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Structure and anticoagulant activity of a sulfated galactan from the red alga, *Gelidium crinale*. Is there a specific structural requirement for the anticoagulant action?

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Abstract—Marine red algae are an abundant source of sulfated galactans with potent anticoagulant activity. However, the specific structural motifs that confer biological activity remain to be elucidated. We have now isolated and purified a sulfated galactan from the marine red alga, *Gellidium crinale*. The structure of this polysaccharide was determined using NMR spectroscopy. It is composed of the repeating structure -4-α-Gal*p*-(1→3)-β-Gal*p*1→ but with a variable sulfation pattern. Clearly 15% of the total α-units are 2,3-di-sulfated and another 55% are 2-sulfated. No evidence for the occurrence of 3,6-anhydro α-galactose units was observed in the NMR spectra. We also compared the anticoagulant activity of this sulfated galactan with a polysaccharide from the species, *Botryocladia occidentalis*, with a similar saccharide chain but with higher amounts of 2,3-di-sulfated α-units. The sulfated galactan from *G. crinale* has a lower anticoagulant activity on a clotting assay when compared with the polysaccharide from *B. occidentalis*. When tested in assays using specific proteases and coagulation inhibitors, these two galactans showed significant differences in their activity. They do not differ in thrombin inhibition mediated by antithrombin, but in assays where heparin cofactor II replaces antithrombin, the sulfated galactan from *G. crinale* requires a significantly higher concentration to achieve the same inhibitory effect as the polysaccharide from *B. occidentalis*. In contrast, when factor Xa instead of thrombin is used as the target protease, the sulfated galactan from *G. crinale* is a more potent anticoagulant. These observations suggest that the proportion and/or the distribution of 2,3-di-sulfated α-units along the galactan chain may be a critical structural motif to promote the interaction of the protease with specific protease and coagulation inhibitors.

Keywords: Sulfated polysaccharides; Sulfated galactans; Carrageenans; Anticoagulant activity; Marine red alga

1. Introduction

Sulfated galactans are among the most abundant non-mammalian sulfated polysaccharides found in nature. They occur in high concentrations, not only in marine red algae (Rhodophyta),[1,2] but are also found in marine invertebrates[3–5] and in sea grass,[6] a group of vascular plants that occur in the marine environment.

Sulfated galactans from marine algae (also known as carrageenans or agarans) are composed of alternating 3-linked β-galactopyranose and 4-linked α-galactopyranose.[1,2] However, considerable structural variation occurs among the polysaccharides obtained from different species and collected at different environments or in different periods of the year. A substantial part or even all the α-galactose residues may exist in the form of

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3,6-anhydro derivatives. Furthermore, various hydroxyl groups may be substituted by sulfate ester, a methyl group or pyruvic acid.[7] These structural variations contribute to the highly heterogeneous and complex nature of the sulfated galactans obtained from marine algae.

In contrast with the algal polysaccharides, sulfated galactans from marine invertebrates have a more homogeneous and simple structure. They are composed of α-L-galactopyranose (the D-isomer is not found) and in most species they are highly branching polymers.[3–5] Sea grass sulfated galactan contains both α- and β-isomers of D-galactose but with a regular sulfation pattern.[6]

Anticoagulant activity is among the most widely studied properties of sulfated polysaccharides. The anticoagulant glycosaminoglycan heparin is an important therapeutic agent for prophylaxis and treatment of thrombosis.[8] Other sulfated polysaccharides, either extracted from marine algae,[9–14] invertebrates[15–17] or obtained by chemical sulfation of natural polysaccharides,[18] have been described as anticoagulant agents. In contrast with heparin, the structural component of the algal sulfated polysaccharides responsible for the anticoagulant activity has not been characterized.

Recently, we undertook a systematic analysis of the anticoagulant activity of sulfated galactans using several polysaccharides from invertebrate and algal origin. We showed that linear sulfated galactans express anticoagulant activity not merely as a function of charge density. The structural basis for this activity certainly depends on the monosaccharide composition, sites of sulfation and/or of the glycosidic linkage and also on the occurrence of disulfated units.[16,17,19,20] Thus, the presence of 2,3-disulfated galactose units in the sulfated galactan from the marine alga, *Botryocladia occidentalis* (see structure in Fig. 1A) has an amplifying effect on the anticoagulant activity.[19,20]

We studied the structural and anticoagulant activity of a sulfated galactan from a different species of red alga, named *Gelidium crinale*. We also analyzed the structure of a 3,6-anhydrogalactose-enriched galactan (Fig. 1B) obtained from the red alga, *Gracilaria cornea*. Comparison among these algal galactans allowed us to propose an approach to distinguish between α-galactose and 3,6-anhydro-α-galactose units based on NMR spectra. The results obtained from the anticoagulant activity of the sulfated galactans from *G. crinale* and *B. occidentalis* suggest that the content of 2,3-disulfated α-galactose units modifies the interaction of the polysaccharide with coagulation inhibitors and their target proteases.

