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A wide diversity of sulfated polysaccharides are synthesized by different species of marine sponges

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Abstract

Sulfated polysaccharides were extracted from four species of marine sponges by exhaustive papain digestion. These compounds were purified by anion-exchange and gel-filtration chromatography. Analysis of the purified polysaccharides revealed a species-specific variation in their chemical composition and also in their molecular masses. In the species *Aplysina fulva* we found a sulfated glucan with a glycogen-like structure. The other three species contained sulfated polysaccharides with variable proportions of galactose, fucose, arabinose and hexuronic acid and also with different degrees of sulfation. Although the complex nature of these polysaccharides did not allow complete structure determination, we detected the occurrence of 4-sulfated residues of fucose and arabinose in the species *Dysidea fragilis*. The biological role of these sulfated polysaccharides requires further investigation. They may be involved in the species-specific aggregation of sponge cells and/or in the structural integrity of sponge, resembling the proteoglycans of mammalian connective tissues.

Keywords: Sulfated polysaccharide; Marine sponge; Glycogen; Sulfated fucose; Sulfated arabinose

1. Introduction

Sulfated polysaccharides are widely distributed among marine invertebrates. Several of these animals possess polysaccharides with unique structures, such as sulfated L-galactans [1,2], sulfated L-fucans [3–5] or glycosaminoglycan-like polymers, but they differ from mammalian compounds due to additional sugar branches [6–8] or to a distinguishing sulfation pattern [9,10].

In the case of marine sponges, several authors have reported the presence of sulfated polysaccharides [11–13]. These compounds have been associated with cell-interaction processes between sponge cells [14–16]. Species-specific reaggregation of dissociated marine sponge cells was the first experimental system to provide direct evidence for the existence of cell recognition and adhesion [17]. The aggregation depends on the presence of a high-molecular-mass sulfated glycoconjugate, named aggregation factor or proteoglycan-like component.

Besides these extensive studies concerning the biological roles of sulfated polysaccharides in marine sponges, the structure (and even the chemical composition) of these compounds remains unclear. The occurrence of glycosaminoglycans in marine sponges was suggested by some studies [18]. Other authors

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reported that the aggregation factor of marine sponges is a sulfated glycoconjugate with a very complex chemical composition. For one species of marine sponge, the presence of 3-sulfated *N*-acetylglucosamine and 3-sulfated galactose were identified on the purified aggregation factor [13].

We now report the isolation and extensive purification of sulfated polysaccharides from four species of marine sponges. Surprisingly we observed a wide variation in the chemical composition of these sulfated polysaccharides. The species *Aplysina fulva* contains a glycogen-like sulfated glucan, as reported previously [19]. The other three species contain complex sulfated polysaccharides, with different proportions of galactose, fucose, arabinose and hexuronic acid. This wide diversity of sulfated polysaccharides in marine sponges may be related with the role of these compounds as a species-specific cell aggregation factor.

2. Experimental

Materials.—The marine sponges *A. fulva*, *Chondrilla nucula* and *Dysidea fragilis* were collected at Forno beach, Arraial do Cabo, on the northern coast of Rio de Janeiro state, Brazil. The marine sponge *Hymeniacidon heliophila* was collected at Urca beach, Rio de Janeiro, Brazil. Dnase I (EC 3.1.21.1), chondroitin 4-sulfate from whale cartilage, chondroitin 6-sulfate from shark cartilage, dextran sulfates (average mass 8 and 500 kDa) and iodomethane were purchased from Sigma; toluidine blue from Fisher; crude papain and dimethyl sulfoxide from E. Merck; 1,9-dimethylmethylene blue and *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide from Aldrich; trifluoroacetic acid from Vetec; agarose from Bio-Rad; and Sepharose CL-4B from Pharmacia. Chondroitin ABC lyase from *Proteus vulgaris* was from Seikagaku American.

