Selective cleavage and anticoagulant activity of a sulfated fucan: stereospecific removal of a 2-sulfate ester from the polysaccharide by mild acid hydrolysis, preparation of oligosaccharides, and heparin cofactor II–dependent anticoagulant activity

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A linear sulfated fucan with a regular repeating sequence of [3]-α-L-Fucp-(2SO4)-(1→3)-α-L-Fucp-(4SO4)-(1→3)-α-L-Fucp-(2,4SO4)-(1→3)-α-L-Fucp-(2SO4)-(1→3)]n is an anticoagulant polysaccharide mainly due to thrombin inhibition mediated by heparin cofactor II. No specific enzymatic or chemical method is available for the preparation of tailored oligosaccharides from sulfated fucans. We employ an apparently nonspecific approach to cleave this polysaccharide based on mild hydrolysis with acid. Surprisingly, the linear sulfated fucan was cleaved by mild acid hydrolysis on an ordered sequence. Initially a 2-sulfate ester of the first fucose unit is selectively removed. Thereafter the glycosidic linkage between the nonsulfated fucose residue and the subsequent 4-sulfated residue is preferentially cleaved by acid hydrolysis, forming oligosaccharides with well-defined size. The low-molecular-weight derivatives obtained from the sulfated fucan were employed to determine the requirement for interaction of this polysaccharide with heparin cofactor II and to achieve complete thrombin inhibition. The linear sulfated fucan requires significantly longer chains than mammalian glycosaminoglycans to achieve anticoagulant activity. A slight decrease in the molecular size of the sulfated fucan dramatically reduces its effect on thrombin inactivation mediated by heparin cofactor II. Sulfated fucan with ~45 tetrasaccharide repeating units binds to heparin cofactor II but is unable to link efficiently the plasma inhibitor and thrombin. This last effect requires chains with ~100 or more tetrasaccharide repeating units. We speculate that the template mechanism may predominate over the allosteric effect in the case of the linear sulfated fucan inactivation of thrombin in the presence of heparin cofactor II.

Key words: anticoagulant activity/heparin cofactor II/selective cleavage of sulfated polysaccharide/stereospecific desulfation/sulfated fucans

Introduction

Sulfated fucans are among the most abundant nonmammalian sulfated polysaccharides found in nature. They occur in brown algae and in marine invertebrates. Algal sulfated fucans have complex and heterogeneous structures, but recent studies revealed the occurrence of some ordered repeat units in the sulfated fucans from several species (Bertea et al., 2003; Mourão, 2004). In contrast, invertebrate polysaccharides have simple, ordered structures, which differ in the specific patterns of sulfation and/or position of the glycosidic linkage within their repeating units (Alves et al., 1997, 1998; Vilela-Silva et al., 1999, 2002).

Sulfated fucans have a wide variety of biological activities, but their potent anticoagulant action is by far the most widely studied. These polysaccharides are potent thrombin inhibitors whose action is mediated by antithrombin or heparin cofactor II. Attempts to identify in the algal polysaccharides specific structural features necessary for their anticoagulant activity have failed due to their complex and heterogeneous structures (Mourão, 2004). As we extend these studies to the invertebrate fucans, we were able to determine that their anticoagulant activity is not merely a consequence of the charge density but depends on specific structural motifs necessary for interaction with coagulation cofactors and their target proteases (Pereira et al., 2002).

The major structural requirement for the interaction of linear sulfated fucans with heparin cofactor II is the presence of 4-sulfated fucosic units. If we replace heparin cofactor II with antithrombin, 2,4-disulfated units are required for interaction with the cofactor. Apparently the presence of exclusively 2-sulfated fucose residues has a deleterious effect on the anticoagulant activity.

A further step for characterizing the anticoagulant activity of the sulfated fucans is to prepare low-molecular-weight derivatives and test their activities in specific assays. In the case of mammalian glycosaminoglycans, specific enzymatic and chemical methods can be used to provide tailored oligosaccharides for biological activity. This approach revealed that a specific sequence of [4]-α-L-IdUA-(2SO4)-(1→3)-β-D-GalNAc-(4SO4)-(1→3)]n, where n = 3 is required for the binding of dermatan sulfate to heparin cofactor II (Maimone and Tollefsen, 1990) and n = 12 is the minimum size required to stimulate the thrombin–heparin cofactor II reaction (Tollefsen et al., 1986). Similar studies...
have been widely used for heparin (Conrad, 1998). However, it is not possible to apply this approach to sulfated fucans due to the absence of enzymes and chemical methods to cleave the polysaccharide and to produce oligosaccharides with well-defined structures. Enzymes that cleave the core of the sulfated fucans, leading to a rapid reduction of the molecular size, have been reported in marine invertebrates and bacteria (Daniel et al., 1999; Furukawa et al., 1992a; Kitamura et al., 1992). However, the limitation for purification of these enzymes and the difficulties to identify different structures varying in their sensitivity to hydrolysis has limited their use for structural elucidation and the preparation of oligosaccharides. Preliminary reports have appeared in the literature concerning sulfatases able to act on sulfated fucans (Furukawa et al., 1992b; Lloyd et al., 1962) and nothing is known about their properties, particularly whether they can act alone or need glycosidases to achieve significant desulfation of sulfated fucans. Only one study has shown a sulfatase able to act specifically on some sulfate groups of sulfated fucan (Daniel et al., 2001). But this enzyme does not act on a long polysaccharide chain and is able to release sulfate groups exclusively from mono- or disaccharides.