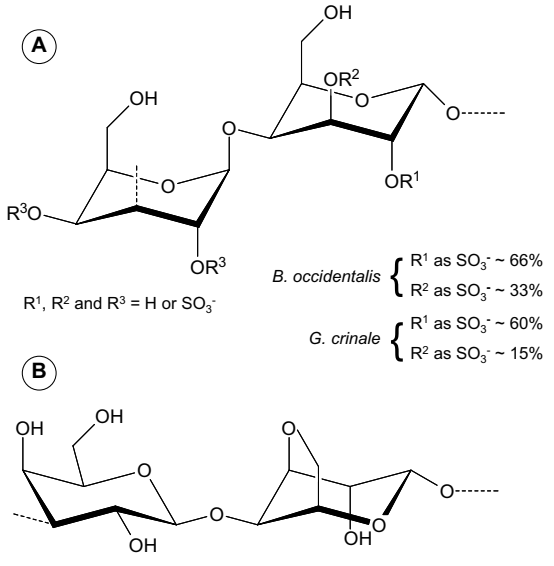

Figure 1. Structures of the galactans from various species of marine red algae. The polysaccharides from *G. crinale* and *B. occidentalis* have the following repeating structure (-3-β-D-Gal*p*-(1→4)-α-D-Gal*p*-(1→), with a variable sulfation pattern (A). NMR analysis indicate the galactans from *G. crinale* and *B. occidentalis* differ in the proportions of 2,3-disulfated and 2-sulfated α-galactopyranose residues. Galactose occurs entirely as D isomer in these polysaccharides. The galactan from *G. cornea* has 3,6-anhydro-α-L-galactose instead of α-galactose units (B). See text and Ref. 19 for the structure of the sulfated galactan from *B. occidentalis*.

2. Experimental

2.1. Sulfated galactans

The marine red alga, *Gelidium crinale*, was collected at Pacheco beach, Caucaia, Ceará, Brazil, separated from other species, washed with distilled water and macerated in liquid nitrogen. This sample was then washed with 25 mM Tris–HCl (pH 7.5) for the extraction of lectin. No sulfated polysaccharide was removed from the tissue during this procedure. Sulfated polysaccharides were extracted from the pellet as described previously.[19] Essentially, the pellet was dried at room temperature (5 g, dry weight), suspended in 250 mL of 0.1 M NaOAC (pH 6.0), containing 1.0 g of papain, 5 mM EDTA and 5 mM cysteine and incubated at 60 °C for 24 h. The incubation mixture was then filtered and the supernatant was saved. The residue was washed with 138 mL of distilled water, filtered again and the two supernatants were combined. Sulfated polysaccharides in solution were precipitated with 16 mL of 10% cetylpyridinium chloride solution. After standing at room temperature for 24 h, the mixture was centrifuged at 2560*g* for 20 min at 5 °C. The sulfated polysaccharides in the pellet were washed with 610 mL of 0.05% cetylpyridinium chloride solution, dissolved with 172 mL of a 2 M NaCl–EtOH (100:15, v/v) mixture and precipitated with 300 mL of abs EtOH. After 24 h at 4 °C the precipitate was collected by centrifugation (2560*g* for 20 min at 5 °C), washed twice with 305 mL of 80%

EtOH and once with the same volume of abs EtOH. The final precipitate was dried at 60 °C overnight. Approximately 120 mg (dry weight) of crude polysaccharides were obtained after these procedures (yield of 2.4%).

A sample of sulfated galactan enriched with 3,6-anhydro- galactose was obtained from the red alga *Gracilaria cornea*, collected at Fleixeiras beach, Trairi, Ceará, Brazil. The algae were cleaned of epiphytes, dried and washed with distilled water (1.5% w/v) under mechanical stirring for 10 min at 20 °C. The residue was removed by centrifugation, mixed again with the same volume of distilled water and maintained at 100 °C for 45 min. The supernatant of this second extraction, which contained most of the sulfated galactan, was lyophilized, weighed and stored. The main components of this polysaccharide are nonsulfated β-galactose and 3,6-anhydro-α-galactose units but it contains also small amounts of sulfate ester at C-6 of the α-galactose units.[21] These sulfate groups were removed from the polysaccharides by alkaline treatment, yielding 3,6-anhydrogalactose.[22] For this treatment, the polysaccharide (200 mg) was dissolved in 100 mL of distilled water, mixed with 20 mg of $NaBH_4$, maintained at room temperature for 16 h, then NaOH was added to a final concentration of 1 M together with 10 mg $NaBH_4$. After 4 h at 80 °C the solution was neutralized with 8 M HCl, dialyzed against distilled water and lyophilized. Approximately 120 mg of polysaccharide was obtained after these procedures (yield of 60%). We will hereby refer to this sample as '3,6-anhydrogalactose-enriched galactan'.