Isolation of acidic polysaccharides.—The sponges were collected from the sea, immersed immediately in acetone, and kept for 24 h at 4 °C. The sponges were cut into small pieces and dried at 60 °C in a oven. The sulfated polysaccharides were extracted from the dried tissues (20 g) by extensive papain digestion, and the extracts were partially purified by cethylpyridinium and EtOH precipitations, using the same methodology described for other tissues [7,19]. About 200 mg (dry weight) of crude extract was obtained after these procedures.

Purification.—The crude extracts of sulfated polysaccharides from the marine sponges (10 mg of each) were applied to a Mono-Q-FPLC column, equilibrated with 20 mM Tris–HCl buffer (pH 8.0). In the case of the *A. fulva*, the crude extract was submitted to a previous purification step using a DEAE-cellulose column eluted with a pH gradient, as described [19]. The polysaccharides were eluted by a linear gradient of 0–3 M NaCl at a flow rate of 0.5 mL/min. Fractions of 0.5 mL were collected and checked by metachromatic assay using 1,9-dimethylmethylene blue [20], phenol–sulfuric acid, [21] and carbazole [22] reactions, and by measuring conductivity. The fractions containing sulfated polysaccharides were pooled, dialyzed against distilled water, lyophilized, and analyzed by agarose and polyacrylamide gels. The Mono-Q purified polysaccharides (20 mg of each) were chromatographed separately on a Sepharose CL-4B column (86 × 1.2 cm). The column was eluted with 0.5 M NaCl in 0.05 M sodium acetate buffer (pH 5.0) at a flow rate of 8 mL/h, and aliquots of 2 mL were collected. The fractions were assayed by the phenol–H_2SO_4 [21] and carbazole reactions [22], and by their metachromatic property [20].

Agarose gel electrophoresis.—The sulfated polysaccharides were analyzed by agarose gel electrophoresis as described [7,19]. Purified sulfated polysaccharides (~ 15 μg) were applied to a 0.5% agarose gel and run for 1 h at 110 V in 0.05 M 1,3-diaminopropane–acetate (pH 9.0). The sulfated polysaccharides in the gel were fixed with 0.1% *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide solution. After 12 h, the gel was dried and stained with 0.1% toluidine blue in 0.1:5:5 AcOH–EtOH–water.

Polyacrylamide gel electrophoresis.—The molecular masses of the sulfated polysaccharides were estimated by polyacrylamide gel electrophoresis. Sulfated polysaccharides (~ 10 μg) were applied to a 6% 1 mm thick

polyacrylamide gel slab in 0.02 M sodium barbital (pH 8.6). After electrophoresis (100 V for 30 min), the gel was stained with 0.1% toluidine blue in 1% AcOH and then washed for about 4 h in 1% AcOH. The molecular mass markers were the low-molecular-mass dextran sulfate (8 kDa), chondroitin 4-sulfate from whale cartilage (40 kDa), chondroitin 6-sulfate from shark cartilage (60 kDa) and high-molecular-mass dextran sulfate ($\sim$500 kDa).

Chemical analysis.—Total hexose and uronic acid were estimated by the phenol–H_2SO_4 reaction [21] and carbazole reaction [22], respectively. After acid hydrolysis of the polysaccharides (6.0 M trifluoroacetic acid for 5 h at 100 °C), sulfate was measured by the $BaCl_2$–gelatin method [23]. The percentages of different neutral sugars were estimated by paper chromatography in 3:2:1 *n*-butanol–pyridine–water for 48 h and by gas–liquid chromatography (GLC) of derived alditol acetates [24].

