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A 2-sulfated, 3-linked α-L-galactan is an anticoagulant polysaccharide

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Dedicated to Professor Derek Horton on the occasion of his 70th birthday

Abstract

Marine alga is an abundant source of sulfated polysaccharides with potent anticoagulant activity. However, several attempts to identify the specific structural features in these compounds, which confer the biological activity, failed due to their complex, heterogeneous structure. We isolated and characterized several sulfated α-L-galactans and sulfated α-L-fucans from marine invertebrates. In contrast to the algal fucans and galactans, these invertebrate polysaccharides have a simple structure, composed of well-defined units of oligosaccharides. We employed two of these compounds to elucidate their structure–anticoagulant action relationship. Our results indicate that a 2-sulfated, 3-linked α-L-galactan, but not an α-L-fucan, is a potent thrombin inhibitor mediated by antithrombin or heparin cofactor II. The difference between the activities of these two polysaccharides is not very pronounced when factor Xa replaces thrombin. Thus, the anticoagulant activity of sulfated galactan and sulfated fucan is not merely a consequence of their charge density. The interaction of these polysaccharides with coagulation cofactors and their target proteases are specific. Identification of specific structural requirements in sulfated galactans and sulfated fucans necessary for interaction with coagulation cofactors is an essential step for a more rational approach to develop new anticoagulant and antithrombotic drugs.

Keywords: Marine invertebrates; Anticoagulant activity; Sulfated polysaccharides; Sulfated galactans; Sulfated fucans

1. Introduction

The anticoagulant activity of mammalian glycosaminoglycans is mediated by specific plasma cofactors, named antithrombin and heparin cofactor II.[1] In the case of heparin, a pentasaccharide sequence, with specific pattern of sugar composition and of sulfation pattern, is required for its interaction with antithrombin.[2,3] Dermatan sulfate is another glycosaminoglycan with anticoagulant activity, but in this case mediated exclusively by heparin cofactor II. A specific sequence of [4-α-L-IdUA-2(SO_4)-1 → 3-β-D-GalNAc-4(SO_4)-1]$_n$, where $n \geq 3$ is required for the binding of dermatan sulfate to the plasma cofactor.[4]

One abundant source of new anticoagulant polysaccharides is marine alga. They contain a variety of sulfated fucans[5–9] and sulfated galactans[10–12] with anticoagulant activity. These compounds are among the most abundant and widely studied of all the polysaccharides from non-mammalian origin. Several attempts to identify in these algal polysaccharides, specific structural features necessary for their anticoagulant activity have been limited by the fact that algal fucans and galactans have complex, heterogeneous structures.[9,12] Their regular repeating sequences are not easily deduced; even high-field NMR is at the limit of its

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resolution, and complete description of their structure is not available at present.[9,12,13] Obviously, identification of specific structural requirements in these algal polysaccharides necessary for interaction with coagulation cofactors is an essential step for a more rational approach to develop new anticoagulant and antithrombotic drugs.

Recently we isolated and characterized several sulfated polysaccharides from invertebrates (mostly from the egg jelly of sea urchins). In contrast to the algal fucans and galactans, these invertebrate polysaccharides have simple, linear structures, composed of well-defined repeating units of oligosaccharides.[13–17] The physiological role of these invertebrate polysaccharides is far distant from blood coagulation. They are either components of the extracellular matrix[18,19] or involved in gamete interaction during fertilization.[14–17]

We now employ two of these invertebrate polysaccharides, with a single 2-sulfated unit, but which differ in their constituent monosaccharide, to elucidate their structure–anticoagulant action relationships. Our results indicate that a 2-sulfated, 3-linked α-L-galactan, but not an α-L-fucan, is a potent anticoagulant polysaccharide.