To overcome the limitation for preparation of low-molecular-weight derivatives from the sulfated fucan, we employ an apparently nonspecific methodology to cleave the polysaccharide, based on mild hydrolysis with acid. We concentrated our work on a sulfated fucan from the sea urchin Lytechinus variegatus, with a regular repeating sequence as follows: [3]-α-L-Fucp-(2SO4)-(1→3)-α-L-Fucp-(4SO4)-(1→3)-α-L-Fucp-(2,4SO4)-(1→n]_n. Four types of fucose units are designated by the letters A–D. The average molecular weight of this fucan is ~100 kDa, and its repeating tetrasaccharide unit has 980 Da. Therefore we estimated that the polysaccharide has an average of 100 tetrasaccharide units. (B) In the first stage of the acid hydrolysis, a sulfate ester is selectively removed from residue A, thereafter designated A’. (C and D) A preferential cleavage of the linkage formed by the nonsulfated fucose residue (A’ unit) occurs in the second stage of the acid hydrolysis. Oligosaccharides with well-defined molecular size are formed, containing nonsulfated fucose units at the reducing ends (A’R).

Fig. 1. Summary of the sulfated fucan structure before (A) and in the course of mild acid hydrolysis (B–D). A sulfated fucan from L. variegatus is a linear polysaccharide, composed of a tetrasaccharide repeating sequence as follows: [3]-α-L-Fucp-(2SO4)-(1→3)-α-L-Fucp-(4SO4)-(1→3)-α-L-Fucp-(2,4SO4)-(1→n]_n. Four types of fucose units are designated by the letters A–D. The average molecular weight of this fucan is ~100 kDa, and its repeating tetrasaccharide unit has 980 Da. Therefore we estimated that the polysaccharide has an average of 100 tetrasaccharide units. (B) In the first stage of the acid hydrolysis, a sulfate ester is selectively removed from residue A, thereafter designated A’. (C and D) A preferential cleavage of the linkage formed by the nonsulfated fucose residue (A’ unit) occurs in the second stage of the acid hydrolysis. Oligosaccharides with well-defined molecular size are formed, containing nonsulfated fucose units at the reducing ends (A’R).
the requirement for interaction of this polysaccharide with heparin cofactor II. The molecular size required for binding to the serpin and to stimulate thrombin inhibition varies markedly between the mammalian glycosaminoglycans and the invertebrate sulfated fucan.

Results and discussion

Mild acid hydrolysis of the sulfated fucan yields oligosaccharides with well-defined molecular size

Mild acid hydrolysis gradually reduces the molecular size of the sulfated fucan, as indicated by metachromasia of fractions eluted from a Superose-6 column (Figure 2A). A similar profile was observed when the fractions were assayed by the phenol–sulfuric acid reaction for fucose (not shown in Figure 2A). Thus the decrease in molecular size by mild hydrolysis is not followed by a substantial disruption of the sulfation pattern of the molecule. Polyacrylamide gel electrophoresis (PAGE) reveals that mild acid hydrolysis produces a wide variety of metachromatic bands with well-defined sizes (Figure 2B). As the time of hydrolysis proceeds from 1 to 6 h, the proportions of bands with higher electrophoretic mobility increase but maintain the same well-defined pattern of narrow electrophoretic bands.

The oligosaccharides produced after 6 h of mild acid hydrolysis of the sulfated fucan were purified by gel chromatography on Bio-Gel P-10 (open circles in Figure 3A). A mixture of oligosaccharides were separated on this column and designated as I–VI. PAGE of the purified fractions (Figure 3B) shows that the oligosaccharides correspond to the well-defined size bands already observed in Figure 2B. As we increased the period of hydrolysis from 6 to 9 h (closed circles in Figure 3A), the proportions of low-molecular-mass oligosaccharides increase, but always with similar size as those obtained after 6 h of hydrolysis.

It is not possible to estimate the molecular masses of oligosaccharides I–VI due to the absence of appropriate standards. However, it is likely that the various metachromatic bands observed on PAGE (Figures 2B and 3B) differ from each other by one tetrasaccharide repeating unit, estimated as 901 Da (Figure 1C). This possibility is also supported by comparison between the electrophoretic mobility of these oligosaccharides with those obtained by chondroitin ABC lyase digestion of mammalian dermatan sulfate (data not shown).

Overall, our results show that mild acid hydrolysis of the sulfated fucan yields oligosaccharides with well-defined molecular size, indicative of preferential cleavage of a specific glycosidic linkage without substantial desulfation. Otherwise, we would expect a mixture of products with a wide dispersion in molecular size, as observed for the majority of sulfated polysaccharides.

Fig. 2. Mild acid hydrolysis of the sulfated fucan. (A) Sulfated fucan from L. variegatus (2 mg) before (closed circles) and after partial acid hydrolysis with 1.25 ml of 0.01 M HCl at 60°C for 1 h (open circles) or 6 h (closed squares) was applied to a Superose-6 and eluted as described under Materials and methods. The fractions were assayed by metachromasia. A similar profile was obtained when the fractions were assayed by the phenol-sulfuric acid reaction (not shown in the panel). (B) Sulfated fucan, before and after mild acid hydrolysis (10 μg each), were analyzed by PAGE, as described under Materials and methods. The molecular weights (kDa) of standard compounds are indicated at the left. These standards are: high-molecular-weight dextran sulfate (±100 kDa), chondroitin 4-sulfate from bovine trachea (~40 kDa), dermatan sulfate from pig skin (~20 kDa), and low-molecular-weight dextran sulfate (~10 kDa).