Desulfation and methylation of the sulfated polysaccharides.—Desulfation of the sulfated polysaccharides was performed by solvolysis in dimethyl sulfoxide, as described previously for desulfation of other types of polysaccharides [1,7]. The native and desulfated polysaccharides ($\sim$5 mg) were subjected to three rounds of methylation as described [25]. The methylated polysaccharides were hydrolyzed with 6 M trifluoroacetic acid for 5 h at 100 °C and reduced with $NaBH_4$, and the alditols were acetylated with 1:1 Ac_2O–pyridine [24]. The alditol acetates of the methylated sugars were dissolved in $CHCl_3$ and analyzed in a gas chromatography–mass spectrometry (GC–MS) unit.

NMR spectroscopy.—^1H spectra were recorded using a Bruker DRX 600 with a triple-resonance 5-mm probe. About 10 mg of each polysaccharide was dissolved in $\sim$0.7 mL of 99.8% D_2O (NMR grade from Sigma). All chemical shifts are relative to internal or external trimethylsilylpropionic acid.

Incubation with yeast amyloglucosidase.—The glycogen-like polysaccharide from *A. fulva* and oyster glycogen were incubated (20 mg of each) with 0.9 U of yeast amyloglucosidase in 0.2 M sodium acetate buffer (pH 5.0) for 22 h at 37 °C. The enzyme-degraded polysaccharides were chromatographed on a Sepharose CL-4B column (86 × 1.2 cm). The column was eluted with 0.5 M NaCl in 0.05 M sodium acetate buffer (pH 5.0) at a flow rate of 8 mL/h, and aliquots of 2 mL were collected. The fractions were assayed by the phenol–H_2SO_4 [21] and by their metachromatic property [20].

Incubation with chondroitin ABC lyase and deaminative cleavage by nitrous acid.—About 100 μg of the sponge polysaccharides were incubated with 0.01 unit of chondroitin ABC lyase in 0.5 M Tris–HCl buffer (pH 8.0) at 37 °C for 8 h. Deaminative cleavage by nitrous acid at pH 4.0 and 2.0 was performed as described [26]. Agarose gel electrophoresis of the control and enzyme- or nitrous acid-incubated polysaccharides was used to assess the enzymatic or deaminative cleavage.

3. Results and discussion

Purification of the sulfated polysaccharides from marine sponges.—The sulfated polysaccharides extracted from four species of marine sponges were purified by a combination of ion exchange on Mono Q-FPLC and gel filtration on Sepharose CL-4B (Fig. 1). These exhaustive fractionation procedures are necessary to achieve purified samples of the polysaccharides for chemical analysis.

Anion-exchange chromatography on Mono Q-FPLC showed a major peak in the cases of the four sponges (Fig. 1(A), (C), (E) and (G)). The phenol–sulfuric acid (○) and carbazole reactions (▲) gave coincident and symmetric peaks, indicating that hexuronic acid and neutral sugars are in the same polymer. Besides these general similarities among the sulfated polysaccharides from the four species of sponges, several differences can be observed. Thus, the sulfated polysaccharide from *H. heliophila* was eluted at lower NaCl concentration than the polysaccharides from the other three species. In the case of *C. nucula* and *D. fragilis*, we observed very narrow peaks than for *A. fulva* and *H. heliophila*, perhaps denoting more homogeneous polysaccharides in terms of molecular mass and/or charge density.

The purity of these sulfated polysaccharides was confirmed by gel filtration on Sepharose CL-4B (Fig. 1(B), (D), (F) and (H)). Some differences were observed on their elution from the column. Polysaccharides from *A. fulva* and *C. nucula* were eluted close to the void volume of the column, but in the case of *D. fragilis* and *H. heliophila*, they were more included in the column. Again, the phenol–sulfuric acid and carbazole reactions gave coincident and symmetric peaks.

Differences among the molecular masses of the sulfated polysaccharides from different species of marine sponge were confirmed by polyacrylamide gel electrophoresis of the purified samples (Fig. 2). The polysaccharides from *C. nucula* and *A. fulva* stayed closed to the origin of the gel, denoting a high molecular mass. On the other extreme was the polysaccharide from *D. fragilis*, which migrates into the gel as a highly disperse band.

