraction F-1 before (*A*) and after desulfation (*B*).

equivalents for methyl α-D-galactopyranoside 3-sulfate[13] (Table IV). On desulfation of fraction F-1, the signals for H-3 (−0.70 p.p.m.), H-2 (−0.24 p.p.m.), and H-4 (−0.36 p.p.m.) are shifted as expected for 3-sulfation[13].

A peak at 1.38 p.p.m. (Fig. 3) was assigned to H-6,6,6 of fucose in the purified fraction F-1. The signal at 4.96 p.p.m. was due to H-1 of α-fucopyranoside (Fig. 3B).

(*c*) ^{13}*C-N.m.r. data.* Six signals are clearly distinguished in the ^{13}C-n.m.r. spectrum of the purified fraction F-1 (Fig. 4); C-1 resonated in the range expected[14,15] for an α-D-galactopyranoside (97.7–101.5 p.p.m.). An intense signal corresponding to non-substituted C-6 confirmed the results of the methylation analysis in showing the absence of 6-substitution. Signals at 68.1 and 72.1 p.p.m. (Fig. 4A), in the region for non-substituted secondary carbons, were assigned to C-2 and C-5, respectively, since, according to the results of methylation analysis (Table III), positions 2 and 5 were neither sulfated nor involved in a glycosidic linkage.

The signals at 76.9 and 77.4 p.p.m. (Fig. 4A) occurred in a region expected for substituted carbons. Taking into account the results of the methylation analysis, these peaks were assigned to C-3 and C-4, respectively. After desulfation, the signal for C-3 was shifted upfield by 7.0 p.p.m. and that for C-4 was shifted downfield by 2.3 p.p.m. as expected for 3-sulfation (Fig. 4B and Table V).

Table V shows the ^{13}C resonances of the purified fraction F-1 before and after

TABLE V

^{13}C chemical shifts (δ, p.p.m.) for native and desulfated fraction F-1

Compound	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
Native F-1	101.2	68.1	76.9	77.4	72.1	60.2
Desulfated F-1	101.4	69.9	69.9	79.7	72.4	61.1
α-D-Gal*p*-(1→4)-Gal*p*-(1→3)[a]	100.4	63.3	71.9	78.8	70.0	61.5
		69.5			69.9	
α-D-Gal*p*-OMe 3-SO_4[b]	100.4	67.4	79.0	68.8	71.6	62.2

[a] Data from Bradbury and Jenkins[14]. [b] Data from Contreras *et al.*[13].

desulfation, and provides additional evidence for the above structural interpretations. Comparison of the ^{13}C resonances of desulfated fraction F-1 with those of 4-*O*-galactopyranosylated methyl α-D-galactopyranoside[14] revealed a close correspondence, this unit being the main structure found in the purified fraction F-1. The peak assigned to C-3 of the fraction F-1, which carried an ester, resembled that for C-3 for 3-sulfated methyl α-D-galactopyranoside[13].

Thus, fraction F-1 from *Clavelina* contained 80% of α-L-galactopyranosyl and 20% of α-L-fucopyranosyl residues. The polysaccharide had a core of 3-sulfated 4-linked α-L-galactopyranosyl residues and the α-L-fucopyranosyl units were present as 3-sulfated 4-linked intra-chain residues. Based on the ^1H-n.m.r. data (inset in Fig. 3B), it is speculated that the fucose residues occurred randomly among the galactose residues. The diversity of signals ascribable to Me-5 of fucose indicated that the fucose residues had several different environments.

In earlier work[2,3], the presence of a small proportion of glucose in fraction F-1 from *Clavelina* sp. was reported. In the present work, the ion-exchange chromatography eliminated neutral polysaccharides, and glucose was not present in the purified fraction F-1.

The F-1 fractions from different species of ascidians differ in the extent of branching[3]. Smith degradation of fraction F-1 from *S. plicata* removed mainly the non-reducing terminal residues, and gave a product that was similar to fraction F-1 from *Clavelina* sp. Thus, the F-1 fractions from the ascidians contained similar cores of a sulfated α-L-galactopyranose.

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