2. Experimental

Sulfated polysaccharides from sea urchin egg jelly. — Extraction.—Adults of *Echinometra lucunter* and *Strongylocentrotus franciscanus* were collected at Rio de Janeiro, Brazil and at La Jolla, CA, USA, respectively. Eggs were spawned into sea water by intracelomic injection of 0.5 M KCl. The crude egg jelly was isolated by the pH 5.0 method and prepared as 30,000*g* supernatant and stored at −20 °C, or lyophilized after dialysis against distilled water.[20] The acidic polysaccharides were extracted from the jelly coat by papain digestion and partially purified by EtOH precipitation, as described previously.[18]

Purification.—The crude polysaccharides (10 mg) from the egg jelly coats were applied to a Mono Q column-FPLC (HR 5/5) (Pharmacia Biotech Inc.) equilibrated in 20 mM Tris–HCl (pH 8.0). The column was washed with 10 mL of the same buffer and then eluted by a linear gradient of 0–4.0 M NaCl in the same buffer. The flow rate of the column was 0.45 mL/min, and fractions of 0.5 mL were collected. Fractions were checked for fucose and sialic acid by the Dubois et al. reaction[21] and by the Ehrlich assay,[22] respectively, and by their metachromasia.[23] The NaCl concentration was estimated by conductivity. Fractions containing the sulfated α-L-galactan or the sulfated α-L-fucan and the sialic acid glycoconjugate were pooled, dialyzed against distilled water, and lyophilized.

Chemical analysis.—Total galactose and fucose were measured by the methods of Dubois et al.[21] and of Dische and Shettles,[24] respectively. After acid hydrolysis of the polysaccharide (5.0 M trifluoroacetic acid for 5 h at 100 °C), sulfate was measured by the $BaCl_2$–gelatin method.[25] The presence of hexoses and 6-deoxyhexoses 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 (GLC–MS) of derived alditols.[26] Optical rotation was measured using a digital polarimeter (Perkin–Elmer model 243-B).

Desulfation and methylation of the polysaccharides.—Desulfation of the sulfated galactan and sulfated fucan was performed by solvolysis in dimethyl sulfoxide, as described previously for desulfation of other types of polysaccharides.[27,28] The native and desulfated polysaccharides (5 mg of each) were subjected to three rounds of methylation, as described previously,[29] with the mod-

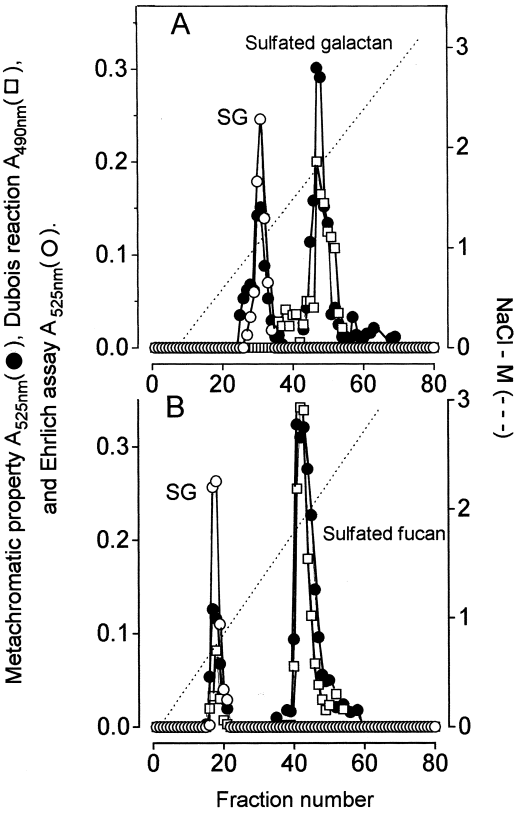

Fig. 1. Purification of the sulfated polysaccharides from sea urchin egg jelly by Mono Q FPLC. The polysaccharides (10 mg) extracted from the egg jelly of *E. lucunter* (A) or *S. franciscanus* (B) was applied to a Mono Q FPLC column (HR5/5) equilibrated with 20 mM Tris–HCl (pH 8.0). The column was developed by a linear gradient of 0–4.0 M NaCl in the same buffer. Fractions were assayed by metachromasia using 1,9-dimethylmethylene blue (●), the Dubois reaction for galactose or fucose (□), and the Ehrlich assay for sialic acid (○). The NaCl concentration was estimated by conductivity (---). Fractions containing the sulfated galactan or sulfated fucan were pooled, dialyzed against distilled water, and lyophilized. SG indicates sialic acid-rich glycoconjugate.