Sulfated polysaccharides from marine sponges differ from mammalian glycosaminoglycans.—Agarose gel electrophoresis in 1,3-diaminopropane–acetate, followed by toluidine blue staining, showed some differences on the mobility of the sulfated polysaccharides from different species of sponge (Fig.

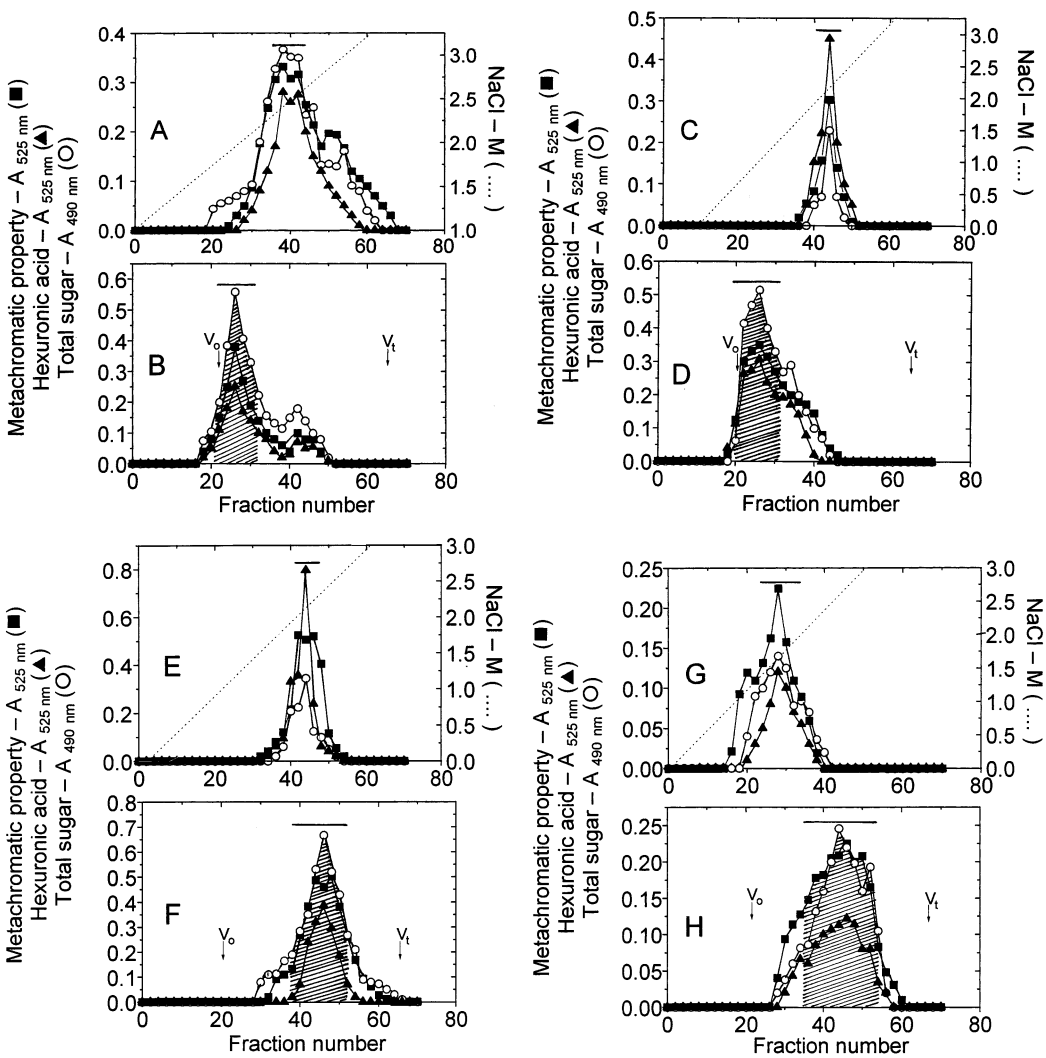

Fig. 1. Purification of the sulfated polysaccharides from marine sponges. The crude polysaccharides from the marine sponges *A. fulva* (A, B), *C. nucula* (C, D), *D. fragilis* (E, F) and *H. heliophila* (G, H) (~ 10 mg of each) were purified by Mono-Q FPLC chromatography (A, C, E and G), as described in Section 2. The partially purified sulfated polysaccharides (~ 20 mg of each) were then chromatographed on a Sepharose CL-4B column (B, D, F and H). Fractions were checked by phenol–H_2SO_4 (○) and carbazole (▲) reactions and for metachromasia (■) and NaCl concentration (---). The fractions indicated by the horizontal bars in the panels were pooled, dialyzed against distilled water, and lyophilized. The fractions corresponding to the purified sponge sulfated polysaccharides are cross-hatched in the panels B, D, F and H.

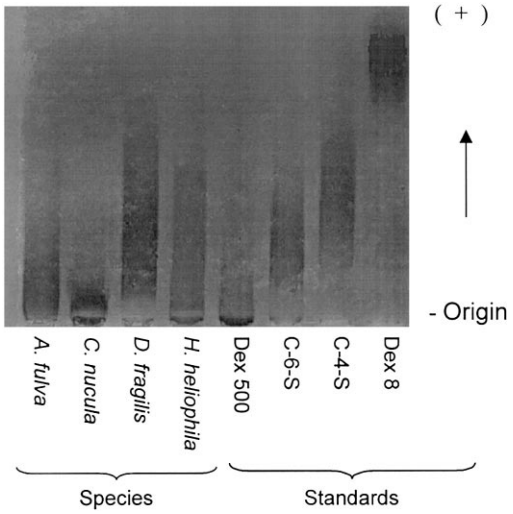

