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Note

A sulfated fucan from the brown alga *Laminaria cichorioides* has mainly heparin cofactor II-dependent anticoagulant activity

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Abstract—The major acidic polysaccharide from the brown alga *Laminaria cichorioides* is a complex and heterogeneous sulfated fucan. Its preponderant structure is a 2,3-disulfated, 4-linked α-fucose unit. The purified polysaccharide has a potent anticoagulant activity, as estimated by APTT assay (~40 IU/mg), which is mainly mediated by thrombin inhibition by heparin cofactor II. It also accelerates thrombin and factor Xa inhibition by antithrombin but at a lower potency. Sulfated fucan from *L. cichorioides* is a promising anticoagulant polysaccharide and a possible alternative for an antithrombotic compound due to its preferential heparin cofactor II-dependent activity.

Keywords: Anticoagulant; Heparin cofactor II; Sulfated polysaccharide; Brown alga; *Laminaria cichorioides*

Sulfated fucans are water-soluble polysaccharides that occur as the major constituent of brown algae. They are obtained in large quantities and are among the most abundant sulfated polysaccharides found in nature.[1] More recently, sulfated fucans were also found in several tissues of marine invertebrates. These polysaccharides have a wide variety of biological properties but the anticoagulant activity is by far the most well studied.[1] Studies with the invertebrate polysaccharides revealed some structure–anticoagulant activity relationship concerning the sulfated fucans.[1,2]

The structures of algal sulfated fucans are complex and heterogeneous. They also vary among species.[1,3] Studies using a sulfated fucan from the brown alga *Fucus vesiculosus* suggested that the antithrombin activity is mediated mainly by heparin cofactor II, with a minor contribution of antithrombin.[4] In contrast, a sulfated fucan from the alga *Ascophyllum nodosum* has an intense antithrombin-mediated anticoagulant activity.[5] Possibly, sulfated fucans from different species may vary in the mechanism of their anticoagulant activity because of their different chemical structure.[6,7] However, it is difficult to compare these results since the anticoagulant assays were performed in different laboratories using distinct protocols, and sulfated fucans were purified using a variety of methodologies.

In the present study, we investigated the structure and anticoagulant activity of a sulfated polysaccharide extracted from the brown alga *Laminaria cichorioides*. The crude acidic polysaccharide showed increasing clotting times in the APTT and TT assays that were proportional to its concentration. No change in the PT clotting time was observed (Table 1). We purified the polysaccharide using an anion-exchange chromatography on Mono-Q (Fig. 1a). The major subfraction (S2) showed strong metachromatic property, high content of sugar and low content of hexuronic acid. Subfraction S1 showed a broad peak, eluted at low salt concentration while subfraction S3 was eluted at high NaCl concentration.

Abbreviations: APTT, activated partial thromboplastin time; PT, prothrombin time; TT, thrombin time

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Table 1. Concentration-dependent anticoagulant activity of crude acidic polysaccharides from *L. cichorioides*

Concentration[a] (ug/mL)	Clotting time[b] (s)		
	APTT	TT	PT
0	40 ± 0.7	10 ± 0.9	14 ± 1.0
10	55 ± 1.1	115 ± 5.8	14 ± 1.0
30	112 ± 2.8	>600	17 ± 2.1
50	231 ± 2.1	>600	23 ± 3.5

[a] The lyophilized crude polysaccharide was dissolved in human platelet-poor plasma (hPPP) to each concentration shown.
[b] Each clotting time represents the average of triplicate experiments using hPPP. Data are shown as means ± SD in s.

Agarose gel electrophoresis showed differences in mobility among the purified subfractions (Fig. 1b). Each subfraction showed very clear bands. S1 and S2 showed a single, homogeneous and almost coincident metachromatic band, while S3 showed an addition second band with higher mobility. The average molecular sizes of the three subfractions were estimated by polyacrylamide gel electrophoresis (Fig. 1c). The unfractionated polysaccharides showed a wide dispersion in their molecular sizes. Subfractions S1 and S2 showed a similar mobility as standard dextran sulfate (~8 kDa) and chondroitin 6-sulfate (~60 kDa), respectively, whereas subfraction S3 remained close to the origin of the gel, denoting a high molecular mass. Gel filtration chromatography on Sephacryl S-400 confirmed the average molecular mass of subfraction S2 as ~60 kDa (not shown).

Chemical analysis (Table 2) revealed that fucose is the major sugar component of subfraction S2 while galactose and glucose prevail in subfractions S1 and S3, respectively. Subfraction S2 has also high sulfate content. Approximately two sulfate esters are present per sugar unit.

Subfraction S2 is the preponderant anticoagulant polysaccharide in the water extract of this alga, with an anticoagulant activity of 40 IU/mg when compared with a heparin standard (193 IU/mg), as determined by the APTT assay (not shown). The crude water extract obtained from this alga has an anticoagulant activity of 25 IU/mg. The anticoagulant activity of the three subfractions has no correlation with their molecular size.