Table 1
Chemical composition and specific optical rotation of the sulfated polysaccharides from the jelly coat of two species of sea urchins

Species	Chemical composition (molar ratios)			$[\alpha]_D^{20°C}$
	Galactose [a]	Fucose [a]	Sulfate [b]	
E. lucunter	1.00	<0.01	1.30	−127°
S. franciscanus	<0.01	1.00	1.14	−57°

[a] Sugars were identified, after acid hydrolysis, by GLC of derived alditol acetates.[26] Total hexose and 6-deoxyhexose were quantified by the Dubois et al.[21] and Dische and Shettles[24] reactions, respectively.

[b] Sulfate was measured by the $BaCl_2$–gelatin method.[25]

ifications suggested by Patankar et al.[30] The methylated polysaccharides were hydrolyzed in 6 M trifluoroacetic acid for 5 h at 100 °C, the component sugars were reduced with borohydride and the resulting alditols were acetylated with 1:1 Ac_2O–pyridine.[26] The alditol acetates of the methylated sugars were dissolved in $CHCl_3$ and analyzed in a GLC–MS unit.

NMR experiments.—^1H and ^{13}C spectra of the sulfated galactan and sulfated fucan were recorded using a Bruker DRX 600 with a triple resonance probe. About 3 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 methanol for ^{13}C.

Clotting assays.—Activated partial thromboplastin time (APTT) clotting assays were performed using normal human plasma according to the manufacturer's specifications, as described by Anderson et al.[31] The clotting times were recorded in a coagulometer (Amelung KC4A). For the APTT assays, the activity was expressed as international units/mg using a parallel standard curve based on the 4th International Heparin Standard (193 international units/mg).

Inhibition of thrombin or factor Xa by antithrombin and heparin cofactor II in the presence of sulfated polysaccharides.—Incubations were preformed in disposable semimicrocuvettes. The final concentrations of reactants included 68 nM heparin cofactor II or 50 nM antithrombin, 15 nM thrombin or factor Xa (all from Diagnostica Stago, Asnières, France) and 0–1000 μg/mL sulfated polysaccharide in 100 μL of 0.02 Tris–HCl, 0.15 M NaCl, and 1.0 mg/mL polyethylene glycol (pH 7.4) (TS/PEG buffer). Thrombin or factor Xa was added last to initiate the reaction. After 60 s incubation at rt, 500 μL of 100 μM chromogenic substrate S-2238 for thrombin or S-2222 for factor Xa (Chromogenix AB, Molndal, Sweden) in TS/PEG buffer was added, and the absorbance at 405 nm was recorded for 60 s. The rate of change of absorbance was proportional to the thrombin or factor Xa activity remaining in the

Table 2
Methylated galactose and fucose derivatives obtained from native and desulfated polysaccharides from the sea urchin jelly coat

Methylated sugar [a] (as alditol acetates)	% of total peak area [b]			
	Sulfated α-galactan from *E. lucunter*		Sulfated α-fucan from *S. franciscanus*	
	Native	Desulfated	Native	Desulfated
2,4-Met_2-Fuc	<1	<1	7	64
2-Met-Fuc	<1	<1	<1	15
4-Met-Fuc	<1	<1	88	6
Fuc	<1	<1	5	15
2,4,6-Met_3-Gal	<1	87	<1	<1
4,6-Met_2-Gal	100	13	<1	<1

[a] After three rounds of methylation, the permethylated polysaccharides were hydrolyzed, and the products analyzed as their alditol acetate derivatives by GLC–MS.

[b] The proportions of the methylated derivatives are based on the area under each peak compared with the total area of galactose or fucose derivatives.