Fig. 2. Polyacrylamide gel electrophoresis of the sponge sulfated polysaccharides. Purified sponge polysaccharides (10 μg of each) were applied to 6% 1 mm thick polyacrylamide gel slabs in 0.02 M sodium barbital (pH 8.6), and run for 30 min at 100 V. After electrophoresis, the sulfated polysaccharides were stained with 0.1% toluidine blue in 1% acetic acid and then washed for about 4 h in 1% acetic acid. The molecular mass markers were high-molecular-mass dextran sulfate (Dex, 500 kDa), chondroitin 6-sulfate from shark cartilage (C-6-S, 60 kDa), chondroitin 4-sulfate from whale cartilage (C-4-S, 40 kDa), and low-molecular-mass dextran sulfate (Dex, 8 kDa).

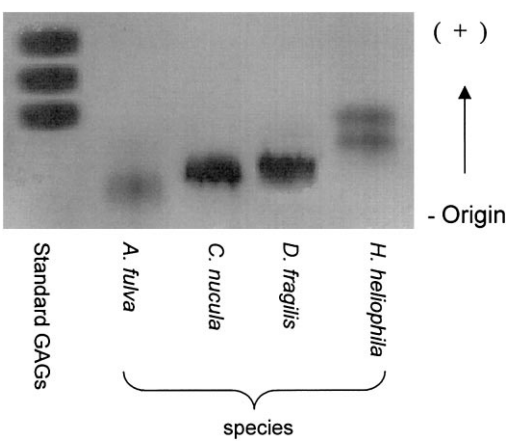

Fig. 3. Agarose gel electrophoresis of the sponge sulfated polysaccharides. Purified sponge polysaccharides (~ 15 μg) and a mixture of standard glycosaminoglycans (GAGs) containing 10 μg of each of chondroitin 4-sulfate (CS), dermatan sulfate (DS), and heparan sulfate (HS) were applied to a 0.5% agarose gel and run for 1 h at 110 V in 0.05 M 1,3-diaminopropane–acetate buffer (pH 9.0). The sulfated polysaccharides were fixed with *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide solution. After 12 h, the gel was dried and stained with 0.1% toluidine blue in 0.1:5:5 acetic acid–ethanol–water.