2.2. Purification of the sulfated galactan from *G. crinale*

The crude sulfated polysaccharide from *G. crinale* (10 mg) was dissolved in 5 mL of 50 mM NaOAc (pH 6.0) and applied to a DEAE-cellulose column (9.0 × 2.0 cm) equilibrated with the same solution. The column was developed by a stepwise gradient of 0 → 3.0 M NaCl in the same solution. The flow rate of the column was 0.5 mL/min. Fractions of 3.0 mL were collected and assayed for sulfated polysaccharide using the metachromatic assay with 1,9-dimethylmethylene blue.[23]

2.3. Chemical analyses

Total galactose was measured by the method of Dubois et al.[24] After acid hydrolysis of the polysaccharide (5.0 M CF_3CO_2H for 5 h at 100 °C), sulfate was measured by the $BaCl_2$/gelatin method.[25] The types of hexose present in the acid hydrolysates were estimated by paper chromatography in 3:2:1 *n*-butanol–pyridine–water for 48 h and by gas–liquid chromatography–mass spectrometry of derived alditols.[26]

2.4. Agarose gel electrophoresis

Sulfated galactans were analyzed by agarose gel electrophoresis as described previously.[19] The sample (~15 μg) was 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 HOAc–EtOH–water (v/v).

2.5. Desulfation of the galactan from *C. crinale*

Desulfation of the sulfated galactan was performed by solvolysis in dimethyl sulfoxide as described previously for desulfation of other types of polysaccharides.[3] Approximately 25 mg of native galactan was used to give ~7 mg of the desulfated polymer. The desulfation reaction was assured by the disappearance of the metachromatic property of the polysaccharide with 1,9-dimethylmethylene blue.[23]

2.6. NMR spectroscopy

^1H and ^{13}C spectra of the native and desulfated galactans were recorded using a Bruker DRX 600 with a triple resonance probe. About 5 mg of each sample was dissolved in 0.5 mL of 99.9% D_2O (CIL). All spectra were recorded at 60 °C with HOD suppression by presaturation. COSY, TOCSY and $^1H/^{13}C$ heteronuclear correlation (HMQC) spectra were recorded using states–TPPI (states–time proportion phase incrementation) for quadrature detection in the indirect dimension. TOCSY spectra were run with 4096 × 400 points with a spin-lock field of about 10 kHz and a mixing time of 80 ms. HMQC were run with 1024 × 256 points and GARP (globally optimized alternating phase rectangular pulses) for decoupling. NOESY spectra were run with a mixing time of 100 ms. Chemical shifts are relative to external trimethylsilylpropionic acid at 0 ppm for ^1H and to MeOH for ^{13}C.

2.7. Clotting assays

Activated partial thromboplastin time (APTT) clotting assays were performed using normal human plasma according to the manufacturer's specifications. The clotting times were recorded in a coagulometer (Amelung KC4A). The activity was expressed as international units/mg using a parallel standard curve based on the 4th International Heparin Standard (193 international units/mg) from the National Institute for Biological Standards and Control, Potters Bar, Herts, UK.

2.8. Inhibition of thrombin or factor Xa by antithrombin and heparin cofactor II in the presence of sulfated polysaccharides

Incubations were performed in micro-plates with 96 wells. The final concentrations of reactants included 10 nM antithrombin or 30 nM heparin cofactor II, 2 nM thrombin or factor Xa and different concentrations of sulfated polysaccharides in 40 μL of TS/PEG buffer (0.02 M Tris/HCl, 0.15 M NaCl and 1.0 mg/mL polyethylene glycol 8000, pH 7.4). Thrombin or factor Xa was added last to initiate the reaction. After 60 s of incubation at 37 °C, 25 μL of TS/PEG buffer containing 0.4 mM chromogenic substrate S-2238 for thrombin or S-2222 for Xa (Chromogenix AB, Mondal, Sweden) was added and the absorbance at 405 nm was recorded for 300 s in a micro-plate reader (Molecular Devices–VMAx). The rate of change of absorbance was proportional to the thrombin or factor Xa activity remaining in the incubation mixtures. No inhibition occurred in control experiments in which thrombin or factor Xa was incubated with antithrombin or heparin cofactor II in the absence of sulfated polysaccharide. In addition, no inhibition was observed when thrombin or factor Xa was incubated with sulfated polysaccharide alone over the range of concentrations tested.

3. Results and discussion

3.1. Purification of the sulfated galactan from *G. crinale*

Anion-exchange chromatography on DEAE-cellulose separated the sulfated polysaccharides from the red alga, *G. crinale*, into three major peaks, named as FI, FII and FIII, eluted from the column with 0.5, 0.75 and 1.0 M NaCl, respectively (Fig. 2A). Minor amounts of sulfated polysaccharides were eluted with 1.25 and 1.5 M NaCl (designated as FIV and FV, respectively) but these fractions were not further analyzed due to scarce material. Agarose gel electrophoresis revealed that only fraction FII contained a single band (Fig. 2B) and therefore it is a purified polysaccharide. This fraction contains exclusively galactose and sulfate.

3.2. Structure of the sulfated galactan from *G. crinale*

We employed NMR analysis to determine the saccharide backbone structure and also to determine the sulfation pattern of the sulfated galactan (fraction F2) from *G. crinale*. For comparative purposes we also used the 3,6-anhydrogalactose-enriched galactan from *G. cornea*. The ^{1}H one-dimensional spectra of these galactans are shown in Figure 3. The chemical shifts in Table 1 are based on the interpretations of TOCSY (Fig. 4A,B), COSY (not shown) and HMQC (Fig. 5) spectra.

