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A novel acidic glycogen extracted from the marine sponge *Aplysina fulva* (Porifera-Demospongiae)

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Abstract

Polysaccharides were extracted from the marine sponge *Aplysina fulva* by papain digestion and precipitation with cetylpyridinium chloride. Eluted from an anion-exchange column with a pH gradient, the sponge polysaccharides yielded two distinct fractions, denoted S1 and S2. The S1 fraction, which was eluted at low pH, constitutes about 0.2% of the sponge dry weight, migrates as a single band on agarose gel electrophoresis, and has a simpler chemical composition than the S2 fraction, which is eluted at more alkaline pH. The S1 fraction was further purified by ion-exchange and gel-permeation chromatography. Methylation, ^{1}H and ^{13}C NMR, amyloglucosidase digestion, specific optical rotation, infrared spectroscopy and the reaction with iodine established that the sponge glucan is a form of glycogen. However, the sponge polysaccharide, unlike oyster and rabbit liver glycogen, has an acidic property that causes it to bind to a DEAE-cellulose column at low ionic strength (pH 5.0). Chemical composition and infrared spectroscopy revealed considerable amounts of sulfate esters (≈ 0.05 moles/mole of glucose) in the sponge molecule. Comparative methylation analysis of the intact and desulfated S1 fraction suggests that about 50% of the non-reducing ends of the sponge polysaccharide are sulfated D-glucose residues, perhaps at position O-4.

Keywords: Acidic glycogen; *Aplysina fulva*

1. Introduction

Glycogen is the main polysaccharide involved in energy storage in animal cells. It is highly branched, has an average molecular weight between 2.7×10^5 and 3.5×10^6 Da,

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and is a homopolymer of α-(1 → 4)-linked glucose residues, with occasional α-(1 → 6) linkages at branchpoints [1,2].

Since the identification of glycogen in yeast [3], glycogen molecules have been isolated from various invertebrate and vertebrate species and analysed for molecular structure [1,4]. The structures of the glycogen molecules from different animal phyla are remarkably similar, and they are always uncharged [5,6]. However, the chemical structure of the glycogen in Porifera remains unknown. Levi [7] identified glycogen histochemically in four sponge species, while Boury-Esnault [8] defined the origin and function of the sponge "gray cells" involved in synthesis, accumulation and transport of glycogen to zones of intense metabolism.

We now describe the extraction, purification and preliminary characterization of the structure of an acidic glycogen from the marine sponge *Aplysina fulva*.

2. Experimental

Materials.—The marine sponge *Aplysina fulva* (Porifera-Demospongiae) was collected at a depth of 5 m off Forno beach in Arraial do Cabo, on the northern coast of Rio de Janeiro state, Brazil. Dermatan sulfate, DNase I (EC 3.1.21.1), yeast amyloglucosidase (E.C.3.2.1.3), D-glucose, D-glucuronic acid, D-glucuronolactone and oyster (Type II) and rabbit liver (Type III) glycogens were purchased from Sigma; Toluidine Blue from Fisher; crude papain and dimethylsulfoxide from Merck; 1,9-dimethylmethylene blue and cetylpyridinium chloride (CPC) from Aldrich; trifluoroacetic acid from Vetec; agarose from Bio-Rad; DEAE-cellulose (DE-52) and paper for chromatography (No. 1) from Whatman; and Sephacryl S-1000 from Pharmacia. Galactan from land snail was a gift from Dr. J. Fontana (Departamento de Bioquímica, Universidade Federal do Paraná, Brazil). A sulfated L-galactan from ascidian was prepared as described [9].