Fig. 3. Size fractionation of the sulfated fucan oligosaccharides. (A) Sulfated fucan (5 mg) partially hydrolyzed with 1 ml 0.01 M HCl at 60°C for 6 h (open circles) or 9 h (closed circles) were applied to a Bio-Gel P-10 column (200 × 0.9 cm), equilibrated with aqueous 10% ethanol, containing 1.0 M NaCl. The column was eluted as described under Materials and methods, and the fractions were assayed by metachromasia. The fractions containing the oligosaccharides (as indicated by the horizontal bars) were pooled, freeze-dried, desalted, and dissolved in distilled water. (B) Intact sulfated fucan, a mixture of unfractonated oligosaccharides, the seven major fractions obtained on Bio-Gel P-10 (10 μg each) were applied to a 10% polyacrylamide gel and analyzed as described in the legend of Figure 2. The molecular weights (kDa) of standard compounds are indicated at the left. See also the legend of Figure 2.
A selective 2-desulfation occurs in the course of mild acid hydrolysis of the sulfated fucan

The $^1$H-nuclear magnetic resonance (NMR) spectrum of the native sulfated fucan shows four well-defined anomeric signals, labeled with the letters A–D (Intact in Figure 4), which correspond to the four types of fucose residues in the sea urchin fucan (see structure in Figure 1A) (Alves et al., 1998). Oligosaccharides II–VI, obtained from gel filtration chromatography after mild acid hydrolysis of the sulfated fucan, show $^1$H spectra very similar with each other but clearly different from the spectrum of the native polysaccharide. The peak corresponding to unit A is not present in any of the oligosaccharides, and a new peak could be assigned as A$'$. Concomitantly, the chemical shift of signal C is slightly to lowfield and overlaps with signal B. Oligosaccharide I, the smallest one, has a more heterogeneous $^1$H-NMR spectrum, indicating that it does not contain a regular repeating structure as do oligosaccharides II–VI.

Total correlation spectroscopy (TOCSY) and $^1$H/$^13$C heteronuclear multiple quantum correlation spectroscopy (HMQC) spectra of oligosaccharides II–VI confirmed that the four anomeric signals correspond to the four units labeled in Figure 4, as exemplified by the native fucan (Figures 5A and 6A) and by oligosaccharide III (Figures 5B and 6B).
Chemical shifts for the four types of fucose residues in oligosaccharide III as well as for the native sulfated fucan are listed in Table I. Residue A′ has H2 at ~0.7 ppm to high-field compared with H2 of residue A in the native fucan (Figure 5A and B), which typically indicates a desulfation at this site. No major differences can be observed among the chemical shifts of residues B, C, and D found in the native fucan and in oligosaccharide III. This observation was further confirmed by the 1H/13C HMQC spectra (Figure 6A and B) and the 13C chemical shifts reported in Table I. Residue A′ has C2 at ~5.8 ppm to highfield compared with C2 of residue A of the native fucan, again as expected for a desulfated site.

The time course of mild acid hydrolysis of the sea urchin fucan was followed by 1D 1H-NMR spectroscopy (Figure 7). The native sulfated fucan was incubated with acid inside the NMR tube and several 1H spectra were obtained from 10 min to 6 h. Using this procedure, we could follow the chemical modifications that occur in the polysaccharide during mild acid hydrolysis. In the first 60 min of hydrolysis, most of the 1H anomeric signal of residue A disappears with a concomitant increase of signal A′, whereas the integral of 1H anomeric signal D does not change (Figure 7B). In the longer periods of hydrolysis, the 1H-NMR spectra remain unchanged. The mixture of oligosaccharides obtained after 1 h of acid hydrolysis was analyzed by TOCSY (Figure 5C). Clearly, we can notice the similarity with the TOCSY spectrum of oligosaccharide III (Figure 5B), except that two spin systems of signal C coexist (designated C and C′), indicative that some 2-sulfation remains on residue A.

In conclusion, mild acid hydrolysis of the sulfated fucan from *L. variegatus* selectively removes 2-sulfate ester from the first fucose unit, which constitutes the regular repetitive tetrasaccharide units of the sea urchin fucan.

### Table I. Proton and carbon chemical shifts (ppm) for residues of α-fucose in native fucan and in oligosaccharide III obtained by mild acid hydrolysis

<table>
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<tr>
<td></td>
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<td>C-5</td>
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The spectra were recorded at 600 MHz in 99.9% D₂O. Chemical shifts are relative to external trimethylsilylpropionic acid at 0 ppm for 1H and to methanol for 13C. Values in boldface indicate positions bearing a sulfate ester.

### Fig. 7. Expansions of the 5.6–4.7 ppm regions of the 1H-NMR spectra at 600 MHz of the sulfated fucan at different periods of mild acid hydrolysis at 60°C. (A) Sulfated fucan (3 mg) was dissolved in 1.0 ml 99.9% D₂O containing 0.01 M HCl in a 5-mm inner diameter NMR tube. This solution was heated at 60°C, and 1H-NMR spectra were recorded from 10 min to 6 h, as indicated in the figure. Signals of the anomeric protons of residues A, A′, and D were integrated, and the integrals are shown in B.
A specific glycosidic linkage is preferentially cleaved by mild acid hydrolysis