3). Sulfated polysaccharides from *C. nucula* and *D. fragilis* showed a single, homogeneous and coincident metachromatic band on agarose gel. In the case of *H. heliophila*, we observed two bands with approximately the same proportion. But, more important, the electrophoretic mobility of the sponge polysaccharides differ from those of standard mammalian glycosaminoglycans. In addition, these polysaccharides were incubated with chondroitin ABC lyase and also submitted to deaminative cleavage by nitrous acid at pH 4.0 and 2.0. The agarose gel electrophoresis of the control and the enzyme- or nitrous acid-incubated polysaccharides indicated these compounds are resistant to all these treatments (not shown), thus differing from all previously described glycosaminoglycans.

Sulfated polysaccharides from different species of marine sponges differ in their chemical composition.—Chemical analysis of the purified sulfated polysaccharides from different species of sponge (Table 1) reveals a heterogeneous composition of sugars, with different proportions of galactose, fucose, arabinose and hexuronic acid. Nevertheless, the sulfated polysaccharide from *A. fulva* differs from the other three species due to its relatively less complex composition, as it contains only glucose and small proportion of hexuronic acid. Therefore this polysaccharide is almost a sulfated glucan. The purified polysaccharides from *C. nucula* and *D. fragilis* have a high sulfate content, while the polymers from *H. heliophila* and, especially from *A. fulva*, are less sulfated.

The sulfated glucan from A. fulva *has a glycogen-like structure.*—The ^{1}H NMR spectrum of the sulfated glucan from *A. fulva* has signals coincident with that of a standard oyster glycogen (Fig. 4). In addition, this polysaccharide is partly digested by yeast amyloglucosidase, as shown in the experiment of Fig. 5. Overall, these experiments indicate that the sulfated glucan from *A. fulva* is a form of glycogen, but it has an acidic property (due to the sulfate esters) that causes it to bind to an anion-exchange column. This compound is similar to an acidic glycogen previously described, in the same species of marine sponge [19], except for its higher sulfate content.

Fucose and arabinose units in the D. fragilis *polysaccharides are 4-sulfated.*—^{1}H NMR spectra (not shown) of the sulfated polysac-

Table 1
Chemical composition of the sulfated polysaccharides from different species of marine sponges

Species	Composition (molar ratios) [a]					
	Glc [b]	Gal [b]	Fuc [b]	Ara [b]	HexUA [b]	Sulfate/total sugar [c]
A. fulva	0.90	nd	nd	nd	0.10	0.70
C. nucula	nd	0.18	0.46	0.11	0.25	2.16
D. fragilis	nd	0.22	0.34	0.19	0.25	2.04
H. heliophila	nd	0.59	0.23	nd	0.18	1.20

[a] nd, not detected.
[b] Sugars were identified, after acid hydrolysis, by GLC of derived alditol acetates [24]. Total hexose and hexuronic acid were quantified by the phenol–sulfuric acid [21] and carbazole [22] reactions, respectively.
[c] Sulfate was measured by the $BaCl_2$–gelatin method [23].

charides isolated from the other three species of marine sponges, showed broader and poorly resolved signals, indicating a clearly heterogeneous chemical structure. It was not possible to assign the peaks, even with two-dimension techniques.

We then employed methylation analysis as an approach to probe the complexity of the sulfated polysaccharide from marine sponge. However, some limitations of this method must be considered. Methylation analysis of sulfated polysaccharides does not always yield reliable proportions of methylated alditols [1,2,5,28,29]. This may be a consequence of steric hindrance due to the sulfate esters, which does not allow complete methylation of these polymers. The more drastic conditions necessary to remove sulfate esters may also destroy some of the methylated derivatives (especially from fucose and arabinose). Nevertheless methylation analysis may offer valuable information concerning the position of the glycosidic linkage and the site of sulfation [1–6].