Isolation of acidic polysaccharides.—Several specimens of *A. fulva* were immersed in 10 volumes of acetone and kept for 24 h at 4°C. The sponges were cut into small pieces and dried at 60°C in an oven. The dried tissue was suspended in 1000 mL of 0.1 M sodium acetate buffer (pH 5.0) containing 4 g crude papain, 5 mM EDTA, and 5 mM cysteine, and incubated at 60°C for 24 h. The incubation mixture was centrifuged (25,000 × *g* for 20 min at 10°C), and the supernatant was boiled at 100°C for 10 min. After cooling, the supernatant was incubated with 1060 Kunitz units of DNase I for 24 h at room temperature. The mixture was then centrifuged (25,000 × *g* for 15 min at 10°C), and the supernatant was precipitated with 125 mL of 10% CPC (v/v). After 24 h at room temperature, the precipitate was separated by centrifugation (1600 × *g* for 15 min) and washed with 300 mL of 0.05% CPC. The final pellet was dissolved in 500 mL of 2 M NaCl–absolute ethanol (10:1.5, v/v) and mixed with 1000 mL of absolute ethanol. After 24 h at 4°C, the precipitated polysaccharides were separated by centrifugation (25,000 × *g* for 30 min at 10°C), and washed with 2 volumes of 80% ethanol and 1 volume of absolute ethanol. Finally, the acidic polysaccharides were dried in a oven at 60°C.

Fractionation of the acidic polysaccharides on DEAE-cellulose columns.—(a) *pH gradient.* About 200 mg of the crude extract from *A. fulva* were applied to a

DEAE-cellulose column (12 cm × 2 cm i.d.) equilibrated with 0.05 M sodium acetate buffer (pH 5.0) and washed with 200 mL of the same buffer. The column was eluted using a pH gradient, prepared by mixing 120 mL of 0.05 M sodium acetate buffer (pH 5.0) adjusted to pH 1.0 by the addition of glacial acetic acid, and 120 mL of the same buffer adjusted to pH 12.0 by the addition of concentrated NaOH. The flow rate of the column was 12 mL/h, and fractions (3.0 mL) were collected. Fractions were assayed by the phenol–sulfuric acid [10] and carbazole [11] reactions and by their metachromatic property [12], and the pH was measured. The two polysaccharide fractions so obtained (S1 and S2) were dialysed against distilled water and lyophilized.

(b) *Salt gradient.* About 50 mg of the S1 fraction were applied to a DEAE-cellulose column (12 × 2 cm) equilibrated with 0.05 M sodium acetate (pH 5.0) and washed with 200 mL of the same buffer. The column was eluted with a linear gradient of NaCl prepared by mixing 120 mL of sodium acetate buffer (pH 5.0) without salt and 120 mL of 1 M NaCl in the same buffer. Fractions were assayed by the phenol–sulfuric acid [10] and carbazole [11] reactions and by measuring conductivity, as well as by absorbance at 280 nm. Fractions testing positive by the phenol–sulfuric acid assay were pooled, dialysed against distilled water, and lyophilized.

(c) *Gel permeation chromatography.*—The native and desulfated ($S1deSO_4$) S1 fractions, as well as oyster glycogen (about 30 mg each), were chromatographed separately on a Sephacryl S-1000 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–sulfuric acid [10] and carbazole [11] reactions. The column was calibrated using blue dextran as a marker for V_0, and cresol red as a marker for V_t. Fractions comprising the single peak were pooled, dialysed against distilled water and lyophilized.

Agarose gel electrophoresis.—Sulfated polysaccharides were analysed by agarose gel electrophoresis as previously described [13]. About 75 μg of sulfated glycans were applied to a 0.5% agarose gel in 0.05 M 1,3-diaminopropane–acetate buffer (pH 9.0); after electrophoresis, the glycans in the gel were fixed with *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide in water and stained with 0.1% Toluidine Blue in acetic acid/50% ethanol (1:1, v/v).

Chemical analysis.—About 500 μg of the sponge polysaccharides were subjected to acid hydrolysis in 6 M trifluoroacetic acid at 100°C for 5 h. After removal of the acid under vacuum, the sugars were spotted on Whatman No. 1 paper and chromatographed in isobutyric acid: M NH_4OH (5:3, v/v) for 24 h or butanol–pyridine–water (3:2:1, v/v) for 48 h [14]. The sugars were visualized by silver nitrate staining. For identification of hexuronic acid residues in the sponge polysaccharide, about 500 μg of the S1 fraction were subjected to acid hydrolysis with 100 μL of 90% formic acid at 100°C under nitrogen in sealed tubes for 22 h [15]. The sugars obtained were spotted on Whatman No. 1 paper and chromatographed in isobutyric acid: M NH_4OH (5:3, v/v) for 24 h. The sugars were visualized by silver nitrate staining. The following standards were utilized: D-glucose, D-glucuronolactone, D-galacturonic acid, D-glucuronic acid, L-iduronic acid (obtained from dermatan sulfate hydrolysis), D-mannuronic and L-guluronic acid (obtained from alginate hydrolysis). After acid hydrolysis (6 M trifluoroacetic acid at 100°C for 5 h), the sulfate content was measured by the $BaCl_2$/gelatin