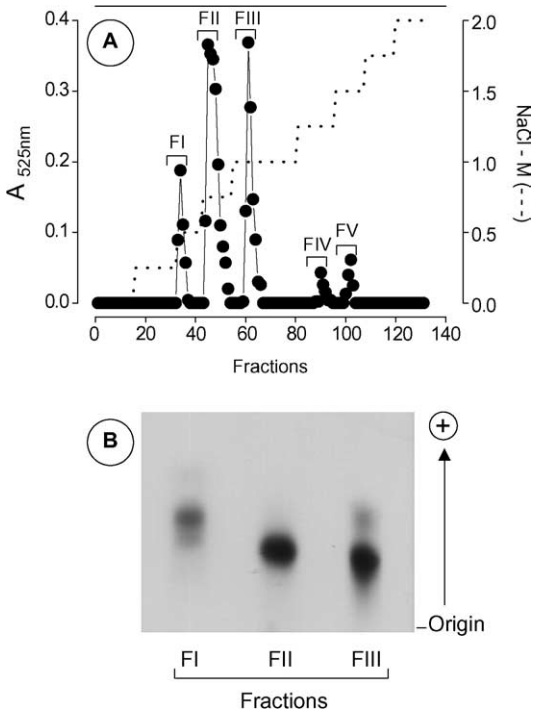

Figure 2. Purification of the sulfated galactans from *G. crinale* by chromatography on DEAE-cellulose and analysis of the purified polysaccharides by agarose gel electrophoresis. (A) The crude sulfated polysaccharide from *G. crinale* (10 mg) was dissolved in 5 mL of 50 mM NaOAc (pH 6.0) and applied to a DEAE-cellulose column (9.0 × 2.0 cm). The column was developed by a stepwise gradient of 0 → 3.0 M NaCl. No polysaccharide was eluted from the column before the NaCl gradient. The fractions were collected from the column and assayed by metachromasia (●) and NaCl concentration (···). Fractions FI, FII and FIII were pooled, dialyzed against distilled water and lyophilized. We obtained 0.5, 2.2 and 1.0 mg of FI, FII and FIII, respectively. (B) The three fractions of sulfated galactans obtained from the DEAE-cellulose column (20 μg of each) were analyzed by agarose gel electrophoresis and stained with toluidine blue, as described under methods. Fractions IV and V were not analyzed due to scarce material.

The desulfated galactan from *G. crinale* shows two main anomeric resonances, one at 5.30 ppm (α unit) and another at 4.40 ppm (β unit). Peak integration demonstrates a 0.53:0.47 ratio (Fig. 3B). The α and β spin systems can be traced based on TOCSY (Fig. 4B), COSY and $^{1}H/^{13}C$ HMQC (Fig. 5) spectra, giving the values presented in Table 1. The C-4 of α-galactose and C-3 of β-galactose residues show strong downfield shifts (10 ppm), indicating that the two residues are 4- and 3-linked, respectively. Linkage information was also obtained in the NOESY spectrum (Fig. 4C). A strong interresidue NOE is seen between β-H-1 and α-H-4, as expected for α-(1→3) and β-(1→4) linkages. These results are compatible with a polysaccharide with the following repeating structure: -4-α-Gal*p*-(1→3)-β-Gal*p*-(1→ (Fig. 1A).

In contrast with the desulfated galactan, the native polysaccharide has very complex NMR spectra (Figs.

