

Theme Issue Article

Effects of polysaccharides enriched in 2,4-disulfated fucose units on coagulation, thrombosis and bleeding

Practical and conceptual implications

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Summary

Sulfated polysaccharides from marine invertebrates have well-defined structures and constitute a reliable class of molecules for structure-activity relationship studies. We tested the effects of two of these polysaccharides, namely a sulfated fucan and a fucosylated chondroitin sulfate, on coagulation, thrombosis and bleeding. The compounds share similar 2,4-disulfated fucose units, which are required for high anticoagulant activity in this class of polymer. These residues occur either as branches in fucosylated chondroitin sulfate or as components of the linear chain in the sulfated fucan. These polysaccharides possess anticoagulant activity but differ significantly in their mechanisms of action. The fucosylated chondroitin sulfate inhibits thrombin by heparin cofactor II, whereas sulfated fucan inhibits thrombin by both antithrombin and heparin cofactor II. In addition, these

polysaccharides also have serpin-independent anticoagulant activities. Fucosylated chondroitin sulfate, but not sulfated fucan, activates factor XII. As a result of the complex anticoagulant mechanism, the invertebrate polysaccharides differ in their effects on experimental thrombosis. For instance, the sulfated fucan inhibits venous thrombosis at lower doses than fucosylated chondroitin sulfate. In contrast, fucosylated chondroitin sulfate is significantly more potent than sulfated fucan in arterial thrombosis. Finally, fucosylated chondroitin sulfate increases bleeding, while sulfated fucan has only a discrete effect. In conclusion, the location of 2,4-disulfated fucose units in the polysaccharide chains dictates the effects on coagulation, thrombosis and bleeding.

Keywords

Sulfated fucans, fucoidan, anticoagulant activity, antithrombotic activity, heparin, invertebrate

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Introduction

Sulfated fucans are widespread polysaccharides in marine brown algae. In terms of total biomass, they are more abundant than the vertebrate glycosaminoglycans (1). These compounds exhibit wide structural diversity and have intriguing biological activities (2–8). In particular, sulfated fucans have been known for some time to act as modulators of coagulation (9). Most of their activities are mediated by both antithrombin and heparin cofactor II.

Determination of the specific structural requirements for interaction with coagulation cofactors is an essential step for the rational development of anticoagulant drugs. However, identifi-

cation of these specific motifs in the algal sulfated fucans has been limited by their complex, heterogeneous structure (10, 11), and relatively few studies have investigated the biological activity of sulfated fucans in terms of molecular structure.

In addition to brown algae, sulfated fucans have been found in marine invertebrates (12). Unlike algal polysaccharides, sulfated fucans from invertebrates have unique structures of linear chains of α -L-fucose in a well-defined repetitive sequence (13). Various species possess sulfated fucans with different numbers of residues in the repeating units that vary among the different species according to the position of glycosidic linkages and the sulfation sites (14). These sulfated carbohydrates consti-

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tute the most reliable class of molecules for structure-activity relationship studies.

Previous studies with the sulfated fucans from invertebrates revealed that the anticoagulant activity is not merely a consequence of their charge density and sulfate content (15, 16). The structural requirement for these polysaccharides to interact with coagulation cofactors and their target proteases are stereospecific. The site of sulfation and/or the position of the glycosidic linkage also affect their activities (15). For instance, the occurrence of 2,4-di-sulfated units amplifies the effect of the anti-thrombin-mediated anticoagulant activity of 3-linked α -L-fucans, and a single 4-sulfated unit is the structural motif for the polymer to enhance the inhibition of thrombin by heparin cofactor II, while the exclusive presence of 2-sulfated residues has a deleterious effect (16).

Investigation of the antithrombotic activity requires the use of in-vivo models of thrombosis, in experimental animals, that mimic the multitude of pathological conditions involved in thrombosis, such as decreased blood flow, a state of hypercoagulation and lesion of the vascular endothelium. So far, only a few studies have reported the antithrombotic activity of the algal sulfated fucans (18–22). Interestingly, the anticoagulant action of these compounds correlates only weakly with their antithrombotic properties (17).

We now report on the effects of a sulfated fucan composed of $[\alpha$ -L-Fuc-2,4(OSO₄)-1 \rightarrow 3- α -L-Fuc-1 \rightarrow 3- α -L-Fuc-2(OSO₄)-1 \rightarrow 3- α -L-Fuc-2(OSO₄)]_n repeating units (Fig. 1A), isolated from the sea cucumber *Ludwigothurea grisea* on coagulation, thrombosis and bleeding, comparing its effects with that of a fucosylated chondroitin sulfate obtained from the same invertebrate (Fig. 1B). These two polysaccharides share the 2,4-disulfated fucose units, but differ in the location of these units, occurring at the non-reducing end branches in the fucosylated chondroitin sulfate or as a component of the linear chain in the sulfated fucan.