method [16] and phosphate was measured as previously described [17]. Sulfate was also identified by paper chromatography after acid hydrolysis of the polysaccharide [18]. The optical rotation of the polysaccharides (5 mg/mL) was measured in a digital polarimeter (Perkin–Elmer model 243-B) and compared with oyster glycogen and D-glucose standards.

Iodine reaction.—The S1 fraction, oyster glycogen, galactan from land snail and sulfated L-galactan from ascidian were tested by the iodine method [19]. Iodine reagent (2.6 mL) was added to 0.4 mL of a solution containing 0.2 mg of polysaccharide. After mixing, a transparent yellow–brown solution was obtained, and its spectrum was recorded by wavelength scanning from 360 to 680 nm in a Hitachi U-2000 spectrophotometer. The extinction coefficient ($E^{1\%}$) was determined at 405 nm.

Desulfation.—Desulfation of the sulfated polysaccharide S1 was performed as previously described [20]. About 20 mg of the S1 fraction in 0.6 mL water was mixed with 1 g (wet weight) of Dowex 50-W (H^+, 200–400 mesh). After neutralization with pyridine, the solution was lyophilized. The resulting pyridinium salt was dissolved in 2 mL of dimethylsulfoxide–methanol (9:1, v/v). The mixture was heated at 80°C for 4 h, and the desulfated products were dialysed against 3 L of distilled water. The extent of desulfation was estimated from the molar ratio of sulfate–total sugars and from the infrared spectrum. About 13 mg of desulfated S1 fraction was obtained.

Methylation analysis.—About 6 mg of each sample were subjected to three rounds of methylation as described [21]. The methylated polysaccharides were hydrolysed with 6 M trifluoroacetic acid for 5 h at 100°C, reduced with borohydride, and the alditols were acetylated with acetic anhydride–pyridine (1:1, v/v). The alditol acetates of the methylated sugars were dissolved in chloroform and analysed on a Hewlett–Packard 5987-A gas chromatography–mass spectrometry unit.

^1H and ^{13}C NMR spectroscopy.—The S1 fraction was analysed by ^1H and ^{13}C NMR at a concentration of approximately 10 mg/mL in deuterium oxide. ^1H NMR spectra (500 NHZ) and ^{13}C NMR spectra (125 MHz) were recorded at 60°C using a Varian Unity 500 spectrometer.

Infrared spectroscopy.—Pellets were formed by packing 100 mg of KBr with 500 μg of native or desulfated sponge polysaccharide or rabbit liver glycogen [22]. Spectra were obtained at room temperature in a Perkin–Elmer 783 infrared spectrophotometer.

Incubation with yeast amyloglucosidase.—The native and desulfated S1, native S2, oyster glycogen and galactan from land snail (500 μg of each) were incubated with 0.9 U of yeast amyloglucosidase [23] in 0.5 mL of 0.2 M sodium acetate buffer (pH 5.0) at 37°C in sealed tubes for different times. At the end of the incubation periods, aliquots (50 μL) were removed from the incubation mixture, heated at 100°C for 5 min to inactivate the enzyme, and then diluted three-fold with distilled water. The amount of reducing sugar (expressed as glucose) in these solutions was estimated by the method of Park and Johnson [24].