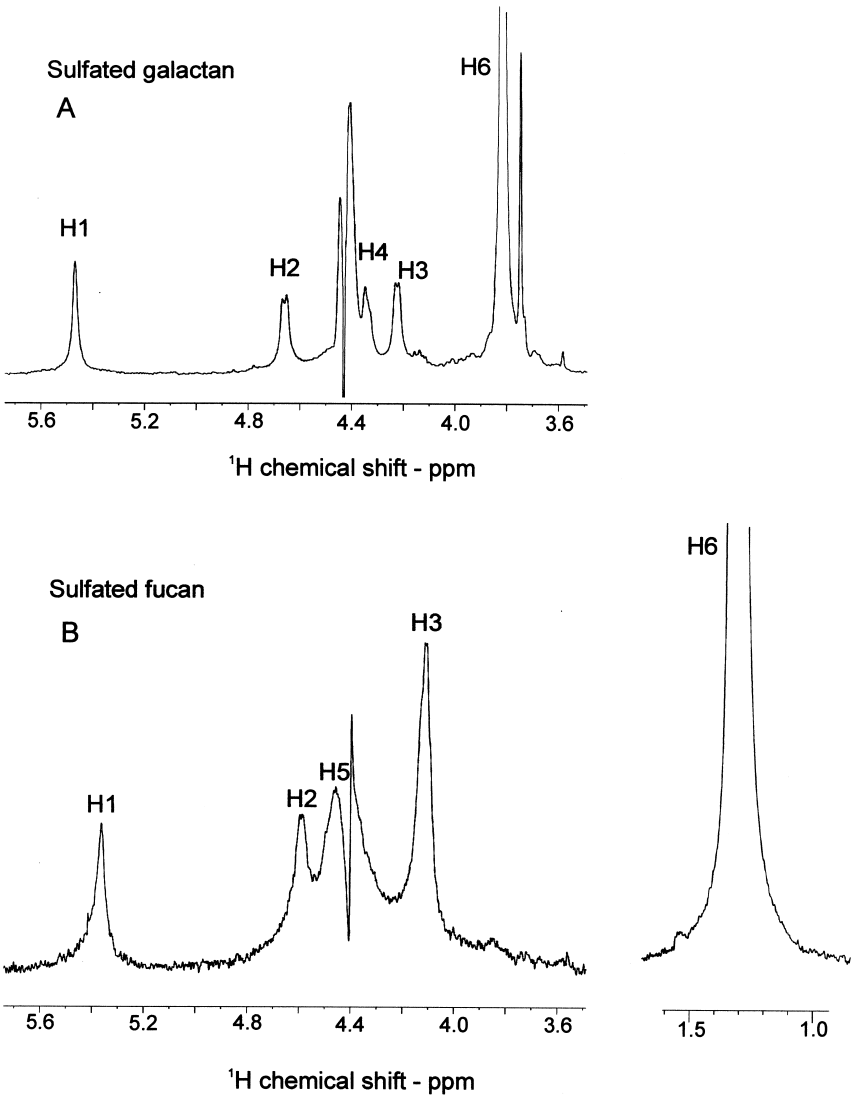

Fig. 2. 1D ^{1}H NMR spectra at 600 MHz of sulfated α-galactan from *E. lucunter* (A) and of sulfated α-fucan from *S. franciscanus* (B). The spectra were recorded at 60 °C for samples in D_2O solution. Chemical shifts are relative to external trimethylsilylpropionic acid at 0 ppm. The residual water has been suppressed by presaturation.

incubation. 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. Nor did inhibition occur when thrombin or factor Xa was incubated with sulfated polysaccharide alone over the range of concentrations tested.

3. Results and discussion

Purification of the sulfated α-L-galactan and sulfated α-L-fucan from the egg jelly coat of the sea urchins.— Sulfated polysaccharides extracted from the egg jelly coat of *E. lucunter* and *S. franciscanus* were purified by anion-exchange chromatography on Mono Q FPLC (Fig. 1). A peak rich in sialic acid was eluted completely up to ~1.0 M NaCl and denominated as a sialic acid-rich glycoconjugate (SG). The structure of this glycoconjugate from other species of sea urchins was characterized previously.[32] It is composed of a polysialic acid chain, containing from four up to more than 40 Neu5Gc residues. The polyNeu5Gc chains are attached to core oligosaccharides.

A second peak, eluted at higher salt concentrations, corresponds to the sulfated galactan (in the case of *E. lucunter*) and sulfated fucan (in the case of *S. franciscanus*). The difference between the concentrations of NaCl necessary to elute these two sulfated polysaccharides from the Mono Q is not very pronounced. However, the sialic acid-rich glycoconjugates from the two species are eluted from the column with well-distinguished concentrations of NaCl.