The molecular size of the oligosaccharides formed from the sulfated fucan decreases as the period of acid hydrolysis proceeds from 1 to 6 h, but each oligosaccharide shows a very narrow band on PAGE (Figures 2 and 3). The preponderant chemical structure of these oligosaccharides remains unchanged, as revealed by 1H-NMR spectra in the time course experiment of Figure 7. These observations suggest that a specific glycosidic linkage of the sulfated fucan is preferentially cleaved by acid hydrolysis; otherwise, we would expect oligosaccharides with a wide variety of molecular size as well as with a heterogeneous sulfation pattern. Identification of the specific linkage cleaved by acid hydrolysis is difficult, because the sulfated fucan contains a single type of glycosidic linkage, that is, α(1→3) linked units (Figure 1). We attempted to determine the cleavage site on the sulfated fucan using NMR spectroscopy. Anomeric protons from reducing terminals of fucose give very faint bands, and it was not possible to identify these signals and to trace spin systems on the 2D 1H-NMR spectra. The presence of only faint bands of terminal reducing fucose units on the 1H-NMR spectra indicates that the oligosaccharides obtained by mild acid hydrolysis of the sulfated fucan have a significant high number of repeating units, certainly =~ 10 tetrasaccharide sequences. This is also supported by the observation that these oligosaccharides migrate on PAGE almost as a dextran sulfate with an average molecular weight of 10 kDa (Figures 2B and 3B). However, 1H/13C HMOC spectra show characteristic anomeric signals of reducing fucose—an equilibrium between α- and β-forms—as exemplified by oligosaccharide II in Figure 8. Comparison between the 1H and 13C chemical shifts of α- and β-anomeric signals of oligosaccharide II with standard compounds (Table II) clearly indicates that they originated from a nonsulfated fucose terminal. This observation indicates that the preferential cleavage site in the course of mild acid hydrolysis is the glycosidic linkage formed between residue A’ (the desulfated fucosyl unit) and residue D (a 4-sulfated fucose unit).

Conclusion concerning the ordered degradation of the sulfated fucan by mild acid hydrolysis

Taken together, the data of Figures 2–8 and Tables I and II indicate that sulfated fucan from the sea urchin *L. variegatus* is degraded in a specific order during mild hydrolysis with acid. In the initial stage of degradation, a 2-sulfate ester is selectively removed from the first fucose unit of a tetrasaccharide (unit A). Thereafter, the molecular size of the polysaccharide is slowly reduced due to a preferential cleavage of the glycosidic linkage formed between the nonsulfated residue (A’ in Figure 1B) and the second, 4-sulfated unit (unit D). It has already been observed for a fucosylated chondroitin sulfate that the susceptibility of fucosyl residues to acid hydrolysis varies depending on the sulfation pattern. But in this case, branches of nonsulfated fucose are more resistant to acid hydrolysis than the sulfated units (Kariya et al., 1990; Mourão et al., 1996). No difference has been observed in the susceptibility of different O-sulfate esters to acid hydrolysis. However, 2-sulfate groups can be removed from heparin with considerable selectively under certain alkaline conditions. These treatments result in loss of 2-sulfate groups without any loss of 6-sulfates (Jaseja et al., 1989; Rej et al., 1989).

Evidence that the ordered degradation of the sulfated fucan from *L. variegatus* by mild acid hydrolysis is determined by its particular pattern of sulfation (and perhaps also by the position of the glycosidic linkage) comes from similar experiments using sulfated fucans from different species of invertebrate and from brown algae (Figure 9). The particular pattern of oligosaccharides with very narrow

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### Table II. Chemical shifts (ppm) for anomeric 1H protons and 13C carbons from reducing terminals of fucosyl units of oligosaccharide II (see Figure 1D) and of standard compounds

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<th>1H chemical shifts–β-Anomer</th>
<th>13C chemical shifts–α-Anomer</th>
<th>13C chemical shifts–β-Anomer</th>
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*Data from Daniel et al. (2001).*

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*The 600 MHz 1H/13C HMOC spectrum was recorded at 60°C. Chemical shifts are referenced to internal trimethylsilylpropionic acid at 0 ppm. Values in boldface indicate chemical shifts from nonsulfated fucosyl terminal units.*

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*Data from Mourão et al. (1996).*

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*Data from Daniel et al. (2001).*
electrophoretic bands on PAGE is observed exclusively with the sulfated fucans from the sea urchin *L. variegatus* and from the brown alga *Laminaria brasiliensis*. The other sulfated fucans were either extensively digested or yielded widely dispersed electrophoretic bands, denoting a heterogeneous mixture of oligosaccharides. Therefore the ordered degradation of the sulfated fucan from *L. variegatus* is in fact determined by its particular structure.

**Sulfated fucan stimulates thrombin inhibition by heparin cofactor II**

The linear sulfated fucan from *L. variegatus* has an anticoagulant activity of 10 IU/mg, as evaluated by the activated partial thromboplastin time (APTT) assay standardized with the International Heparin Standard (220 IU/mg). Inhibition of thrombin by antithrombin is only marginally stimulated by the sulfated fucan (open squares in Figure 10A). As we replaced antithrombin by heparin cofactor II, the polysaccharide enhances thrombin inhibition with a sigmoid curve similar to that observed for mammalian dermatan sulfate (closed squares and closed circles in Figure 10A, respectively). However, the concentration of heparin cofactor II required to ensure the formation of an effective inhibitory complex varies significantly between assays performed with invertebrate sulfated fucan (Figure 10B) and mammalian dermatan sulfate (Figure 10C). Total thrombin inhibition occurs with 0.2 mM heparin cofactor II in the presence of mammalian dermatan sulfate, whereas ~ a fivefold higher concentration of this cofactor is required for total thrombin inhibition in the presence of the invertebrate sulfated fucan. One possibility that could explain the requirement for the higher concentration of heparin cofactor II for thrombin inhibition in the presence of sulfated fucan is that the serpin is preferentially cleaved by the protease rather than forming a covalent complex. This aspect requires future investigation.