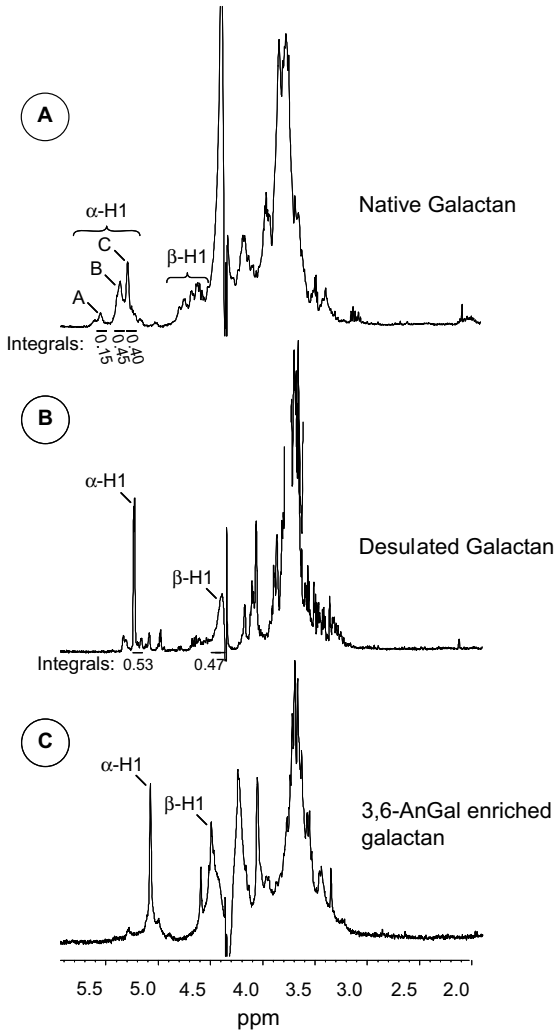

Figure 3. 1D ^{1}H NMR spectra at 600 MHz of the native (A) and desulfated (B) galactans from *G. crinale* and of the 3,6-anhydrogalactose-enriched galactan from *G. cornea* (C). The spectra were recorded at 60 °C for samples in D_2O solution. Chemical shifts are relative to external trimethylsilylpropionic acid at 0 ppm. The residual water has been suppressed by presaturation. The anomerics assigned by the $^{1}H/^{13}C$ HMQC (see Fig. 5) are labelled in the panels. Signals designated as A, B and C refer to those produced by α-(1→3) units. The integral listed under the H1 regions of residues A, B and C in the native galactan and the α-H1 and β-H1 of the desulfated polysaccharide were normalized to a total number of anomeric protons.

3A, 4A and 5A) due to its heterogeneous sulfation pattern. Several anomeric resonances were observed. Three of them (denominated as A, B and C) are between 5.6 and 5.2 ppm, in agreement with α-anomeric protons. A major β-anomeric signal is observed at 4.45 ppm. Two-dimensional assignment techniques (TOCSY and COSY) were used to trace spin systems, but only partial assignments were obtained. However, some information about the sulfation sites of this polysaccharide was easily deduced. An intense signal at ~3.8 ppm (and at ~63 ppm in the ^{13}C-dimension) indicates nonsubstituted H-6. Since this signal does not increase after desulfation of the polysaccharide, we can conclude that there is no significant sulfation at position 6. For β-anomeric residues, no unambiguous cross-peak could be found. In this region several overlap resonances exist hampering the assignment strategy.

The three α-anomeric signals certainly denote different sulfation patterns of the α-galactose unit. We can deduce the sulfation sites by analogy with the results obtained with a similar sulfated galactan from *B. occidentalis* (Ref. 19). Furthermore the TOCSY spectrum (Fig. 4A) shows that H-2 of residues A and B are ~0.8 downshifted from the desulfated value, confirming sulfation in this position. No signal of nonsulfated H-3 is observed in the spin system of residues A. Overall, these observations indicate that residues A, B and C are 2,3-disulfated, 2-sulfated and nonsulfated α-galactose units, respectively. The integrals of the three α-H-1 resonances in the ^{1}H spectrum suggest a 0.15:0.45:0.40 ratio of residues A:B:C. Of course, these integrals are derived from poorly resolved signals and thus require careful interpretation.

It remains to be clarified of the possible occurrence of 3,6-anhydro-α-galactose in the sulfated galactan from *G. crinale*. Most of the literature on NMR data of algal sulfated galactan is based on ^{13}C NMR spectra obtained by analogy with standard compounds.[28–30] Two-dimensional assignment techniques have not been applied to these compounds due to their complex spectra, especially the ^{1}H NMR. For comparative purposes we now employed a 3,6-anhydrogalactose-enriched galactan obtained from *G. cornea*. As the desulfated galactan from *G. crinale*, this polysaccharide shows two main anomeric signals, and spin systems can be traced on TOCSY (Fig. 4B) and HMQC (Fig. 5C) spectra, giving the values presented in Table 1. Clearly the ^{1}H and ^{13}C chemical shifts for the β-system are almost coincident for the desulfated galactans from *G. crinale* and *G. cornea*. In contrast, the α-system differs due to a downfield shift of 8 ppm of C-3 and C-6 of the 3,6-anhydro-α-galactose compared with the α-galactose unit. ^{1}H chemical shifts are always downfield shifts for the 3,6-anhydro-α-galactose residues (−0.28, −0.59, −0.42, −0.40 and −0.24 ppm for H-2, H-3, H-4, H-5 and H-6, respectively), except for a slight upfield shift of H-1 (+0.15 ppm). Clearly, 2D NMR spectra distinguish these two types of α-galactose units found in algal galactans and assure that the galactan from *G. crinale* contains no 3,6-anhydro-α-galactose residues.

It remains to be clarified whether the 3,6-anhydro ring of galactose is disrupted during the desulfation reaction with DMSO. In this case the absence of 3,6-anhydro-α-galactose residues in the galactan from *G. crinale* is an artifact. In order to clarify this aspect we submitted the galactan from *G. cornea* to the same conditions used for solvolysis with DMSO. The NMR spectra of the galactan were not modified after this reaction (not

Table 1. ^{1}H and ^{13}C chemical shifts for residues of galactose on desulfated galactans from marine algae