Chemical analysis of the purified sulfated polysaccharides revealed galactose or fucose as the only sugar in *E. lucunter* and *S. franciscanus*, respectively (Table 1). Both polysaccharides have high sulfate content. The slight difference in sulfate content between the galactan and fucan must be interpreted carefully. Possible the assay for sulfate is not accurate enough to distinguish small differences in sulfate content. The strongly negative specific rotation of the polysaccharides is compatible with residues of α-L-galactopyranose or α-L-fucopyranose. The enantiomeric form of L-galactose was determined based on the resistance of this sugar to oxidation by D-galactose oxidase. Fucose occurs in the *S. franciscanus* fucan entirely in the L-enantiomeric form since this sugar is totally oxidized by L-fucose dehydrogenase.

The sulfated α-galactan and sulfated α-fucan are composed of 2-sulfated, 3-linked units.—Methylation of the native sulfated galactan from *E. lucunter* yields 4,6-di-*O*-methylgalactose, whereas 2,4,6-tri-*O*-methylgalactose is the predominant methyl ether derivative from the desulfated galactan (Table 2). This indicates a linear polysaccharide composed of 3-linked and 2-sulfated galactopyranoside residues.

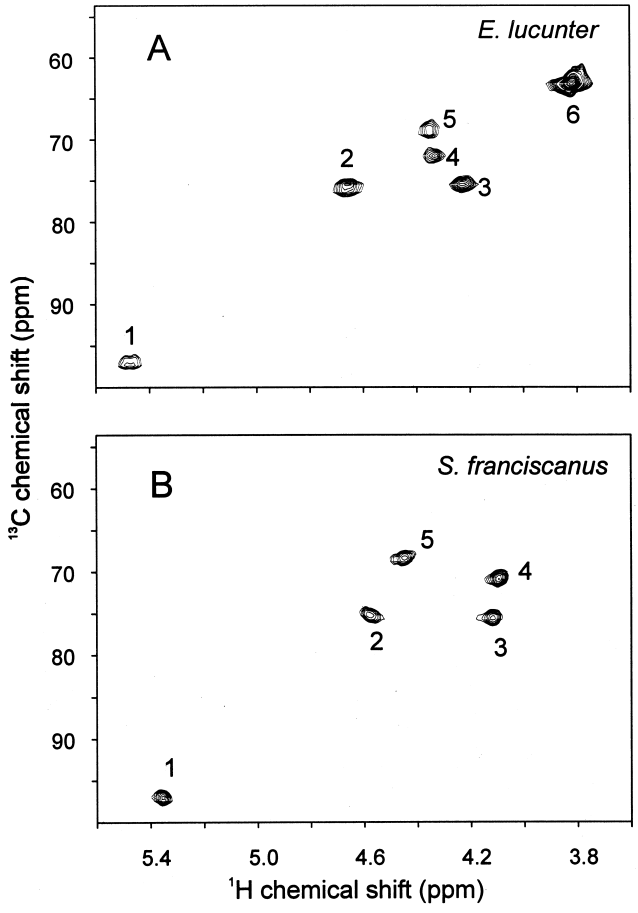

Fig. 3. ^{1}H–^{13}C HMQC spectra of sulfated α-galactan from *E. lucunter* (A) and sulfated α-fucan from *S. franciscanus* (B). The assignment was based on TOCSY and COSY spectra. The values of chemical shifts reported in Table 3 are relative to external trimethylsilylpropionic acid at 0 ppm for ^{1}H and to methanol for ^{13}C.

In a similar way, the sulfated fucan from *S. franciscanus* yields 4-*O*-methylfucose, whereas 2,4-di-*O*-methylfucose is the predominant methyl ether derivative from the desulfated fucan. Again, these results are consistent with a linear polysaccharide composed mainly of 3-linked and 2-sulfated fucose residues. Possibly 2-*O*-methylfucose and non-methylated fucose obtained from the desulfated fucan are products of an incomplete methylation reaction, whereas the formation of 4-*O*-methylfucose indicates some 2-sulfated residues still present after the solvolytic desulfation process.