Materials and methods

Sulfated polysaccharides

The sulfated fucan and the fucosylated chondroitin sulfate were extracted from the sea cucumber *L. grisea* freshly collected from Guanabara Bay, Rio de Janeiro. The extraction and purification were carried out as described previously (15, 23). The purity and structure of the sulfated fucan were checked by agarose gel electrophoresis and Nuclear Magnetic Resonance (NMR) spectroscopy as previously described (15). Chondroitin 6-sulfate from shark cartilage was obtained from Sigma (St. Louis, MO, USA). Heparin used was the 5th International Standard (229 IU/mg)

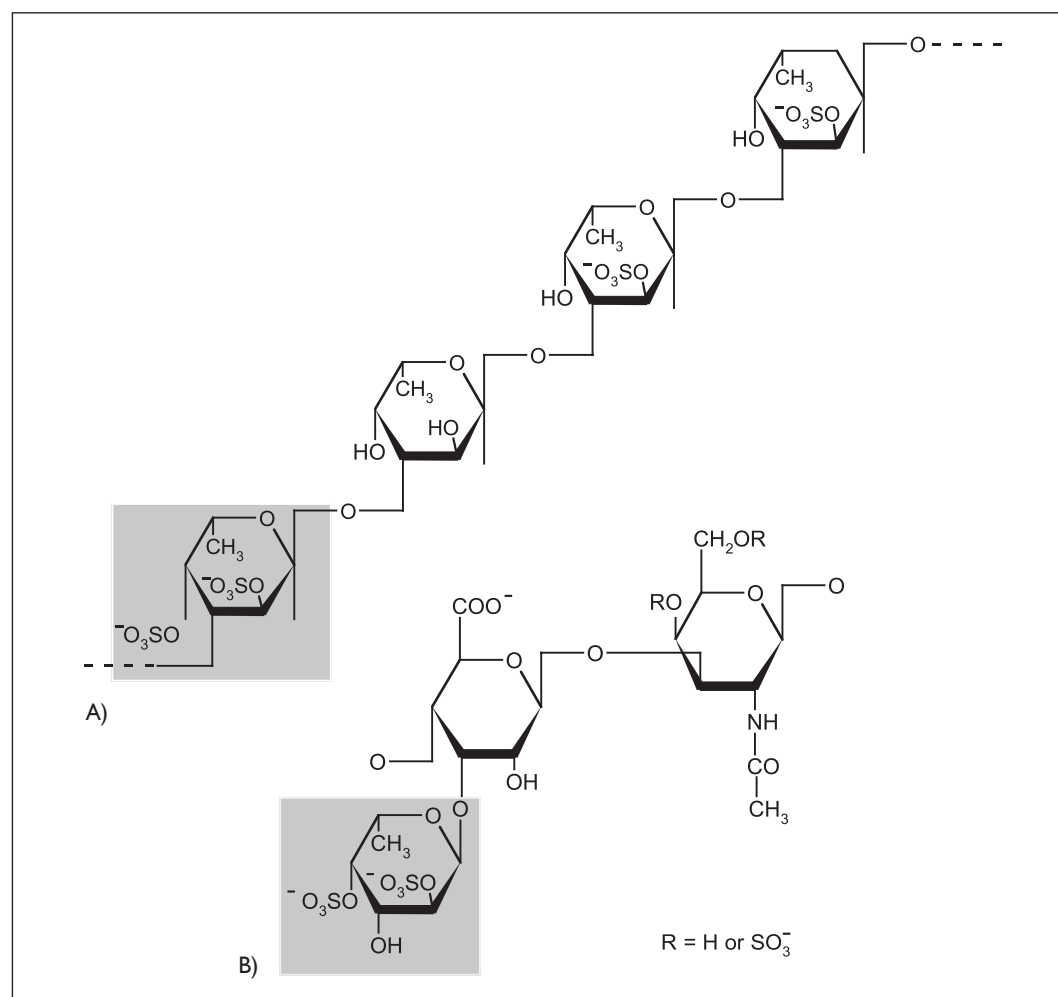


Figure 1: Structure of the sulfated polysaccharides from the sea cucumber *L. grisea*. A) The sulfated fucan is composed of $[\alpha$ -L-Fuc-2,4(OSO₄)-1 \rightarrow 3- α -L-Fuc-1 \rightarrow 3- α -L-Fuc-2(OSO₄)-1 \rightarrow 3- α -L-Fuc-2(OSO₄)]_n repeating units. B) Fucosylated chondroitin sulfate has a chondroitin sulfate-like backbone, but contains branches of 2,4-disulfated fucose units linked to the β -D-glucuronic acid residues on the polysaccharide core. The galactosamine units from the central core have a complex sulfation pattern. Approximately 12% are 4,6-disulfated, 53% 6-sulfated, 4% 4-sulfated and 31% are non-sulfated residues (39). These two sulfated polysaccharides also differ in their molecular size. Fucosylated chondroitin is approximately 40 kDa while sulfated fucan has a higher molecular size (>100 kDa). See References 23 and 39 for further details. The 2,4-disulfated fucose units in these two polysaccharides are highlighted in gray.

from the National Institute for Biological Standards and Control (Potters Bar, UK). Low-molecular-weight heparin (LMWH) (155 and 27 anti-Xa and anti-IIa units/mg, respectively) was a kind gift from Cristália Produtos Químicos e Farmacêuticos, São Paulo, Brazil.