Polysaccharide	Unit	^{1}H chemical shifts[a] (ppm)					
		H-1	H-2	H-3	H-4	H-5	H-6
Desulfated galactan from *G. crinale*	4-α-Gal-1	5.30	3.84	3.95	**4.24**	4.18	3.80
	3-β-Gal-1	4.40	3.78	**3.76**	4.13	3.73	3.80
3,6-Anhydrogalactose-enriched galactan from *G. cornea*	4-α-3,6-AnGal-1	5.15	4.12	*4.54*	**4.66**	4.58	*4.04*
	3-β-Gal-1	4.57	3.63	**3.73**	4.12	3.76	3.77
Desulfated galactan from *B. occidentalis*[b]	4-α-Gal-1	5.29	3.85	3.95	**4.22**	4.16	3.78
	3-β-Gal-1	4.40	3.78	**3.75**	4.12	3.73	3.78
Galactan from *C. nipae*[c]	4-α-3,6-AnGal-1	5.09	4.12	*4.50*	NR	4.68	*4.20*
	3-β-Gal-1	4.61	3.62	**3.82**	4.10	3.65	NR
		^{13}C-chemical shifts[a] (ppm)					
		C-1	C-2	C-3	C-4	C-5	C-6
Desulfated galactan from *G. crinale*	4-α-Gal-1	102.9	72.9	71.0	**81.2**	75.1	63.3
	3-β-Gal-1	105.6	72.8	**83.0**	70.8	77.4	63.3
3,6-Anhydrogalactose-enriched galactan from *G. cornea*	4-α-3,6-AnGal-1	100.4	71.4	*77.9*	**82.0**	79.4	*71.1*
	3-β-Gal-1	104.5	72.1	**84.1**	71.4	77.7	63.4
Desulfated galactan from *B. occidentalis*[b]	4-α-Gal-1	102.9	71.5	70.9	**80.9**	73.9	63.0
	3-β-Gal-1	105.3	72.3	**82.7**	70.4	77.5	63.0

[a] Chemical shifts are relative to external trimethylsilylpropionic acid at 0 ppm for ^{1}H and MeOH for ^{13}C. Values in boldface indicate glycosylated positions and those in italic indicate positions involved in the 3,6-anhydro-α-galactose ring.
[b] See Ref. 19.
[c] See Ref. 27.

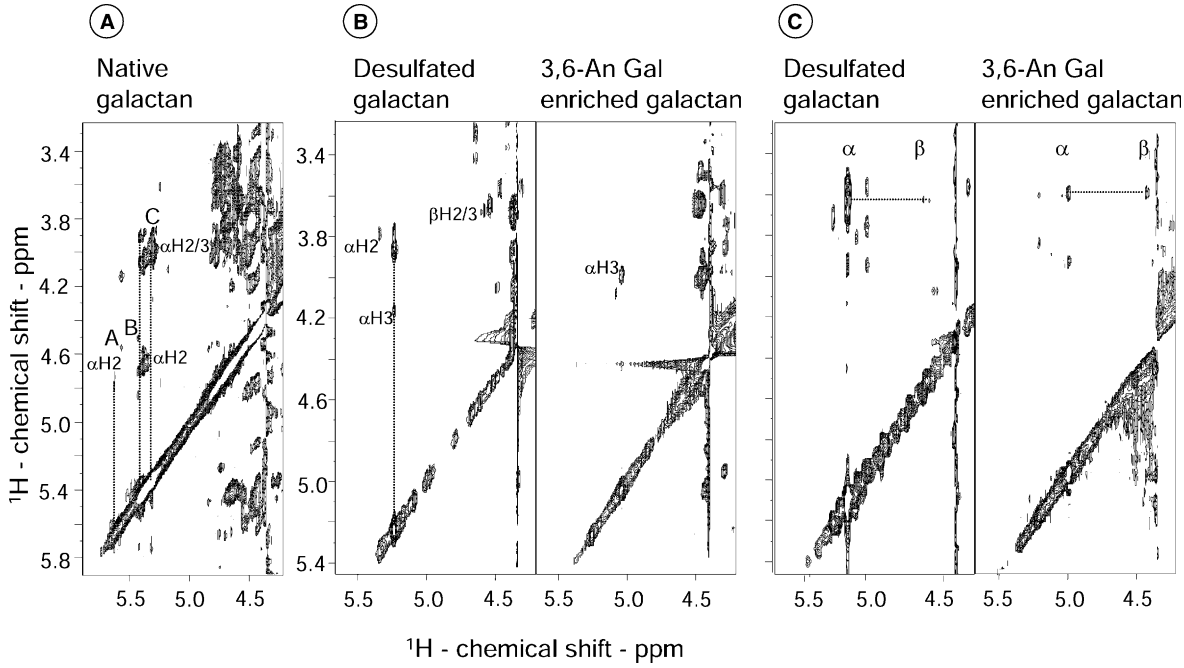

Figure 4. Strips from the TOCSY (A and B) and NOESY (C) spectra of the native (A) and desulfated galactan from *G. crinale* and of the 3,6-anhydrogalactose-enriched galactan from *G. cornea* (B and C) at 600 MHz, 60 °C, in D_2O. The spin systems for α-units are traced in the TOCSY spectra. The strips of NOESY spectra of desulfated galactans in comparison with the TOCSY show the NOEs from the α-H1 and H3 of the β unit as expected for α-(1→3) and β-(1→4) alternating linkages.

shown) therefore assuring that the 3,6-anhydro ring resists the conditions used for the desulfation of the polysaccharide.