3. Results and discussion

Fractionation and chemical analysis of the sponge polysaccharides.—The polysaccharides were extracted from the acetone powder of *A. fulva* by papain digestion and

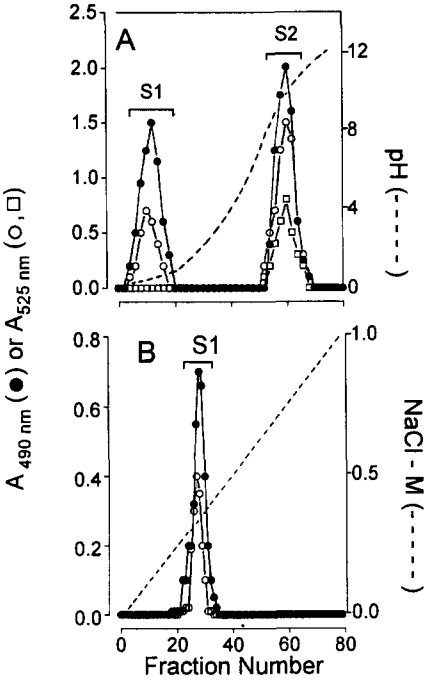

Fig. 1. Fractionation of the acidic polysaccharides extracted from the marine sponge *A. fulva*. In A, about 200 mg of the crude extract were applied to a DEAE-cellulose column and eluted with a pH gradient (1.0 to 12.0). Fractions were analysed by the phenol–sulfuric acid (●) and carbazole (○) reactions and for (□) metachromatic properties (left axis); pH (- - -) was also measured (right axis). Fractions at low pH were pooled and were denoted S1; fractions at the more alkaline pH were denoted S2. In B, the S1 fraction (50 mg) was reapplied to a DEAE-cellulose column and eluted with a salt gradient (0 to 1 M NaCl). Fractions were analysed by the phenol–sulfuric acid (●) and carbazole (○) reactions (left axis), and NaCl concentration (- - -) was measured (right axis).

cetylpyridinium chloride precipitation. Typically, about 250 mg of crude extract were obtained from 40 g of dry tissue. Therefore, approximately 0.5% of the sponge (dry weight) consists of acidic polysaccharides. They were fractionated on a DEAE-cellulose column eluted with a pH gradient (Fig. 1A). Fractions 5 to 20 and 52 to 68 were pooled and denoted S1 and S2, respectively. Both S1 and S2 contain hexoses, hexuronic acid and sulfate, but only S2 is metachromatic. Agarose gel electrophoresis shows S1 as a single band while S2 has various components with the Toluidine Blue staining characteristics of highly sulfated polysaccharides such as glycosaminoglycans (Fig. 2). About 50 mg of the S1 fraction were applied to a DEAE-cellulose column and eluted with a linear salt gradient (0–1 M NaCl). The fractions were monitored by the phenol–sulfuric acid [10] and carbazole [11] reactions, and a single peak was obtained (Fig. 1B). Gel permeation chromatography of this material (30 mg) on a Sephacryl S-1000 column yielded a single peak with high molecular weight (Fig. 3A) very similar to that of oyster glycogen (Fig. 3B). In both cases, the phenol–sulfuric acid and carbazole reactions gave

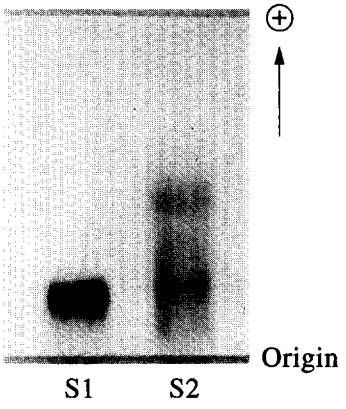

Fig. 2. Agarose gel electrophoresis of the S1 and S2 fractions. About 75 μg of each fraction were applied to a 0.5% agarose gel and run for 1 h at 110 V in 50 mM 1,3-diaminopropane–acetate buffer (pH 9.0). The polysaccharides in the gel were fixed with 0.1% cetyltrimethylammoniun bromide and stained with 0.1% Toluidine Blue in acetic acid–50% ethanol (1:1, v/v).

two coincident and symmetric peaks, indicating that hexuronic acid and glucose are in the same polymer.