The proposed structures for the sulfated galactan and sulfated fucan were confirmed by ^{1}H and ^{13}C NMR spectra. The one-dimensional ^{1}H (Fig. 2) and ^{1}H–^{13}C HMQC spectra (Fig. 3) of the sulfated galactan and sulfated fucan show a single anomeric signal and agreeing with the presence of a homopolymer of galactose or fucose.

The assignment of ^{1}H peaks was achieved by analysis of ^{1}H COSY and ^{1}H TOCSY (not shown). All spectra have the simplicity compatible with the presence of homopolymers and the chemical shifts are presented in Table 3. The carbon chemical shifts were obtained using the ^{1}H–^{13}C HMQC spectra (Fig. 3). The HMQC was interpreted using the information obtained in the COSY and TOCSY spectra and the values of ^{13}C chemical shifts are reported in Table 3.

The position of sulfation was deduced from the strong downfield shifts in H-2 of both polysaccharides. The position of the glycosidic linkage is not easily deduced from the ^{1}H chemical shifts, but from the ^{13}C chemical shifts, based on the interpretation of the HMQC spectra (Fig. 3), as C-3 shows the characteristic shift of a 3-linked galactan or fucan (Table 3).

NMR analysis shows no sign of disulfated residues, although one cannot rule out a small proportion of these units, especially in the case of the sulfated fucan. The small proportion (5%) of unmethylated fucose obtained from the native fucan (Table 2) may indicate small amounts of 2,4-disulfated residues.

Overall, the combination of methylation and NMR spectroscopic analysis confirm the preponderant structure of the sulfated α-L-galactan and sulfated α-L-fucan from *E. lucunter* and *S. franciscanus*, respectively, as shown in Fig. 4.

The 2-sulfated, 3-linked α-L-galactan is an anticoagulant polysaccharide.—The APTT assay indicate that the 2-sulfated, 3-linked α-L-galactan from *E. lucunter*, but not the α-L-fucan from *S. franciscanus*, has a distinguished potent anticoagulant activity (Table 4). The sulfated α-galactan enhances thrombin or factor Xa inhibition by antithrombin or heparin cofactor II with

Table 3
^{1}H and ^{13}C chemical shifts, $^{3}J_{H,H}$ and $^{1}J_{C,H}$ in the NMR spectra of the sulfated α-L-galactan and sulfated α-L-fucan from sea urchins

Polysaccharide	^{1}H chemical shifts (ppm) [a]					
	H-1	H-2	H-3	H-4	H-5	H-6
Sulfated α-L-galactan	5.47	**4.66**	4.23	4.33	4.35	3.82
Sulfated α-L-fucan	5.33	**4.55**	4.10	4.07	4.42	1.25
	^{13}C chemical shifts (ppm) [a]					
	C-1	C-2	C-3	C-4	C-5	C-6
Sulfated α-L-galactan	97.2	**76.2**	75.9	72.5	69.5	63.8
Sulfated α-L-fucan	96.8	**75.5**	75.9	71.2	68.6	17.3
	$^{3}J_{H,H}$ (Hz)					
	H-1,H-2	H-2,H-3	H-3,H-4	H-4,H-5	H-5,H-6	
Sulfated α-L-galactan	3.0	10.5	ND [b]	ND	4.5	
Sulfated α-L-fucan	3.1	9.5	ND	ND	4.9	
	$^{1}J_{C,H}$ (Hz)					
	C-1,H-1	C-2,H-2	C-3,H-3	C-4,H-4	C-5,H-5	C-6,H-6
Sulfated α-L-galactan	177.5	151.3	146.2	146.5	146.8	143.3
Sulfated α-L-fucan	177.2	148.7	144.6	140.9	147.1	125.3

[a] Chemical shifts are referenced to internal trimethylsilylpropionic acid at 0 ppm for ^{1}H and to methanol for ^{13}C. Values in boldface indicate positions bearing sulfate ester. The assignments were based on TOCSY, NOESY and ^{1}H–^{13}C HMQC spectra.
[b] ND, not determined.

similar sigmoid curves as observed for heparin or dermatan sulfate (Fig. 5(A and B)). Although higher concentrations are required to achieve the same effect as heparin, the IC_{50} for thrombin or factor Xa inhibition can be easily determined, as reported in Table 4. For the sulfated α-fucan from *S. franciscanus*, we observed a dramatic shift to the right of its effect on thrombin and total inhibition is not achieved in the range of concentrations used in our experiments (Fig. 5(A and B)). When the target protease is factor Xa instead of thrombin, the difference between the activity of the two polysaccharides is not very pronounced (Fig. 5(C)).