Activated partial thromboplastin time

Activated partial thromboplastin time (APTT) (Biolab-Merieux AS, Rio de Janeiro, Brazil) was performed according to the manufacturer's specifications.

Recalcification time

Clotting assays were carried out in 96-well plates. First, normal human plasma samples (90 μ l) were mixed with different amounts of the sulfated polysaccharides in 0.15 M NaCl (10 μ l) and warmed for 60 seconds (s) at 37°C. Then, 100 μ l of 0.25 M CaCl₂ was added, and the transmittance at 405 nm was recorded for 720 s (Plate reader Thermo-max, America Devices). The rate of change of transmittance was proportional to clot formation.

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

Incubations were performed in 96-well plates. The final concentrations of the reactants included 10 nM antithrombin or 15 nM heparin cofactor II, 2 nM thrombin or factor Xa and 0–1,000 μ g/ml sulfated polysaccharide in 40 μ l of TS/PEG buffer (0.02 M Tris/HCl, 0.15 M NaCl, and 1.0 mg/ml polyethylene glycol 8,000, pH 7.4). When the same set of experiments was performed with diluted rat plasma in TS/PEG buffer (1:15), the addition of heparin cofactor II or antithrombin was omitted. Thrombin or factor Xa was added last to initiate the reaction. After 60 s of incubation at 37°C, 25 μ l of chromogenic substrate S-2238 for thrombin or S-2222 for factor Xa (Chromogenix AB, Mondal, Sweden) was added, and the absorbance at 405 nm recorded for 120 s (Plate reader Thermo-max, America Devices). The rate of change of absorbance was proportional to the thrombin or factor Xa activity remaining in the 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. In addition, no inhibition was observed when thrombin or factor Xa was incubated with sulfated polysaccharide alone over the range of concentrations tested. Thrombin activity was defined as 100% in control samples lacking sulfated polysaccharides. The IC₅₀ is the concentration of a given sulfated polysaccharide required for 50% inhibition of thrombin or factor Xa activities.

Activation of factor XII in the presence of sulfated polysaccharides

Assays of activation of factor XII were carried out in 96-well plates. Normal human plasma was diluted with three volumes of TS buffer and samples (40 μ l) were incubated with different concentrations of sulfated polysaccharide (30 μ l). After 60 s of incubation at 37°C, 30 μ l of chromogenic substrate 0.3 mM S-2302 (Chromogenix AB, Mondal, Sweden) was added, and the absorbance at 405 nm was recorded for 300 s (Plate reader Thermo-max, America Devices). The S-2302 is a chromogenic substrate

for plasma kallikrein, which is activated from its precursor prekallikrein by active factor XII. The method to determine the activity is based on the difference in absorbance between the p-nitroanilide formed and the original substrate. The rate of p-nitroanilide formation, i.e. the increase in absorbance per second at 405 nm, is proportional to the enzymatic activity. No activation occurred in control experiments in the absence of sulfated polysaccharide.

Assessment of antithrombotic properties

Venous thrombosis

Antithrombotic activity was investigated in rats with rabbit brain thromboplastin as the thrombogenic stimulus (24). We followed the institutional guidelines for animal care and experimentation. Rats (both sexes, ~200 g body weight, 5 animals per dose) were anaesthetised with an intramuscular injection of 100 mg/kg body weight of ketamine (Cristália, São Paulo, Brazil) and 16 mg/kg body weight of xylazine (Bayer AS, São Paulo, Brazil). Different doses of polysaccharides were infused into the right carotid artery and allowed to circulate for 5 minutes (min). The inferior vena cava was isolated and brain thromboplastin (5 mg/kg body weight) from Biolab-Merieux AS (Rio de Janeiro, Brazil) was slowly injected intravenously; after 1 min, 0.7 cm of isolated vena cava was clamped off using distal and proximal sutures. After 20 min stasis, the thrombus formed inside the occluded segment was carefully pulled out, washed with phosphate-buffered saline (PBS), dried for 1 hour at 60°C and weighed. Mean thrombus weight was obtained by the average weight from each group and then expressed as the percentage of the thrombus weight in the absence of polysaccharide administration.

Arterial thrombosis

Carotid artery thrombosis was induced using a modified method of Eitzman et al. (25). Rats (both sexes, ~200 g body weight) were anaesthetised with a mixture of ketamine and xylazine, as previously described, and secured in the supine position. The right common carotid artery was isolated through a midline cervical incision, and an ultrasonic flow probe (model 0.5 VB; Transonic Systems Inc., Ithaca, NY, USA) was applied. Different concentrations of sulfated polysaccharides were slowly injected into the left carotid and allowed to circulate for 5 min. Rose Bengal (90 mg/kg body weight; Fisher Scientific Co., Fair Lawn, NJ, USA) was injected into the inferior vena cava and, immediately after that, a 1.5 mW, 540 nm laser beam (Melles Griot Inc., Carlsbad, CA, USA) was applied to the right carotid artery from a distance of 6 cm. The flow in the carotid artery was monitored until complete occlusion occurred.