In conclusion, NMR analysis indicates that the sulfated galactan from *G. crinale* (fraction FII) is a linear polysaccharide, containing alternating α-(1→3)- and β-(1→4)-galactopyranose units. A variable sulfation pattern confers high heterogeneity to this polysaccharide. Nevertheless, it is clear that 2,3-disulfated residues occur as ~15% of the total α units of the galactan. The proportion of this unit is lower than that in a similar sulfated galactan from the algae *B. occidentalis* (Fig. 1A).

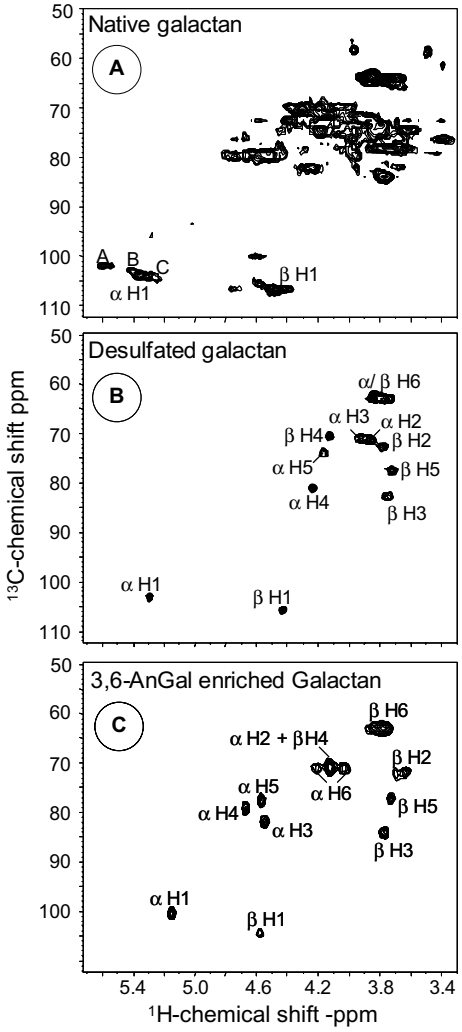

Figure 5. $^{1}H/^{13}C$ HMQC spectra of the native (A) and desulfated (B) galactan from *G. crinale* and of the 3,6-anhydrogalactose-enriched galactan from *G. cornea* (C). The assignments were based on TOCSY and COSY spectra. The values of chemical shifts reported in Table 1 are relative to external trimethylsilylpropionic acid at 0 ppm for ^{1}H and to MeOH for ^{13}C. The anomeric resonances were identified by their characteristic carbon chemical shifts.

3.3. Comparison between the anticoagulant activity of the sulfated galactans from *G. crinale* and *B. occidentalis*

The APTT assays indicate that the sulfated galactan from *G. crinale* has anticoagulant activity but is less potent than a similar polysaccharide from *B. occidentalis* (65 and 90 IU/mg compared with a heparin standard, respectively, Fig. 6). The difference in the anticoagulant activity of these two sulfated galactans may be ascribed to the discrete variation of their sulfation patterns since both polysaccharides have a similar saccharide chain (Fig. 1A).

We further evaluated the anticoagulant activity of the sulfated galactans using assays with purified proteases and coagulation inhibitors (Fig. 7). Surprisingly, we

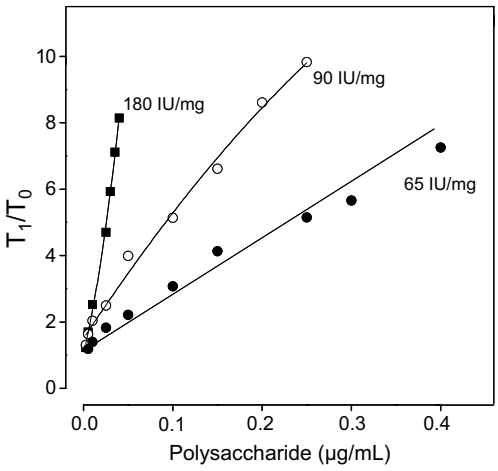

Figure 6. APTT clotting assays versus concentration of the sulfated galactans. The clotting assays were performed using normal human plasma at different concentrations of sulfated galactans from *G. crinale* (●), *B. occidentalis* (○) and heparin standard (■). The activity is expressed as international units/mg using the parallel standard curve with heparin.

observed marked differences in the effect of these two sulfated galactans, depending on the protease or coagulation inhibitor employed in the assays. Both sulfated galactans showed almost coincident curves for thrombin inhibition in the presence of antithrombin, with similar sigmoid curves as observed for heparin (Fig. 7A). However, when we replaced antithrombin by heparin cofactor II, we observed a dramatic shift to the right of the effect of the sulfated galactan from *G. crinale* on thrombin inhibition (Fig. 7B). When the target protease is factor Xa instead of thrombin, higher concentrations of both sulfated galactans are required to obtain the same effect as with heparin (Fig. 7C), the effect of the sulfated galactan from *G. crinale* being more pronounced than the polysaccharide from *B. occidentalis*.[†]