**Size dependence of the sulfated fucan required for thrombin inhibition mediated by heparin cofactor II**

None of the oligosaccharides I–VI obtained by mild acid hydrolysis and purified by gel chromatography on Bio-Gel P-10 (Figure 3) show any anticoagulant activity in the APTT and the thrombin inhibition assays. Furthermore, the mixture of oligosaccharides obtained after 1 h of mild hydrolysis of sulfated fucans with different structures. (A) Structures of sulfated fucans and sulfated galactan used in the experiment. Sulfated polysaccharides isolated from invertebrates consist of 1- or 4-linked α-L-fucose or α-L-galactose units with 2-sulfate/4-sulfate substitution. The minimum repeating unit of each polysaccharide is shown. The sulfated fucan from the brown alga *L. brasiliensis* has not a clear repetitive unit. (B) the sulfated fucan (100 µg of each) was dissolved in 100 µl of distilled water (–) or aqueous 0.01 M HCl solution (+) and heated at 60°C for 6 h. The pH of the solution was then adjusted to 7.0 with the addition of 100 µl ice-cold 0.01 M NaOH. The samples (10 µg of each) were then analyzed by PAGE as described in the legend of Figure 2.
hydrolysis, ranging from ~ 10 kDa to ~ 50 kDa (Figure 2), has only a modest anticoagulant activity (<1.0 IU/mg, based on APTT assay). These observations indicate that sulfated fucan requires fragments with longer size than mammalian dermatan sulfate to enhance thrombin inhibition by heparin cofactor II. The minimum sizes for complete thrombin inhibition in assays using mammalian dermatan sulfate (Tollefsen et al., 1986) and heparin (Bray et al., 1989; Sié et al., 1988) are 12–14 and 26 monosaccharide units, respectively (Table III).

We further investigated this aspect using heparin cofactor II affinity chromatography. A sample of intact sulfated fucan was applied to a heparin cofactor II affinity column

![Graph](https://example.com/graph.png)

**Fig. 10.** Dependence on the concentration of invertebrate sulfated fucan (A and B) or mammalian dermatan sulfate (A and C) for inactivation of thrombin by heparin cofactor II (A–C) or antithrombin (A). (A) Heparin cofactor II (1 µM) (closed squares, closed circles) or antithrombin (50 nM) (open squares) was incubated with thrombin in the presence of various concentrations of invertebrate sulfated fucan (closed and open squares) or mammalian dermatan sulfate (closed circles). After 60 s, the remaining thrombin activity was determined with a chromogenic substrate (Å405 nm/min). Open circles indicate incubation with various concentrations of sulfated fucan in the absence of coagulation inhibitor. (B and C) Incubation with increasing concentrations of sulfated fucan (B) or dermatan sulfate (C) in the presence of the indicated concentrations of heparin cofactor II.

![Graph](https://example.com/graph.png)

**Fig. 11.** Heparin cofactor II affinity chromatography of intact and low-molecular-weight sulfated fucans. (A and B) Intact sulfated fucan (A) and the low-molecular-weight fragments obtained after mild acid hydrolysis for 1 (closed circles in B) or 6 h (open circles) (70 µg of each) were applied to a heparin cofactor II column (HiTrap NHS-activated 1 ml, coupled with 7.5 mg heparin cofactor II). The column was washed with 15 ml 20 mM Tris-HCl (pH 7.4), 0.05 M NaCl at a flow rate of 1 ml/min. The fractions were checked for their metachromatic properties. (C) Fractions obtained on the affinity column of the fragments from 1 h acid hydrolysis of the sulfated fucan were pooled as indicated in B, desalted, and the retained (R) and nonretained (non-R) fractions, as well as the native sulfated fucan (intact) and the low-molecular-weight fragments obtained after 6 h mild hydrolysis (6 h) (10 µg of each) were run on 10% PAGE, as described in the legend of Figure 2. (D) Effect of the native fucan and of the retained and nonretained fractions obtained from the affinity column (B) on the inactivation of thrombin by heparin cofactor II. This experiment was as described in the legend of Figure 10, except that fixed concentrations of sulfated fucan (50 µg/ml) and of heparin cofactor II (1 µM) were used in the assays.

**Table III.** Comparison between the structural requirements for the heparin cofactor II (HCII)–mediated thrombin inhibition of sulfated fucan and mammalian glycosaminoglycans

<table>
<thead>
<tr>
<th></th>
<th>Sulfated fucan</th>
<th>Dermatan sulfate</th>
<th>Heparin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structural motif</td>
<td>[Fucp-(4SO₄)₆]⁺</td>
<td>[IdUA-(2SO₄)-GalNAc-(4SO₄)]⁺</td>
<td>No specific structure but high charge regions of the molecule³</td>
</tr>
<tr>
<td>Minimum molecular size</td>
<td>~ 178 monosaccharide units (~ 40 kDa)²</td>
<td>6 monosaccharide units²</td>
<td>4 monosaccharide units²</td>
</tr>
<tr>
<td>Interaction with HCII</td>
<td>~ 408 monosaccharide units (~ 100 kDa)²</td>
<td>12–14 monosaccharide units²</td>
<td>26 monosaccharide units²</td>
</tr>
</tbody>
</table>

²See Pereira et al. (2002).
³See Maimone and Tollefsen (1990).
⁴We calculated the number of monosaccharide units for sulfated fucan activity assuming 901 and 980 Da as the molecular weight of tetrasaccharide repeating units of the degraded and native sulfated fucan, respectively (see Figure 1B).
⁵See Tollefsen et al. (1986).
⁶See Hurst et al. (1983) and Maimone and Tollefsen (1988).
⁸See Sié et al. (1988) and Bray et al. (1989).
were heterogeneous, because only part of the oligosaccharides were retained on the column (closed circles in Figure 11B). On the other hand, the fragments obtained after 6 h of mild acid hydrolysis of the sulfated fucan do not bind at all to the heparin cofactor II affinity column (open circles in Figure 11B).