Chemical analysis of *A. fulva* polysaccharides in the S2 fraction revealed the presence of highly sulfated polysaccharides with a heterogeneous composition of sugars, whereas S1 contains only glucose, hexuronic acid and sulfate groups (Table 1). The possibility that low amounts of the S2 fraction might be contaminating S1 was excluded by the observation that galactose and fucose, present in high amounts in S2, are absent from the S1 fraction (Table 1) [1]. The positive optical rotation of S1 (+150°) resembles that reported for a glycogen extracted from mussel (+140°) [25]. After desulfation, the S1 fraction presents an optical rotation very close to that of other animal glycogens (Table 1). These values, together with the NMR data (Table 2) and enzymatic degradation by amyloglucosidase (Fig. 4), support the occurrence of α-D-glucopyranosyl units in the S1 fraction. Indeed, the glucose obtained by acid hydrolysis of S1 is totally oxidized by D-glucose oxidase (not shown). The hexuronic acid residue was identified as glucuronic acid (not shown) after hydrolysis with formic acid and paper chromatography using commercial and prepared standards (see Experimental section). Interestingly, we also detected hexuronic acid by the carbazole reaction in oyster glycogen (Fig. 3B) and rabbit liver glycogen (Table 1). This observation suggests that sponge, rabbit and oyster glycogen are heteropolysaccharides.

Comparison between sponge glucan and other animal glycogens.—The positive iodine reaction of the S1 polysaccharide and the characteristic infrared band at 820 cm^{-1} (not shown) suggest structural similarities with oyster glycogen. Both polysaccharides give a yellowish-brown colour with maximum absorption at 405 nm. The distinct

[1] The complexities of the chemical composition (Table 1) and NMR spectra (not shown) of the S2 fraction indicate a complex structure for this polysaccharide, which is currently under investigation.

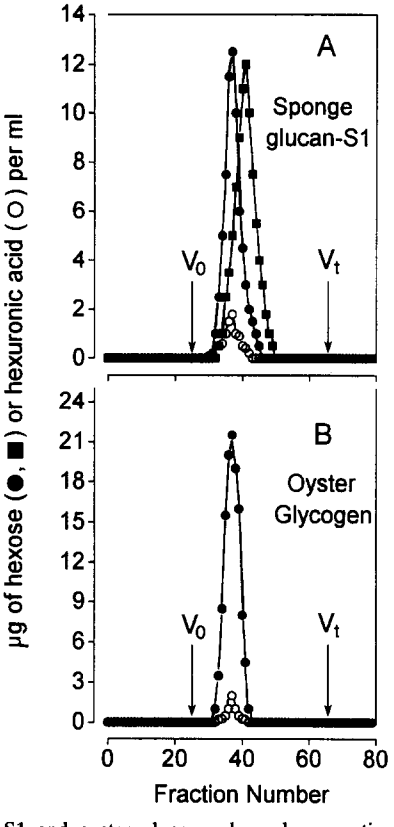

Fig. 3. Comparison of sponge S1 and oyster glycogen by gel permeation chromatography on Sephacryl S-1000. In A, about 30 mg of native S1 (●) and 30 mg of desulfated S1 (■) were applied to a Sephacryl S-1000 column and eluted with 0.5 M NaCl in 0.05 M sodium acetate buffer (pH 5.0). In B, the same procedure was applied to 30 mg of standard oyster glycogen. Fractions were assayed by the (● or ■) phenol sulfuric acid and carbazole (○) reactions.

extinction coefficients (oyster glycogen, $E^{1\%}_{405} = 45.4\ cm^{-1}$; sponge glucan, $E^{1\%}_{405} = 95.9\ cm^{-1}$) suggest differences in the external chains or in the degree of branching of these polysaccharides [19]. In addition, methylation analysis of native and desulfated S1 and oyster glycogen showed in all three cases a high proportion of 2,3,6-tri-*O*-methylglucose, which indicates the presence of linear portions made up of (1 → 4)-linked glucopyranose units (Table 3). Hydrolysis of the permethylated polyssacharides yielded 2,3,4,6-tetra-, 2,3,6-tri- (mainly) and 2,3-di-*O*-methyl-D-glucose. Like oyster glycogen, the sponge polysaccharide yields 2,3-di-*O*-methylglucose, which is characteristic of branch points with glucose units in (1 → 6)-linkage (Table 3). All three polysaccharides yielded 2,3,4,6-tetra-*O*-methylglucose, which reflects the presence of glucose at the non-reducing ends, as described for various animal glycogens [26]. The ^{1}H and ^{13}C NMR chemical shifts for the S1 polysaccharide were compared with those established for rabbit liver glycogen [27] (Table 2). The principal anomeric resonance (5.38 ppm)