Bleeding effect

Wistar rats (both sexes, ~200 g body weight) were anaesthetised with a combination of xylazine and ketamine, as previously described. A cannula was inserted into the right carotid artery for administration of different doses of sulfated polysaccharides. After the polysaccharides had circulated for 5 min, the tail was cut 3 mm from the tip and carefully immersed in 40 ml distilled water at room temperature. Blood loss was determined 60 min later by measuring the hemoglobin dissolved in the water using a spectrophotometric method, as described by Herbert et al. (26).

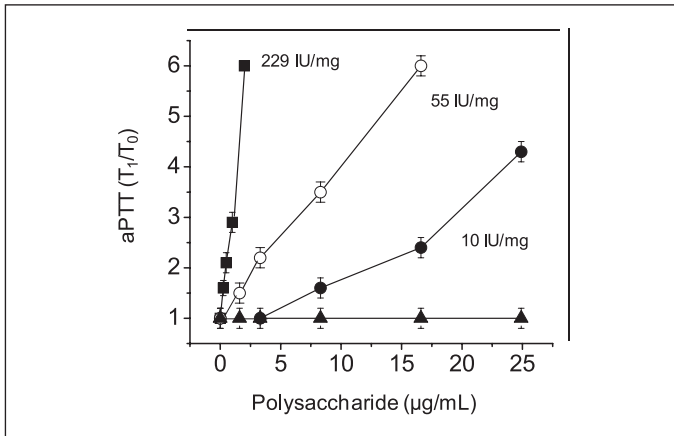


Figure 2: In-vitro anticoagulant activity of sulfated polysaccharides. Citrated human plasma samples were incubated with different concentrations of sulfated fucan (●), fucosylated chondroitin sulfate (○), unfractionated heparin standard (■) and vertebrate chondroitin 6-sulfate (▲) [mean ± SEM, n=3]. The anticoagulant activity was expressed as T_1/T_0 , which is the ratio between the clotting time in the presence and absence of sulfated polysaccharide. Numbers in the panel show anticoagulant activity as IU/mg, compared with unfractionated heparin standard.

The volume of blood was deduced from a standard curve based on absorbance at 540 nm.

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Differences in mean values were analysed using the Mann-Whitney U test. When more than one group was compared with one control, significance was evaluated using one-way analysis of variance (ANOVA). $P < 0.01$ was considered statistically significant.

Results

Anticoagulant activity

Sulfated fucan and fucosylated chondroitin sulfate from *L. grisea* have anticoagulant activities of 10 and 55 IU mg^{-1} by APTT, compared with a value 229 IU mg^{-1} for standard unfractionated heparin (UFH) (Fig. 2). Assays with purified serpins and coagulation proteases (Fig. 3) revealed significant differences in the effects of these two sulfated polysaccharides on the coagulation system. The action of fucosylated chondroitin sulfate was mainly via potentiation of heparin cofactor II inactivation of thrombin (open circles, Fig. 3A), with ~10-fold higher potency than sulfated fucan or heparin. When heparin cofactor II was replaced by antithrombin, a significant decrease in the activity of fucosylated chondroitin sulfate was observed (Fig. 3C). In contrast, sulfated fucan potentiated antithrombin and heparin cofactor II inhibition of thrombin in approximately the same range of concentrations (closed circles, Fig. 3A and C). Assays with factor Xa showed a shift to the right of the inhibitory curves for these two polysaccharides and total inactivation of the protease was not achieved (Fig. 3B). No inhibition of thrombin or factor Xa by sulfated fucan or fucosylated chondroitin sulfate was observed