The molecular size of the oligosaccharides obtained from the heparin cofactor II column was determined by PAGE (Figure 11C). Clearly, binding of the sulfated fucan to heparin cofactor II depends on the molecular size. A comparison of the ability of the retained and nonretained fragments of sulfated fucan to stimulate thrombin inhibition by heparin cofactor II is shown in Figure 11D. We tested a single concentration of oligosaccharide due to the small amount of available material. Under the conditions of this assay, total inhibition of thrombin activity occurs in the presence of 50 μg/ml of intact sulfated fucan. But the retained oligosaccharides cause inhibition of only ~ 45% of the thrombin activity even at high concentration of heparin cofactor II (1.0 mM), whereas no inhibition of thrombin occurs in the presence of the nonretained oligosaccharides. In control experiments, neither the retained nor the nonretained oligosaccharides inhibit thrombin in the absence of heparin cofactor II. These results indicate that the oligosaccharides retained on the heparin cofactor II affinity column (average molecular weight of ~ 40 kDa) are less effective than the native fucan (average molecular weight of ~ 100 kDa) on thrombin inhibition.

The experiments based on the interaction of sulfated fucan oligosaccharides with heparin cofactor II-Sepharose were limited by the saturation of the heparin cofactor II affinity column with a very low amount of the carbohydrate (~ 70 μg). Therefore, we explored an alternative approach to obtain larger amounts of oligosaccharides with different activities toward heparin cofactor II. We initially prepared sulfated fucan oligosaccharides with different molecular size by gel chromatography (Figure 12A and B) and then tested the effect of the various fractions on thrombin inhibition assays (Figure 12C). In fact, we obtained fractions of oligosaccharides with molecular size similar to those from the heparin cofactor II affinity column (Figure 12B). Again, in contrast with the native sulfated fucan, which achieves total thrombin inhibition at a concentration of ~ 10 μg/ml (closed circles in Figure 12C), the sulfated fucan oligosaccharides with average size of ~ 40 kDa causes only 30% thrombin inhibition in the presence of heparin cofactor II even at a concentration of 100 μg/ml (closed squares in Figure 12C). No inhibition of thrombin occurs in the presence of oligosaccharides with molecular size lower than ~ 40 kDa (open circles in Figure 12C).

Overall, a linear sulfated fucan requires significantly longer chains than mammalian glycosaminoglycans to achieve complete thrombin inhibition with heparin cofactor II. Thus dermatan sulfate and heparin require 6 and 4 monosaccharide units to bind heparin cofactor II, respectively, whereas sulfated fucan needs ~ 178 monosaccharide units for this interaction (Table III). Furthermore, a slight decrease in the molecular size of the sulfated fucan dramatically reduces its effect on thrombin inhibition in the presence of heparin cofactor II. Sulfated fucan of ~ 40 kDa binds to heparin cofactor II but is unable to efficiently stimulate the thrombin–heparin cofactor II reaction. This last effect
requires a molecular size $\approx 100$ kDa ($\approx 408$ monosaccharide units).

**Possibility that the heparin cofactor II-dependent anticoagulant activity of the sulfated fucan is related to a rare and specific local structure in the polysaccharide chain**

An aspect we need to consider is related to the possibility that the anticoagulant activity of the sulfated fucan is related to a rare and specific structure in the polysaccharide chain, rather than to the bulk pattern of sulfation. This point has been well clarified in the case of mammalian glycosaminoglycans (Conrad, 1998). Thus heparin preparations with similar bulk structure may vary in their antithrombin-dependent anticoagulant activity due to different proportions of a specific pentasaccharide sequence, which is almost undetectable on analysis of the native molecule (Conrad, 1998; Lindahl et al., 1983; Thumberg et al., 1982). If this is the case for the binding of sulfated fucan to heparin cofactor II, we would expect at least one binding site in the $\approx 100$-kDa native polymer, because the polysaccharide is totally retained on the heparin cofactor II affinity column (Figure 11A). If the average molecular size decreases to $\approx 40$ kDa and $\approx 10$ kDa after 1 and 6 h mild acid hydrolysis by random cleavage, $\approx 50\%$ and $\approx 10\%$ of the sulfated fucan should be retained on the affinity column, respectively. Certainly this is not the case for the sulfated fucan (Figure 11B). Furthermore, we would not expect a molecular size dependence for the retention of sulfated fucan on the heparin cofactor II affinity column, as in Figure 11C, if the binding is related to a specific local structure rather than to the bulk sulfation pattern.

Another possibility is that a specific sequence of oligosaccharide is selectively removed from the sulfated fucan during mild acid hydrolysis. Unless the oligosaccharide is located at the reducing or nonreducing ends, mild acid hydrolysis must be selective for the two linkages at the extreme ends of the oligosaccharide. Furthermore, the released oligosaccharide must be inactive; otherwise, we would detect significant anticoagulant activity on the mixtures obtained after 1 or 6 h hydrolysis. Finally, PAGE should reveal minor components formed in the course of mild hydrolysis besides those related to the tetrasaccharide repeating unit of the sulfated fucan (Figures 2 and 3).

Finally, we may consider that the rare and specific sequence is constituted by a fucose unit with a particular sulfation pattern, which is extremely labile to acid hydrolysis. However, we already demonstrated that 4-sulfated fucans, not 2-sulfated ones, are heparin cofactor II–dependent anticoagulant polysaccharides (Pereira et al., 2002). It is unlikely that a rare and active sulfation sequence is always present in 4-sulfated fucans and absent in the 2-sulfated ones.

Therefore our observations suggest that the heparin cofactor II–dependent anticoagulant activity of the linear sulfated fucan is in fact related to the bulk structure, rather than to a rare and specific oligosaccharide sequence. Of course, to provide unequivocal evidence for this, we would have to make a synthetic polymer and show that it had the same properties as the natural polysaccharide. This is not a feasible experiment due to the highly complex nature of the sulfated fucans.