Table 1
Chemical composition of native and desulfated S1, native S2 and oyster glycogen

Polysaccharide	Glc [a]	Gal [a]	Fuc [a]	HexUA [a]	SO_4 /total sugar [b]	$[\alpha]_D^{20°C}$ (deg)
Native S1	0.90 [c]	< 0.01	< 0.01	0.10	0.05	+150
Desulfated S1	0.90 [c]	< 0.01	< 0.01	0.10	< 0.01	+190
Native S2	< 0.01	0.46	0.38	0.16	0.48	+86
Oyster glycogen	0.92	< 0.01	< 0.01	0.08	< 0.01	+196
Rabbit liver glycogen	0.95	< 0.01	< 0.01	0.05	< 0.01	+198

[a] Sugars were identified, after acid hydrolysis, by paper chromatography. Total hexose and hexuronic acid were quantified by the phenol–sulfuric acid [10] and carbazole [11] reactions, respectively.
[b] Sulfate was measured by the BaCl/gelatin method [16].
[c] Glucose occurs entirely in the D-enantiomeric form since this sugar was totally oxidized by D-glucose oxidase.

corresponds to H-1 (1 → 4) with a smaller resonance at 4.98 ppm corresponding to H-1 (1 → 6). The NMR data confirm the structural resemblance between S1 and glycogen.

Unique properties of A. fulva *acidic glucan.*—Differences between the S1 polysaccharide from sponge and standard glycogens can be visualized by agarose gel electrophoresis. The S1 polysaccharide migrates on agarose gels and is stained by Toluidine Blue (Fig. 2), whereas standard glycogens do not show these properties (not shown). In addition, sulfate ester was detected in the sponge glucan by chemical analysis (Table 1), paper chromatography and infrared spectrophotometry. The 1240 cm^{-1} absorption band characteristic of S=O groups can be observed in native S1, but disappears in the desulfated S1 (not shown). In rabbit liver glycogen, on the other hand, ester sulfate groups are not detected by chemical analysis (Table 1), and the 1240 cm^{-1} band is absent from the infrared spectrum (not shown). The acidic property of the sponge glucan cannot be attributed to the small amount of hexuronic acid present, since we found

Table 2
^1H and ^{13}C chemical shifts (ppm) of sponge polysaccharide S1 and rabbit liver glycogen

^1H Chemical shifts [a] (ppm)			^{13}C Chemical shifts [a] (ppm)		
Assignment	S1	Rabbit liver glycogen [b]	Assignment	S1	Rabbit liver glycogen [b]
H-1 α-(1 → 6)	4.99	4.98			
H-1 α-(1 → 4)	5.38	5.38	C-1	100.5	100.5
H-2	3.67	3.66	C-2	72.1	72.3
H-3	3.98	3.97	C-3	73.8	74.0
H-4, 5, 6a, 6b	3.86	3.85	C-4	78.0	78.0
H-4 [c]	3.46	3.44	C-4[c]	70.1	70.1
			C-5	72.1	72.0
			C-6	61.3	61.3

[a] 500 MHz ^1H and 125 MHz ^{13}C NMR spectra were recorded at 60°C in 99% deuterium oxide. ^{13}C Chemical shifts are relative to external trimethylsilane at 0 ppm; ^1H Chemical shifts are relative internal sodium 3-(trimethylsilyl) propionate-d_4.
[b] Data from Zang et al. [27].
[c] Non-reducing end unit.