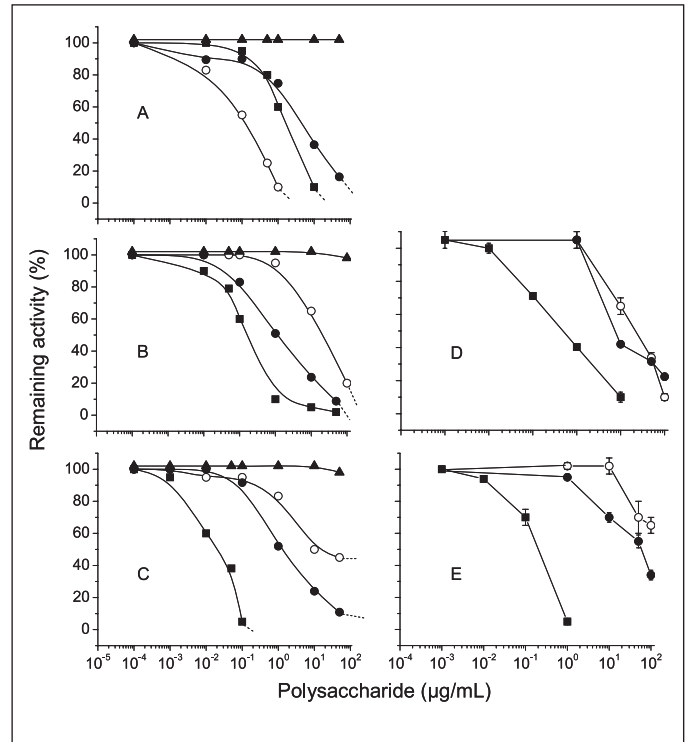


Figure 3: Concentration dependence of the sulfated polysaccharide for inactivation of thrombin by heparin cofactor II (A), antithrombin (C) or rat plasma (D) and inactivation of factor Xa by antithrombin (B) or rat plasma (E). Antithrombin or heparin cofactor (10 nM) were incubated with thrombin or factor Xa (2 nM) in the presence of increasing concentrations of sulfated fucan (●), fucosylated chondroitin sulfate (○), unfractionated heparin (■) and vertebrate chondroitin sulfate (▲). After 60 s, the remaining thrombin or factor Xa activity was determined with a chromogenic substrate. Substrate hydrolysis was detected using a Thermomax Microplate Reader equipped with a microplate mixer and heating system. Reactions were recorded continuously at 405 nm for 5 min at 37°C. The panels show the mean ± SD, n=3. D and E Pooled rat diluted plasma (1:15) was incubated with increasing concentrations of sulfated fucan (●), fucosylated chondroitin sulfate (○) and unfractionated heparin (■). Thrombin (D) or factor Xa (E) was added last to initiate the reaction. After 60 s of incubation at 37°C, the remaining thrombin or factor Xa activity was determined as described above.

in the absence of antithrombin or heparin cofactor II (not shown). Vertebrate chondroitin 6-sulfate, devoid of the sulfated fucose branches, had no effect on the coagulation assays (closed triangles, Fig. 3). When the same set of experiments was performed using diluted rat plasma as a source of antithrombin and heparin cofactor II, we clearly confirmed that the action of fucosylated chondroitin sulfate is mainly via potentiation of thrombin inhibition (Fig. 3D, open circles). Sulfated fucan possess a more balanced effect on thrombin and factor Xa (Figs. 3D and E, closed circles). We observed a shift to the right of the curves for both sulfated polysaccharides from sea cucumber when assays were performed with diluted rat plasma compared with assays with purified serpins. This observation suggests the interaction of the sulfated polysaccharides with other plasma proteins, preventing the binding to serpins.

We also investigated serpin-independent anticoagulant activity of these sulfated polysaccharides using antithrombin- and heparin cofactor II-free plasma. Addition of sulfated fucan or fucosylated chondroitin sulfate to either normal or serpin-free plasma increased the clotting time with similar potency (Table 1). For heparin, the effect was abolished in the serpin-free plasma, as expected. In antithrombin-free plasma, the clotting times were even higher after the addition of sulfated fucan or fucosylated chondroitin sulfate compared with normal control plasma. A possible explanation for this observation is that sulfated polysaccharides, free from sequestration with antithrombin, yield a more potent heparin cofactor II-mediated anticoagulant effect. Clearly, these results indicate that the invertebrate polysaccharides also possess a serpin-independent anticoagulant effect. For fucosylated chondroitin sulfate, we already demonstrated that this effect is due to inhibition of factor Xa and thrombin generation by the tenase and prothrombinase complexes, respectively (27). It is possible that sulfated fucan has a similar effect.

In conclusion, sulfated fucan and fucosylated chondroitin sulfate have a serpin-mediated anticoagulant activity. The effect of fucosylated chondroitin sulfate is primarily due to potentiation of heparin cofactor II. In contrast, sulfated fucan has a more balanced effect on thrombin inhibition, which is mediated by both antithrombin and heparin cofactor II. In addition, these two sulfated polysaccharides have a serpin-independent anticoagulant effect.

Activation of factor XII

Since factor XII is a coagulation system component that may be activated by sulfated polysaccharides (28), we investigated the effect of the polysaccharides on factor XII. Figure 4A shows that fucosylated chondroitin sulfate (open circles) induced factor XII activation whereas sulfated fucan (closed circles), similar to heparin, was inactive.

Our previous study with algal sulfated galactans demonstrated that activation of factor XII was associated with a prothrombotic effect on venous thrombosis (29). This effect may be observed using in-vitro clotting assays based on the recalcification time. Testing of sulfated fucan and fucosylated chondroitin sulfate on this assay showed exclusively anticoagulant effect, as indicated by $T_1/T_0 > 1$ (Fig. 4B).

Antithrombotic activity

Sulfated fucan and fucosylated chondroitin sulfate inhibited thrombus formation in a venous model in rats, but at distinct doses (Fig. 5A). At low doses (up to 1.0 mg/kg body weight), sulfated fucan was an effective antithrombotic agent, similar to LMWH. Fucosylated chondroitin sulfate also exhibited an antithrombotic effect, but required higher doses to achieve the same effect as with sulfated fucan, LMWH or UFH. Thus, sulfated fucan and fucosylated chondroitin sulfate inhibited ~60% and ~10% of the thrombus weight, respectively, at a dose of 1.0 mg/kg body weight (Fig. 5A). Sulfated fucan produced a dose response curve nearly coincident to that of LMWH, especially at low doses.