**Speculation concerning the mechanism of anticoagulant action of the sulfated fucan**

It is well established that dermatan sulfate and heparin accelerate thrombin inactivation by heparin cofactor II through an allosteric mechanism in which the acidic N-terminal domain of the coagulation inhibitor interacts with exosite I of thrombin (Hortin et al., 1989; Mitchell and Church, 2002; Ragg et al., 1990; Rogers et al., 1992; Van Deerlin and Tønnesen, 1991). In a more recent study (Verhamme et al., 2004), it was proposed that the inactivation mechanism combines this allosteric effect with a template mechanism in which the glycosaminoglycan forms a temporary noncovalent ternary complex with the protease and the inhibitor. The template effect involves the preferential formation of a thrombin–glycosaminoglycan complex, which then interacts with heparin cofactor II rather than a cofactor–glycosaminoglycan interaction with thrombin (Verhamme et al., 2004). A template mechanism was also proposed for the antithrombin-dependent anticoagulant activity of low-affinity heparin, that is, heparin fragments devoid of the specific pentasaccharide sequence with high affinity for antithrombin (Streusand et al., 1995). It is still unclear what is the contribution of the allosteric and of the template effect to the overall mechanism of glycosaminoglycan-catalyzed thrombin inactivation by heparin cofactor II.

We hypothesize that linear sulfated fucan requires significantly longer chains than mammalian glycosaminoglycans to efficiently inactivate thrombin through heparin cofactor II because the template mechanism predominates over the allosteric effect in the case of the invertebrate polysaccharide. We already proposed a similar effect for sulfated galactan inactivation of thrombin mediated by antithrombin. In this case, the activation conformational change in antithrombin is less important for the anticoagulant activity of sulfated galactan than for heparin (Melo et al., 2004).

We also speculate that different mechanisms may be involved in the action of sulfated fucan on heparin cofactor II–mediated thrombin inactivation. The polysaccharide may hold together more than one molecule of the protease and/or of the plasma cofactor in a noncovalent complex. In this case, the efficacy can be attributed to the longer chains that occur in the sulfated fucan. In fact, the observation that higher concentrations of heparin cofactor II are required for effective thrombin inactivation by sulfated fucan than by mammalian dermatan sulfate (Figure 10) suggests different stoichiometries for the reactions catalyzed by these two polysaccharides.

Our results concerning the heparin cofactor II–mediated anticoagulant activity of sulfated fucan may help design new drugs with specific actions on coagulation and thrombosis. Furthermore, this sulfated polysaccharide may be used as a research reagent to clarify different mechanisms for thrombin inactivation, which remain obscure in the case of mammalian glycosaminoglycans.
Materials and methods

Sulfated fucan

Eggs from mature females of the sea urchins were spawned into filtered sea water after intracelomic injection of 0.5 M KCl (~ 5 ml per specimen). The egg jelly was separated by pH shock, as described previously (SeGall and Lennarz, 1979). Sulfated polysaccharides were extracted from the egg jelly by protease digestion (Albano and Mourão, 1986). Sulfated fucans were purified by anion exchange chromatography, and their purity was checked by agarose gel electrophoresis and NMR spectroscopy (Alves et al., 1997, 1998; Vilela-Silva et al., 1999, 2002). A sulfated fucan from the brown alga L. brasiliensis was prepared as described (Pereira et al., 1999).

Depolymerization of sulfated fucan by mild acid hydrolysis

Sulfated fucan (10 mg) was dissolved in 1.25 ml 0.01 M HCl and maintained at 60°C for different periods of time. The pH of the solution was then adjusted to 7.0 with the addition of ~ 1.25 ml ice-cold 0.01 M NaOH. The products formed in the course of the mild acid hydrolysis were separated by gel chromatography and analyzed by PAGE, as described shortly.

Gel filtration chromatography

The products formed in the course of the mild acid hydrolysis of the sulfated fucan (2 mg) were applied to a Superose-6 (HR 10/30) column (Amersham Biosciences, Little Chalfont, U.K.), linked to a high-pressure liquid chromatography (HPLC) system from Shimadzu (Tokyo), equilibrated with 0.2 M NH₄HCO₃ (pH 8.0). The column was eluted with the same solution at a flow rate of ~ 3 ml/h and fractions of 0.5 ml were collected and assayed by metachromasia using 1,9-dimethylmethylene blue (Farndale et al., 1986) and by the phenol–sulfuric acid reaction (Dubois et al., 1956). The fractions containing the low-molecular-weight sulfated fucans were pooled and freeze-dried.

The oligosaccharides formed after 6 or 9 h of mild acid hydrolysis of the sulfated fucan (5 mg) were fractionated on a Bio-Gel P-10 (Bio-Rad Laboratories, Hercules, CA) column (200 × 0.9 cm), equilibrated with aqueous 10% ethanol, containing 1.0 M NaCl. The column was eluted with the same solution at a flow rate of ~ 3 ml/h and fractions of 1.0 ml were collected and assayed by metachromasia using 1,9-dimethylmethylenblue (Farndale et al., 1986) and by the phenol–sulfuric acid reaction (Dubois et al., 1956). The elution volume of blue dextran and cresol red indicates the position of the column, respectively. The fractions containing the various oligosaccharides (as indicated by the positive metachromatic assay) were pooled, freeze-dried, and dissolved in 2.0 ml distilled water. These solutions were desalted on Superdex peptide column (Amersham Biosciences, Piscataway, NJ), linked to a HPLC system. Fractions of 0.5 ml were collected and their conductivities determined. The oligosaccharides were detected by metachromasia (Farndale et al., 1986). Fractions containing the desalted oligosaccharides were pooled and freeze-dried.