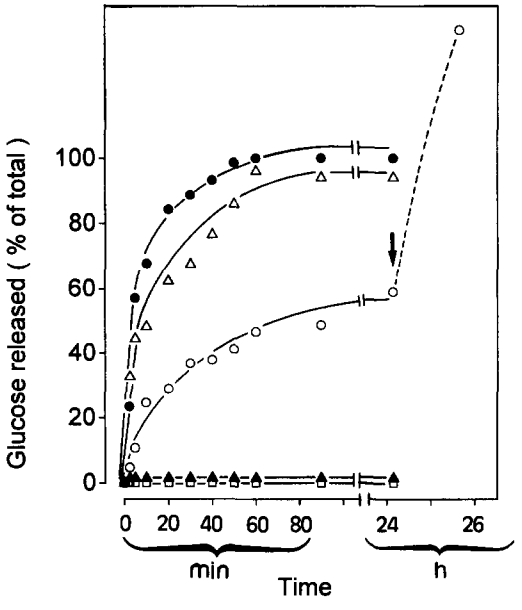

Fig. 4. Degradation of the native and desulfated polysaccharides with yeast amyloglucosidase. Native S1 (○), desulfated S1 (△), native S2 (▲), oyster glycogen (●) and galactan from land snail (□) (500 μg of each) were incubated with 0.9 U of yeast amyloglucosidase in 0.5 mL of 0.2 M sodium acetate buffer (pH 5.0) at 37°C for different times. At the end of the incubation periods, aliquots of 50 μL were removed and reducing sugar was estimated by the method of Park and Johnson (see Experimental section). At the point indicated by the arrow, 500 μg of oyster glycogen were added to the incubation mixture and an additional sample was removed after 2 h.

similar amounts of these residues in oyster glycogen, which is not acidic. The binding of intact and desulfated sponge glucan to an ion-exchange column at low pH was compared with that of oyster and rabbit liver glycogens (Fig. 5). In this experiment, each polysaccharide was applied to a DEAE-cellulose column and eluted stepwise with buffers of increasing ionic strength. The intact S1 was retained by the column at low ionic strength while the oyster and rabbit liver glycogens were not. Desulfation of the S1 fraction drastically reduced the ionic interaction with the DEAE-cellulose column (Fig. 5).

Comparison of the tetra-*O*-methyl derivatives obtained from native and desulfated S1 shows that the proportion of 2,3,4,6-tetra-*O*-methylglucitol increases after desulfation (Table 3). This result could be ascribed to the cleavage of (1 → 4)-linkages of the central core during chemical desulfation. This possibility was excluded by the analysis of the molecular mass of the S1 fraction after desulfation (Fig. 3A). The reduction of the molecular mass is not sufficient to explain the increase in the tetra-*O*-methyl derivative. Therefore, the methylation experiment suggests the presence of sulfate ester at the non-reducing terminal glucose residues in the native molecule, possibly at position O-4. Since the proportion of tetra-*O*-methyl derivatives in desulfated S1 is twice that of native S1, ≈ 50% of the non-reducing terminal glucose residues may contain the sulfate

Table 3
Methylation analysis of native and desulfated S1 from *A. fulva*, and comparison with oyster glycogen

Methylated sugars [a] (as alditol acetates)	Retention time [b] (min)	S1 Polysaccharide		Oyster glycogen [c] (mol%)
		Native [c] (mol%)	Desulfated [c] (mol%)	
2,3,4,6-Me_4-Glc	16.3	9.9	19.9	12.8
2,3,6-Me_3-Glc	20.8	77.5	68.3	77.9
2,3-Me_2-Glc	25.1	12.6	11.8	9.3

[a] After three rounds of methylation, the permethylated polysaccharides were hydrolysed and the products analysed as their alditol acetate derivatives by GLC and GLC-mass spectrometry.
[b] Retention times are relative to the initial time of the injection of the sample.
[c] The proportions of the methylated sugars are based on the area under each peak compared with the total area of all three methyl derivatives. These values were corrected for molar response according to the effective carbon response theory [28].

ester. The higher proportion of di-*O*-methyl derivatives in native and desulfated sponge glucan indicates more extensive branching compared to oyster glycogen (Table 3). The sulfated glucose units were not identified in the ^{1}H and ^{13}C NMR spectra possibly due to the occurrence of small proportions of these units in the polysaccharide [2].