The methylation studies of the *D. fragilis* polysaccharide indicate the presence of arabinose units linked glycosidically through position (1→3) and sulfated at position 4. That is, 2,4-di-*O*-methylarabinose is the methyl ether derivative obtained from the desulfated polysaccharide, whereas 2-*O*-methylarabinose is the methyl ether from the native compound (Table 2). Methylation analysis also indicates the presence of fucose units sulfated at position 4. That is, the derivatives containing methyl ether at position 4 are absent in the native polysaccharide but constitute approximately 35% of the total products formed after desulfation of the polysaccharide (2,4-tri-*O*-methylfucose + 2,3,4-tri-*O*-methylfucose). Finally, the presence of 2,3,4-tri-*O*-methylfucose after methylation of the desulfated polysaccharide indicates this compound is a branched polymer with fucosyl units at nonreducing ends. It was not possible to draw conclusions concerning the galactose residues from the *D. fragilis* polysaccharide. Both the native and desulfated compounds yielded high propor-

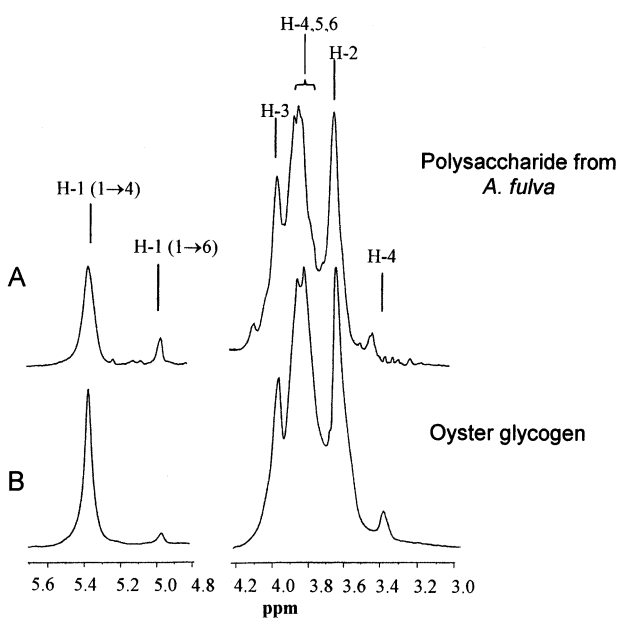

Fig. 4. Expansions of the 5.7–4.8 and 4.2–3.0 regions of the ^1H NMR spectra at 600 MHz of *A. fulva* polysaccharide (A) and standard oyster glycogen (B). The spectra were recorded at 60 °C for samples in D_2O solution. Chemical shifts are relative to internal or external trimethylsilylpropionic acid at 0 ppm. The chemical shifts of the standard glycogen (B) are similar to those reported previously [27], for rabbit liver glycogen. The H-4 signal at 3.44 ppm refers to nonreducing end units.

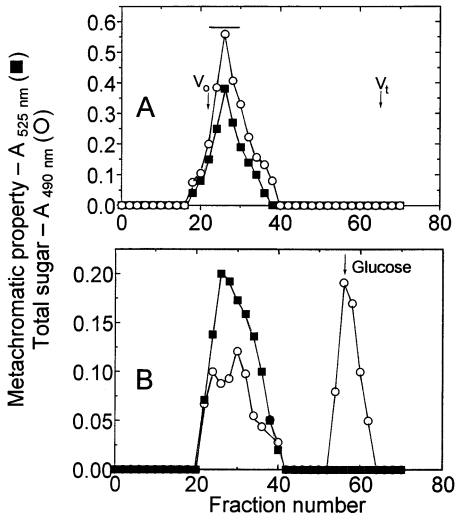

Fig. 5. Degradation of the *A. fulva* polysaccharide with yeast amyloglucosidase. *A. fulva* polysaccharide, before (A) and after (B) exhaustive digestion with yeast amyloglucosidase (20 mg of each) were chromatographed on a Sepharose CL-4B column (86 × 1.2 cm). The column was eluted with 0.5 M NaCl in 0.05 M sodium acetate buffer (pH 5.0) at a flow rate of 8 mL/h, and aliquots of 2 mL were collected and assayed by the phenol–sulfuric acid (○) and by their metachromasy (■). The arrow in (B) indicates the elution of standard glucose.