PAGE

The native and low-molecular-weight derivatives of the sulfated fucan (10 µg of each) were applied to a 10% 1-mm-thick polyacrylamide gel slab in 0.02 M sodium barbital (pH 8.6) and run for 30 min at 100 V. After electrophoresis the sulfated fucans were stained with 0.1% toluidine blue in 1% acetic acid and washed for about 4 h in 1% acetic acid. The molecular masses of the low-molecular-weight fragments of the sulfated fucan were estimated by comparison with the electrophoretic mobility of standard compounds (Pavao et al., 1998; Pereira et al., 1999; Sautos et al., 1992). The standards used were high-molecular-weight dextran sulfate (≥ 100 kDa), chondroitin 4-sulfate from bovine trachea (~ 40 kDa), dermanan sulfate from pig skin (~ 20 kDa), and low-molecular-weight dextran sulfate (~ 10 kDa).

NMR experiments

¹H and ¹³C spectra of the native sulfated fucan and its low-molecular-weight derivatives were recorded using a Bruker DRX 600 apparatus with a triple resonance probe. About 3 mg of each sample was dissolved in 0.5 ml 99.9% D₂O (Cambridge Isotope Laboratory, Cambridge, MA). All spectra were recorded at 60°C with HOD suppression by presaturation. In some experiments sulfated fucan (3 mg) was dissolved in 0.5 ml 0.01 M HCl, prepared in 99.9% D₂O. The solution was put into an NMR tube and maintained at 60°C. 1D ¹H-NMR spectra were recorded from 10 min to 6 h. Correlation spectroscopy, TOCSY, and ¹H/¹³C HMOC spectra were recorded using states-time proportion phase incrementation for quadrature detection in the indirect dimension. TOCSY spectra were run with 4046 × 400 points with a spin-lock field of ~ 10 kHz and a mixed time of 80 ms. HMOC spectra were run with 1024 × 256 points and globally optimized alternating phase rectangular pulses for decoupling. Chemical shifts are relative to external trimethylsilyl-propionic acid at 0 ppm for ¹H and to methanol for ¹³C.

Anticoagulant activity measured by APTT

APTT clotting assays were carried out by the method of Anderson et al. (1976) using normal human plasma. In these assays, plasma samples (90 µl) were mixed with different amounts of sulfated polysaccharide in 0.9% NaCl (10 µl), warmed for 60 s at 37°C and then 100 µl prewarmed APTT reagent (Biomerieux, Rio de Janeiro, Brazil) was added and allowed to incubate for 2 min at 37°C. Prewarmed 0.25 M CaCl₂ (100 µl) was then added, and the APTT recorded as the time for clot formation in a coagulometer (Amelung KC4A, Lemgo, Germany). The activity was expressed as IU/mg using a parallel standard curve based on the fourth International Heparin Standard (220 U/mg) from the National Institute for Biological Standards and Control (Potters Bar, U.K.).

Inhibition of thrombin by heparin cofactor II or antithrombin in the presence of sulfated fucan

Incubations were performed in disposable UV semimicrocuvettes. The final concentrations of the reactants included 0.2–1.0 µM plasma-derived heparin cofactor II or 50 nM antithrombin, 15 nM bovine thrombin, and 0–1000 µg/ml sulfated fucan in 50 or 100 ml TS/PEG buffer.
(0.02 M Tris–HCl, 0.15 M NaCl, and 1 µg/ml polyethylene glycol, pH 7.4). Thrombin was added last to initiate the reaction. After a 60-s incubation at room temperature, 250 or 500 µl TS/PEG buffer containing 100 µM chromogenic substrate S-2238 (Chromogenix AB, Monstad, Sweden) was added, and the absorbance at 405 nm was recorded for 120 s. The change of absorbance was proportional to the thrombin activity remaining in the incubation. No inhibition occurred in control experiments in which thrombin was incubated with heparin cofactor II or antithrombin in the absence of sulfated fucan. In addition, no inhibition was observed when thrombin was incubated with sulfated fucan alone over the range of concentration tested.

Heparin cofactor II affinity chromatography

HiTrap NHS activated HP (5 ml) from Amersham Biosciences was mixed with 7.3 mg heparin cofactor II dissolved in 5 ml 200 mM NaHCO3, containing 50 mM NaCl (pH 8.3), and coupling was carried out as described by the manufacturer. The immobilized heparin cofactor II maintained its expected binding specificities, because a dermatan sulfate sample with a high heparin cofactor II activity, obtained from the ascidida Styela plicata (Pavão et al., 1998), bound to the heparin cofactor II affinity column, whereas both a dermatan sulfate with no heparin cofactor II activity, obtained from the ascidian Ascidia nigra (Pavão et al., 1998), and vertebrate chondroitin 4-sulfate did not bind to the heparin cofactor II column. Intact sulfated fucan and its low-molecular-weight fragments (70 µg of each) were applied to the immobilized heparin cofactor II affinity column. Intact sulfated fucan and its low-molecular-weight fragments (70 µg of each) were applied to the immobilized heparin cofactor II affinity column. Fractions of 1.0 ml were collected and the absorbance at 405 nm was recorded for 120 s. The change of absorbance was proportional to the thrombin activity remaining in the incubation. No inhibition occurred in control experiments in which thrombin was incubated with heparin cofactor II or antithrombin in the absence of sulfated fucan. In addition, no inhibition was observed when thrombin was incubated with sulfated fucan alone over the range of concentration tested.

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Acknowledgments

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Abbreviations

APTT, activated partial thromboplastin time; HMQC, heteronuclear multiple quantum correlation spectroscopy; HPLC, high-pressure liquid chromatography; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; TOCSY, total correlation spectroscopy.

References