Table 4
APTT and IC_{50} of the sulfated α-galactan and sulfated α-fucan for thrombin or factor Xa inhibition in the presence of antithrombin or heparin cofactor II

Polysaccharide	APTT [b] (IU/mg)	IC_{50} (μg/mL) [a]		
		Thrombin/antithrombin	Thrombin/heparin cofactor II	Factor Xa/antithrombin
Sulfated α-galactan	20	3	6	20
Sulfated α-fucan	2	>500	>500	250
Heparin [c]	193	0.02	0.5	0.02
Dermatan sulfate	4	>500	0.7	>500

[a] These values were obtained from the inhibition curves shown in Fig. 5.
[b] The activity is expressed as international units/mg using a parallel standard curve based on the International Heparin Standard (193 units/mg).
[c] International standard.

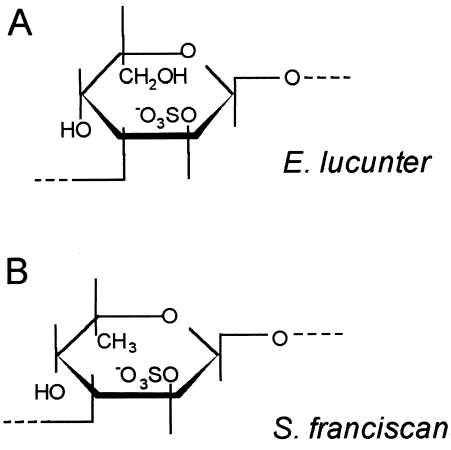

Fig. 4. Deduced structures of the sulfated α-L-galactan (A) and sulfated α-L-fucan (B) from *E. lucunter* and *S. franciscanus*, respectively. Both polysaccharides are 3-linked with a regular sulfation at the 2-position, but differ in their constituent monosaccharides. The structures were deduced from the methylation (Table 2) and NMR spectroscopic (Figs. 2 and 3, Table 3) analysis.

Therefore, differences in the effect of these two polysaccharides cannot be ascribed exclusively to variation in their affinities for antithrombin. The differences observed result from a more complex and still unclear effect of the polysaccharide on the complex formed between the plasma cofactor and its target protease.

The differences in anticoagulant activity observed between the sulfated α-galactan and sulfated α-fucan cannot be ascribed to variation in the size of their chains, since these two polysaccharides do not diverge in their elution from a Superose 6-FPLC column (not shown). Both polysaccharides have a molecular mass of ~ 100 kDa.

Finally, it is possible to argue that the anticoagulant activity of the sulfated galactan is determined by short oversulfated sequences, as described for heparin's interaction with antithrombin,[2,3] rather than by the bulk polysaccharide structure. But heparin has an additional anticoagulant mechanism, resulting from bridging the protease and the antithrombin molecules.[33] This last effect depends on the bulk structure of the polysaccharide. We attempted to clarify this aspect in the case of the sulfated galactan. Our approach was to decrease the molecular size of the polysaccharide by mild-acid hydrolysis and to separate the fragments by gel filtration. A slight decrease in the molecular mass of the sulfated galactan dramatically reduced its effect on thrombin in the presence of antithrombin. The polysaccharide always required fragments with much higher molecular masses than heparin to inactivate the protease (Pereira, M. S.; Melo, F. R.; Mourão, P. A. S. unpublished results). This observation suggested that the sulfated galactan accelerates antithrombin–proteinase reaction