tions of nonmethylated galactose, even after three rounds of methylation. This may be a consequence of steric hindrance that does not allow for complete methylation of these residues. Nevertheless, both the native and desulfated compounds yielded high proportions of 2-*O*- and 3-*O*-methylgalactose, whereas tri- and tetramethylated derivatives were not observed (not shown in Table 2).

4. Conclusions

Sulfated polysaccharides extracted from four species of marine sponges by exhaustive protease digestion showed a wide diversity in their chemical composition (Table 1). *A. fulva* contains a sulfated glucan with a glycogen-like structure (Figs. 4 and 5), as already observed for another polysaccharide fraction from the same species of marine sponge [19]. The other three species contain sulfated polysaccharides with different proportions of galactose, fucose, arabinose and hexuronic acid and also with variable proportions of sulfation. Amino sugars were not detected in these polymers. These sulfated polysaccharides also vary in the molecular masses (Fig. 1(B), (D), (F) and (H), Fig. 2) and in their electrophoretic mobilities on agarose gel (Fig. 3).

In the case of the species *D. fragilis*, we demonstrated that the fucose and arabinose residues are 4-sulfated (Table 2), but it was not possible to determine the sulfation or linkage pattern of the galactose residues. This is the first report about the structure of the sulfated units in sponge polysaccharides, except for the work of Spillmann and co-workers [13], which demonstrated the occurrence of 3-sulfated *N*-acetylglucosamine and 3-sulfated galactose units in the aggregation factor of a different species of marine sponge.

A sulfated glycoconjugate is an essential requirement for species-specific cell aggrega-

Table 2
Methylated fucose and arabinose derivatives obtained from native and desulfated polysaccharides from *D. fragilis*

Methylated sugars [b] (as alditol acetates)	Retention time [c] (min)	*D. fragilis* polysaccharides [a]	
		Native [d] (mol%)	Desulfated [d] (mol%)
2,3,4-Me_3-Fuc	22.07	nd	15
2,3-Me_2-Fuc	25.95	26	36
2,4-Me_2-Fuc	26.47	nd	20
2-Me-Fuc	28.79	74	29
2,4-Me_2-Ara	25.23	nd	100
2-Me-Ara	28.50	100	nd

[a] nd, not detected.
[b] After three rounds of methylation, the permethylated polysaccharides were hydrolyzed and the products analyzed as their alditol acetate derivatives by GLC–MS.
[c] Retention times on DB-1 capillary column relative to the initial time of the injection of the sample.
[d] The proportions of the methylated derivatives are based on the area under each peak compared with the total area of fucose or arabinose derivatives.

tion in marine sponge, as extensive studies in the literature point out (see Section 1). However, the structure of this sulfated glycoconjugate and variation in the molecule depending on the sponge species, has not yet been reported in the literature. The sulfated polysaccharides described in this work may be involved in the aggregation of marine sponge cells. Alternatively, these compounds may be essential for maintaining the structural integrity of the marine sponges, resembling the structural function of the glycosaminoglycans (and proteoglycans) in the connective tissues of vertebrates. These aspects require further investigation.

Finally, another unique aspect of the marine sponge polysaccharides is the occurrence of arabinose in the species *C. nucula* and *D. fragilis*. This is a common sugar in plant, bacterial, and fungi glycoconjugate but not in animal tissues.

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