These results indicate that discrete variations in the sulfation patterns of the polysaccharides results in significant modification of their anticoagulant activity. The presence of 2,3-disulfated α-galactose units is required for the anticoagulant activity of the sulfated galactans.[19,20] But, as a plausible explanation for the results of Figure 7, we may propose that the proportion and/or distribution of these units along the polysaccharide may be critical depending on the protease or coagulation cofactor tested. In this case, the differences in sulfation of the galactans from *G. crinale* and *B. occidentalis* do not limit the thrombin inhibitory reaction when

[†]The differences in anticoagulant activity observed between the sulfated galactans from *G. crinale* and *B. occidentalis* cannot be ascribed to variation in the size of their chains, since these two polysaccharides have a similar migration on polyacrylamide gel electrophoresis, used to estimate the molecular weight of these compounds. See Refs. 3–5 for the methodology.

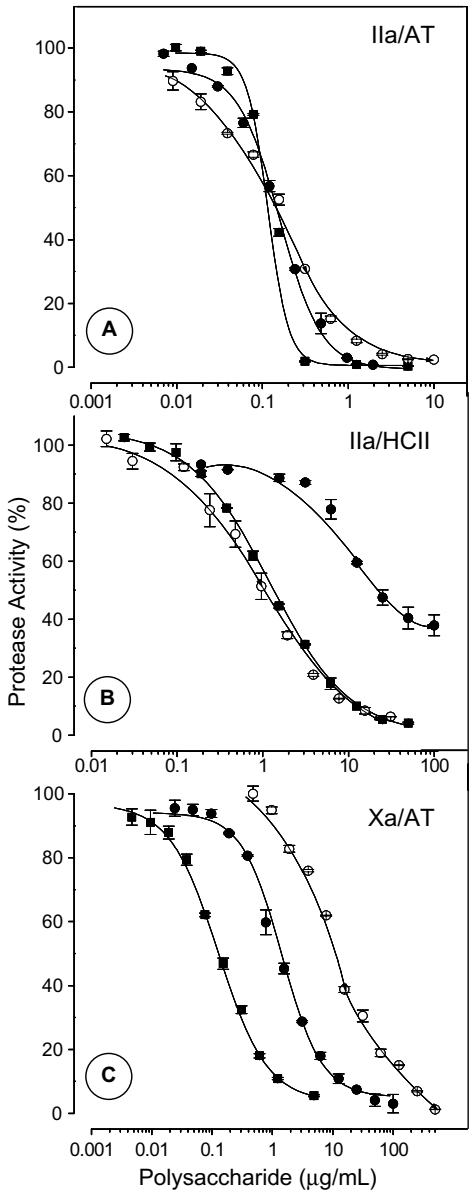

Figure 7. Dependence on the concentration of sulfated galactan for inactivation of thrombin (A and B) or factor Xa (C) by antithrombin (A and C) or heparin cofactor II (B). Antithrombin (10 nM) or heparin cofactor II (30 nM) were incubated with thrombin or factor Xa (2 nM of each) in the presence of various concentrations of the sulfated galactan from *G. crinale* (●) or *B. occidenatlis* (○) and heparin (■). After 60 s of incubation at 37 °C, the remaining thrombin or factor Xa activity was determined with a chromogenic substrate ($A_{405\,nm}$/min). AT, antithrombin, HCII, heparin cofactor II, IIa, thrombin, Xa, factor Xa.

antithrombin is used as cofactor (Fig. 7A), but it is critical when heparin cofactor II replaces antithrombin (Fig. 7B). When factor Xa is used as the target protease, the higher amounts of 2,3-disulfated α-galactose units in the galactan from *B. occidentalis* may be disturbing the formation of the inhibitory complex and a more favorable effect is obtained with the less sulfated polysaccharide from *G. crinale*.

3.4. Major conclusions

We purified and characterized the structure of a sulfated galactan from the red alga, *G. crinale*. This polysaccharide has a similar saccharide backbone as another galactan previously studied in our laboratory, but they differ mainly in the amount of 2,3-disulfated α-galactose units. Comparison between the anticoagulant activity of these two polysaccharides reveals that the structural requirements for the interaction of sulfated galactans with coagulation inhibitors and their target proteases are not merely a consequence of their charge density. The occurrence of 2,3-disulfated α-galactose units is important, but the optimal proportion of these units for the effect of the polysaccharide on coagulation assays varies depending on the protease and coagulation inhibitor. For the sulfated fucans, we observed a different sulfation requirement for the anticoagulant activity.[17] In this case, 2,4- and 4-sulfated units are necessary for thrombin inhibition in the presence of antithrombin and heparin cofactor II, respectively. Therefore, the sulfation pattern required for the anticoagulant activity depends also on the nature of the saccharide chain.

Finally, our approach that combines structural analysis of sulfated polysaccharides with specific biological assays is a useful tool to investigate anticoagulant activity in mammals. This protocol may help to design new drugs with specific actions on coagulation and thrombosis. New compounds with obvious practical applications may be found.

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