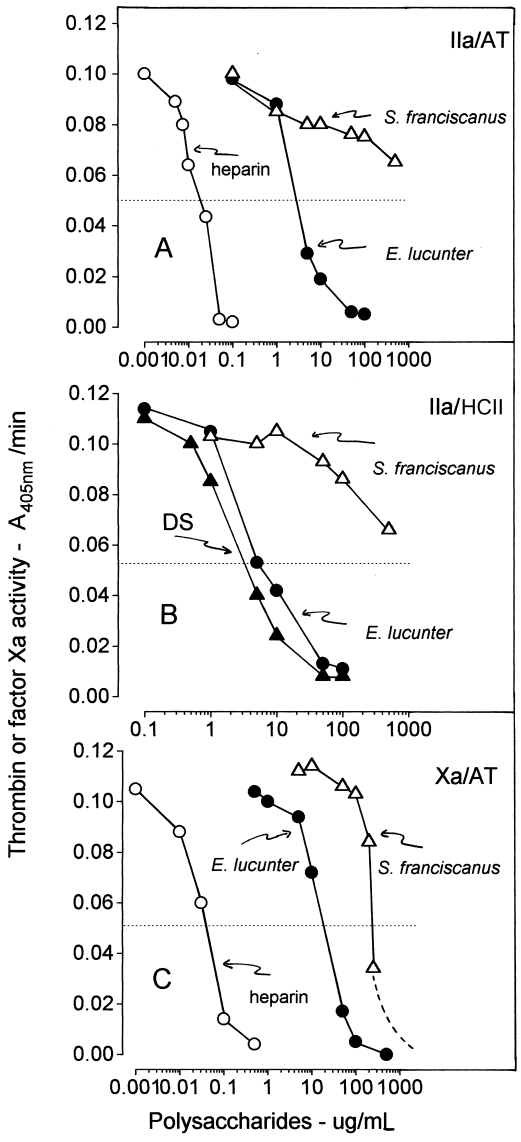

Fig. 5. Dependence on the concentration of 3-linked, 2-sulfated α-L-galactan and α-L-fucan for inactivation of thrombin (A and B) or factor Xa (C) by antithrombin (A and C) or heparin cofactor II (B). Antithrombin (50 nM) or heparin cofactor II (68 nM) were incubated with thrombin (15 nM) or factor Xa (15 nM) in the presence of various concentrations of the sulfated α-L-galactans from *E. lucunter* (●), sulfated α-L-fucans from *S. franciscanus* (△), heparin (○) and dermatan sulfate (▲). After 60 s, the remaining thrombin or factor Xa activity was determined with a chromogenic substrate (A_{405nm}/min). DS, dermatan sulfate.

mostly by the bridging mechanisms, as determined by the bulk polysaccharide structure.

Major conclusions.—Our results indicate that the structural requirements for interaction of sulfated polysaccharides with coagulation cofactors are stereospecific and have no relation with the charge density of the polysaccharide. This conclusion comes from comparison between the active sulfated α-L-galactan from *E. lucunter* and the almost inactive sulfated α-L-fucan from *S. franciscanus*.

The conformational analysis of these two polysaccharides is an important route to follow. The differences in chemical structure may in fact determine spacing between sulfate groups required to match the interval between basic amino acid residues in the protein chain. Scalar coupling constants are very similar for the sulfated α-L-galactan and the sulfated α-L-fucan (Table 3) and their values cannot explain the difference in biological activity. Furthermore, it indicates that the average conformation is the same for both polysaccharides. A more detailed study, including molecular dynamics, is necessary to correlate structure with the biological activity. Some polysaccharides give NMR spectra with line widths of few Hz, while others, with similar molecular mass, give spectra with broad lines. This implies the molecular dynamics must depend strongly on details of the local stereochemistry.[34]

Traditionally, determination of macromolecular structure by NMR depends on constraints whose information is local and is restricted to atoms close in space and of stable conformations. However, polysaccharides are flexible and with a linear shape. This results in incomplete constraints to define their relative orientation. In addition, several of them have high molecular mass hampering structure studies. New strategies are being developed, such as residual dipolar coupling, that can contribute to structure studies of these macromolecules,[35,36] including the sulfated α-L-galactan and sulfated α-L-fucan described in this study.

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