The influence of the sulfate groups on the rate of degradation of the native and desulfated polysaccharides incubated with yeast amyloglucosidase is shown in Fig. 4. In this experiment, native and desulfated S1, native S2, oyster glycogen, and galactan from landsnail were incubated with the enzyme at different times. The oyster glycogen was totally degradated to glucose by the amyloglucosidase, but the native sponge glucan was only partially degradated (about 55%). The addition of 500 μg of glycogen after 24 h incubation of native S1 with the amyloglucosidase showed that the enzyme was still active (Fig. 4, arrow). After desulfation, however, the sponge glucan was totally degradated by the enzyme, suggesting that the sulfated glucose units present at the non-reducing ends of the native S1 are responsible for the decrease in the degradation rate. The controls, native S2 and galactan from land snail, were not degradated by the amyloglucosidase.

4. Conclusion

The acidic polysaccharide (S1) extracted from the marine sponge *A. fulva* has a glycogen-like structure. It contains mainly (1 → 4)-linked α-D-glucopyranose units, and some residues are branched at the O-6 position ending in non-reducing terminal

[2] It is difficult to assign signals in the NMR spectra to sulfated units when these residues are present in small proportions. We already reported an example of this problem in the NMR analysis of a sulfated galactofucan [29]. In this polysaccharide, the signals assigned to the small amounts of sulfated fucose residues ($\approx$ 10% of total sugar units), which are randomly distributed among sulfated galactose units, could not be identified in the spectra.

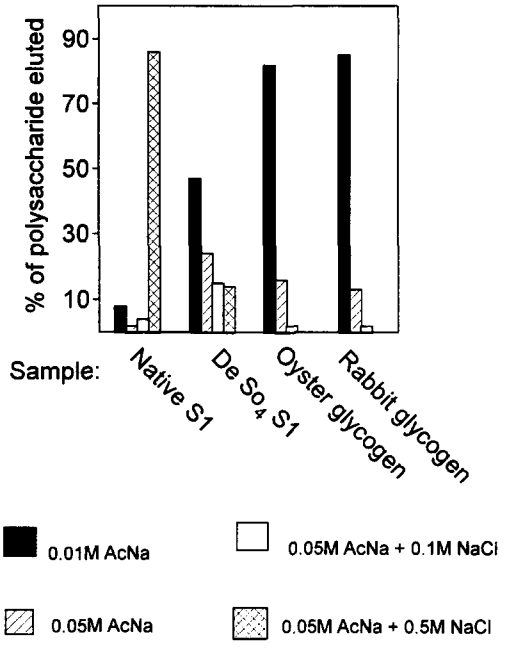

Fig. 5. Ionic interaction with DEAE-cellulose column of native, partially desulfated S1, desulfated S1, oyster and rabbit liver glycogens. About 2 mg of each polysaccharide were applied to a DEAE-cellulose column (10×0.5 cm) and eluted step-wise using 2 mL each of the following solutions (in the order given): 0.01 M sodium acetate buffer (pH 5.0) (**1**); 0.05 M of sodium acetate buffer (**2**); 0.1 M NaCl in sodium acetate buffer (**3**); 0.5 M NaCl in sodium acetate buffer (**4**). Fractions (2 mL) were collected and analysed by the phenol–sulfuric acid reaction. The left axis shows the relative percentages of polysaccharides which eluted from each column at different ionic strengths.

α-D-glucopyranose units. However, about 50% of the non-reduced residues are sulfated, possibly at O-4, conferring acidic properties to the sponge polymer. Substitution with sulfate groups has not been reported in any other vertebrate and invertebrate glycogen. Phosphate was not detected in the sponge glycogen. A few glucuronic acid units are present, but the contribution of these residues to the molecular structure is not clear at present. Since a similar amount of hexuronic acid is also detected in oyster and rabbit glycogen, it cannot be responsible for the acidic property of sponge glycogen; this unique characteristic must be due to the presence of sulfate ester groups.

It is not yet possible to consider the acidic glycogen of *A. fulva* as a common trait of the phyllum *Porifera*. Likewise, the biological significance of the unusual acidic nature of this polysaccharide is unknown at present. We have demonstrated that the presence of sulfate ester groups at the non-reducing end of this polysaccharide reduces the rate of its degradation by yeast amyloglucosidase (Fig. 4). Thus, it is possible that these sulfate groups render the sponge glycogen more resistant to the degradative enzymes of microorganisms that inhabit the sponge canals.

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