Surprisingly, in an experimental model of arterial thrombosis, fucosylated chondroitin sulfate showed a significantly more potent antithrombotic effect than sulfated fucan (Fig. 5B). Fucosylated

Table 1: Concentrations of sulfated polysaccharides required to double APTT in normal, antithrombin- and/or heparin cofactor II-free plasmas. Results are representative of three different experiments. * $p < 0.01$ or **not significant for normal versus serpin-depleted plasmas.

Sulfated polysaccharide	Plasma (values in $\mu\text{g/ml}$)			
	Normal	Anti-thrombin-free	Heparin cofactor II-free	Antithrombin+ heparin cofactor II-free
Heparin	0.68	ND	0.43**	>10.0*
Sulfated fucan	7.7	7.0*	7.8**	8.3**
Fucosylated chondroitin sulfate	2.8	2.08*	2.6**	3.0**

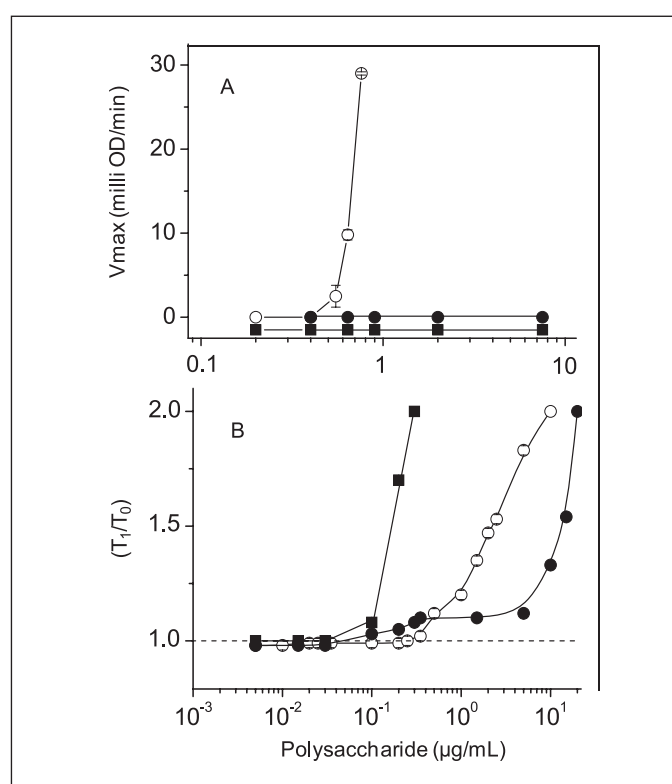


Figure 4: Factor XII activation (A) and anticoagulant activity based on recalcification time (B). Citrated human plasma samples were incubated with different concentrations of sulfated fucan (●), fucosylated chondroitin sulfate (○) or unfractionated heparin (■) and used to measure either factor XII activation (A) or recalcification time (B) (mean \pm SEM, $n=3$). In the assays for factor XII activation, after 10 s of incubation at 37°C, 0.3 mM chromogenic substrate for plasma kallikrein was added. The increase in absorbance at 405 nm was expressed in milli optical density/min (mean \pm SEM, $n=3$). In the recalcification time assay, 90 μl -samples of normal human plasma were mixed with different amounts of sulfated polysaccharides for 60 s at 37°C. Then 100 μl of 0.25 M CaCl_2 was added and the transmittance at 405 nm was recorded for 720 s. The anticoagulant activity was expressed as T_1/T_0 , which is the ratio between the clotting time in the presence and absence of sulfated polysaccharide. The broken line ($T_1/T_0 = 1$) indicates no effect of the sulfated polysaccharide on coagulation.

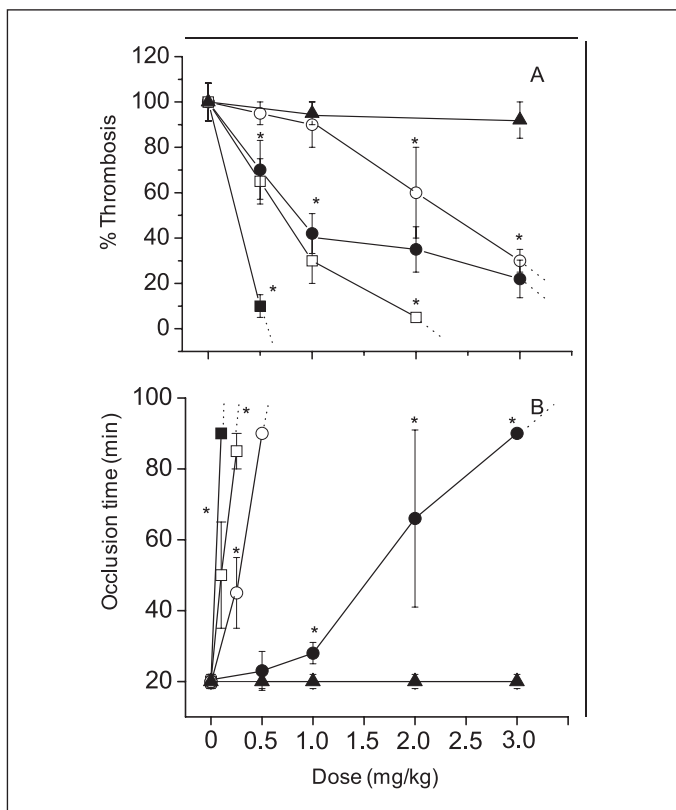


Figure 5: Antithrombotic effect of sulfated polysaccharides.

Assays were carried out in rats with different intravascular doses of sulfated fucan (●), fucosylated chondroitin sulfate (○), unfractionated heparin (■), low-molecular-weight heparin (□) and vertebrate chondroitin 6-sulfate (▲). A) Venous antithrombotic activity was investigated using a stasis + hypercoagulability thrombosis model in vena cava of rats. Different doses of sulfated polysaccharide were administered and allowed to circulate for 5 min. Then, thromboplastin (5 mg/kg body weight) was slowly injected intravenously and 0.7 cm of the isolated vena cava segment was tied off. After 20 min stasis, the thrombus formed was dried and weighed. The results were expressed as % of the weight (mean \pm SEM, $n=5$ * $p < 0.01$ vs. control), 100% representing absence of any inhibition of thrombosis (thrombus weight in the absence of sulfated polysaccharide administration). B) Arterial thrombosis model induced in carotid artery of rats by laser irradiation. Different doses of sulfated polysaccharides were administered and allowed to circulate for 5 min. A 1.5-mW, 540-nm laser beam was applied to the artery from a distance of 5 cm. Rose Bengal dye (80 mg/kg body weight) was then injected into the cava vein, and the flow in the vessel was monitored by an ultrasonic flow probe until complete occlusion occurred. The time for complete artery occlusion was the average time for each dose expressed as percentage of the time in the absence of sulfated polysaccharide (mean \pm SEM, $n=5$ * $p < 0.01$ vs. control).

sylated chondroitin sulfate exhibited a dose response curve almost coincident to that of LMWH. As a negative control, vertebrate chondroitin 6-sulfate showed no antithrombotic effect.

Bleeding effect

To evaluate the effect of sulfated polysaccharides on bleeding, we used the bleeding time assay. In this model, UFH, but not LMWH, increased the loss of blood significantly (Fig. 6). Fuco-

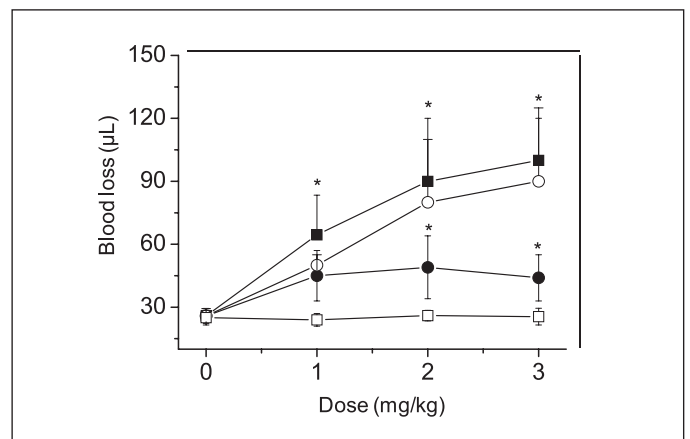


Figure 6: Bleeding effect. Different doses of sulfated fucan (●), fucosylated chondroitin sulfate (○), unfractionated heparin (■) and low-molecular-weight heparin (□) were infused into rats. After 5 min, the rat's tail was cut 3 mm from the tip and immersed in 40 ml of distilled water at room temperature. Blood loss was determined 60 min later by measurement of the haemoglobin in the water. The results were expressed in μL of blood loss (mean \pm SEM, $n=5$ * $p < 0.01$ vs. control). For clarity, only one SEM bar is shown.

sylated chondroitin sulfate had an effect similar to that of UFH. In contrast, sulfated fucan slightly increased the loss of blood.

Discussion

Several authors have attempted to identify structural motifs necessary for the anticoagulant and antithrombotic activities using either chemically oversulfated or desulfated algal sulfated fucan (30, 31). The activity increases with increasing sulfate content and decreases when the native sulfation pattern is disrupted by partial desulfation (32). In order to avoid the size dispersion observed for native high-molecular-weight algal sulfated fucan, several low-molecular-weight derivatives were prepared. The anticoagulant activity decreases with a decrease in the molecular size of the polysaccharide (30). The main obstacle that continues to persist is that most of the low-molecular-weight derivatives still have complex and heterogeneous structures.

This problem may be overcome with the use of invertebrate sulfated fucans. The regular and well-defined units in these compounds reveal clearly which type of glycosidic linkage, sulfation position and sequence of residues are responsible for the specific interaction with blood coagulation proteins that triggers the coagulation process (1).

We now employed two invertebrate polysaccharides that share 2,4-disulfated fucose residues. This is a structural motif in sulfated fucans that favors anticoagulant activity (15). Both polysaccharides possess anticoagulant activity, as expected, but differ significantly in the anticoagulant mechanism. Sulfated fucan potentiates antithrombin inhibition of thrombin. This observation is in line with our previous proposal that the occurrence of 2,4-di-sulfated units has an amplifying effect on the antithrombin-mediated anticoagulant activity of 3-linked α -L-fucans (13). Surprisingly, when occurring as branches, they do not favor interaction with antithrombin in a similar way. The

anticoagulant effect of fucosylated chondroitin sulfate is mainly via potentiation of heparin cofactor II. The decreased effect of sulfated fucan through this serpin may be attributed to the observation that 2-sulfated fucose units, which intercalate with the 2,4-disulfated units in the polysaccharide chain (Fig. 1A), have a deleterious effect on thrombin inhibition through heparin cofactor II (16). Sulfated fucan and fucosylated chondroitin sulfate differ in their molecular sizes (>100 vs. ~40 kDa). Our results do not allow us to determine the contribution of the molecular size to the biological activities of these polysaccharides, but both compounds have a significantly larger size than UFH (~15 kDa), possibly indicating that their sizes do not limit activity.

Another intriguing observation is that fucosylated chondroitin sulfate, a polymer with an average molecular size of ~40 kDa, activates factor XII while sulfated fucan, a high-molecular-weight polysaccharide (>100 kDa), is inactive (Fig. 4A). Activation of this factor has been associated with high-molecular-weight polysaccharides (28). Clearly, our results indicate that, beyond molecular size, specific structures are necessary for activation of factor XII by sulfated polysaccharides. The importance of activation of this factor has been highlighted by the observation that chemically oversulfated chondroitin sulfate, found as a contaminant of heparin preparations, activates factor XII and induces severe hypertension, associated with kallikrein release, when administered by intravenous injection (33).

The differences in the effects of the two invertebrate polysaccharides on thrombosis are even more intriguing. Sulfated fucan inhibits thrombus formation in a venous thrombosis model at lower doses than fucosylated chondroitin sulfate does, despite its lower anticoagulant activity. Therefore, it is not possible to correlate anticoagulant activity *in vitro* and antithrombotic effect *in vivo*. Venous thrombosis is mostly associated with blood coagulation and the consequent deposits of fibrin and erythrocytes in regions of stasis or low shear stress (34). It is possible that the more balanced anticoagulant effect of sulfated fucan, activating both antithrombin and heparin cofactor II, favors its antithrombotic effect compared with fucosylated chondroitin sulfate, which acts almost exclusively through heparin cofactor II. Another possibility is that activation of factor XII by fucosylated chondroitin sulfate and the consequent prothrombotic stimulus counterbalance the anticoagulant effect of the polysaccharide. In contrast, sulfated fucan does not activate factor XII.

In arterial thrombosis, we observed the opposite effect; fucosylated chondroitin sulfate is considerably more active as an antithrombotic than sulfated fucan. Arterial thrombosis occurs in regions of moderate shear stress, mainly through adhesion and aggregation of platelets on the luminal surface of damaged

vessels (35). The potent action of fucosylated chondroitin sulfate on arterial thrombosis cannot be attributed to inhibition of platelet aggregation since the polysaccharide is devoid of this effect (not shown, see [36]). We still do not have an explanation for this result. It is possible that the higher blood flow in arteries compared to veins requires antithrombotic polysaccharides that can interact with the vessel wall and prevent the beginning of thrombosis in the interface between the vessel wall and blood. In this case, fucosylated chondroitin sulfate could interact with higher affinity than sulfated fucan with other molecules from the arterial wall. However, more significantly, our results indicate that it is possible to obtain sulfated polysaccharides that act specifically by preventing venous or arterial thrombosis. This observation may help to develop specific and potent antithrombotic agents. Our results do not allow us to determine the contribution of the molecular size to the antithrombotic activity of these compounds. Nevertheless, as we already discussed for the anticoagulant activity, both invertebrate polysaccharides have a significantly larger size than UFH, indicating that their sizes do not limit the activity.

Finally, these two invertebrate polysaccharides also differ in their effect on bleeding. It has been suggested that the removal of all antithrombin-dependent actions of heparin could reduce the bleeding risk (27). Dermatan sulfate, an anticoagulant that acts exclusively through heparin cofactor II, has no effect on bleeding (37). These previous observations suggest a more favorable profile with respect to bleeding for fucosylated chondroitin sulfate than for sulfated fucan. Unexpectedly, fucosylated chondroitin sulfate increases bleeding, like UFH, while sulfated fucan is almost inactive in this respect. Therefore, it is difficult to predict the haemostatic actions of a sulfated polysaccharide based exclusively on the effects *in vitro*. Each polysaccharide requires testing on models *in vivo*. In fact, we have already observed that sulfated galactans, with antithrombin-mediated anticoagulant activity similar to UFH, have almost no effect on bleeding (38).

In conclusion, we studied two invertebrate polysaccharides, which share similar 2,4-disulfated fucose residues, the structural motif associated with high anticoagulant activity. The occurrence of these units as branches or as components of a linear polysaccharide chain determines different effects on coagulation, thrombosis and bleeding. Our results provide essential data for a more rational development of antithrombotic drugs from sulfated fucans.

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