We attempted to determine the major structural component of the sulfated fucan from *L. cichorioides* using NMR and methylation analysis. The assignment of the major peaks was achieved by analysis of ^{1}H one-dimensional NMR, ^{1}H COSY, ^{1}H TOCSY, and $^{1}H/^{13}C$ HMQC spectra. The spectra of subfraction S2 showed broader and poorly resolved signals indicating a clearly heterogeneous chemical structure (data not shown). However, peaks clearly attributable to α-anomeric protons were identified in the ^{1}H NMR and $^{1}H/^{13}C$ HMQC spectra. The signals in the vicinity of 5.5 ppm of the ^{1}H NMR spectra showed the preponderance of α-anomeric protons. Comparison between chemical shifts observed

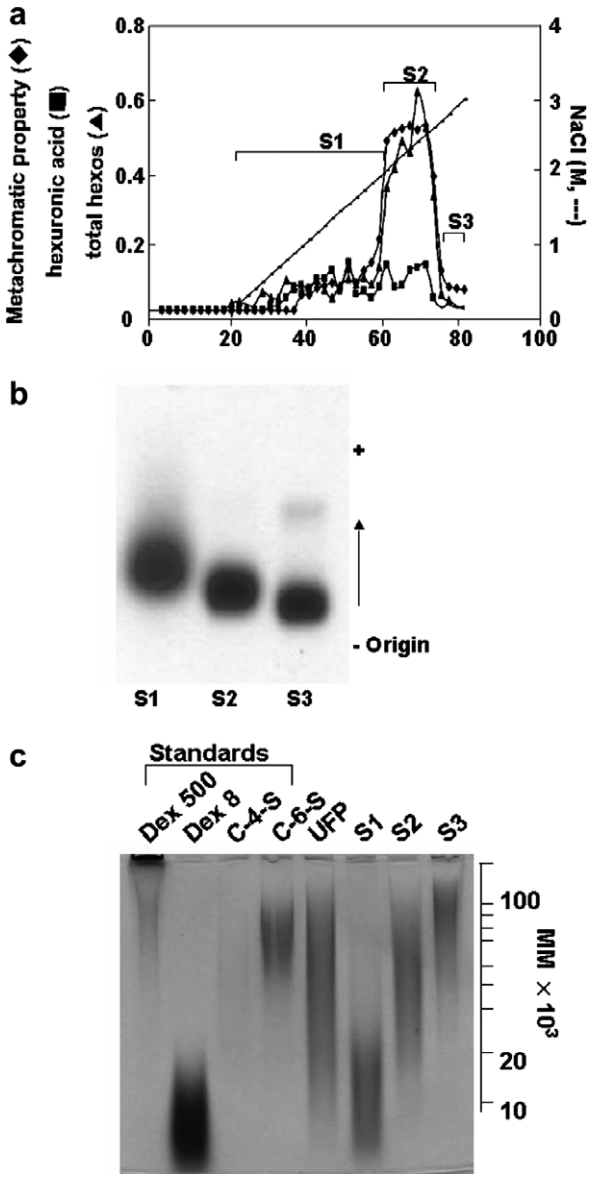

Figure 1. Purification of the sulfated polysaccharides from the brown alga *L. cichorioides* by anion-exchange chromatography (a), analysis of the purified fractions by agarose gel electrophoresis (b) and estimation of their average molecular masses by polyacrylamide gel electrophoresis (c). (a) Acidic polysaccharides (~60 mg) obtained from the brown alga *L. cichorioides* were applied to Mono Q-FPLC column and purified as described under 'Experimental'. Fractions were checked by the phenol–H_2SO_4 (▲) and carbazole (■) reactions, for metachromasia (◆) and NaCl concentration (---). The fractions were pooled into three subfractions, denominated as S1, S2, and S3 (see horizontal bars in the panel). The purified subfractions (~15 μg) were analyzed by agarose gel electrophoresis (b) and by polyacrylamide electrophoresis (c). For this last experiment, the molecular markers used were low-molecular-weight dextran sulfate (Dex 8) (8 kDa), chondroitin 4-sulfate from whale cartilage (C-4-S) (40 kDa), chondroitin 6-sulfate from shark cartilage (C-6-S) (60 kDa) and high-molecular-weight dextran sulfate (Dex 500) (~500 kDa). UFP, unfractionated polysaccharide.

for the preponderant residue of subfraction S2 with the literature data indicates that this polysaccharide

Table 2. Chemical composition of the various subfractions of polysaccharide from *L. cichorioides*

Subfraction[a]	Chemical composition (as molar ratios)					Sulfate/total sugar (molar ratios)
	Fucose	Galactose	Mannose	Glucose	Xylose	
S1	0.12	0.54	0.28	0.06	0	0.09
S2	0.60	0.36	0	0.03	0.01	2.19
S3	0.15	0.22	0	0.63	0	1.38

[a] These subfractions were obtained after anion-exchange chromatography on Mono Q-FPLC (see Fig. 1a).

Table 3. ^{1}H Chemical shifts of subfraction S2 from the brown alga *L. cichorioides*

Compound	Proton		
	H-1	H-2	H-3
Our sample	5.55	**4.70**	**5.00**
Literature data			
[4-Fuc-2,3(diSO_4-1-)][a]	5.45	**4.68**	**4.85**
[4-Fuc-2(SO_4)-1][a]	5.29	**4.61**	4.20
[4-Fuc-1][b]	5.05	3.89	4.07

The ^{1}H spectrum was recorded at 400 MHz, 60 °C in 99.8% D_2O. Chemical shifts are referenced to internal trimethylsilylpropionic acid. Values of chemical shifts indicating sulfation sites are boldface.

[a] See Ref. 3.

[b] See Ref. 8.

has mainly 2,3-disulfate, 4-linked α-fucose units (Table 3).

No signals for galactose were identified in the NMR spectra although this sugar is a substantial component of the algal polysaccharide. However, NMR is not appropriate to identify a small component even at substantial amounts. For example, on a sulfated galactan, which contains 24% of fucose units, no signals of this sugar were assigned on the NMR spectra.[9] Furthermore, H-4 and H-5 signals of fucose were not identified on the NMR spectra. In a previous study using sulfated fucan from different species of brown alga, we had a similar difficulty in identifying these signals.[3]

When subfraction S2 was submitted to three rounds of methylation a variety of methylated derivatives was obtained, mainly mono- and dimethylated fucosyl derivatives. In fact, methylation of sulfated polysaccharides does not always yield reliable proportions of methylated alditols.[3] But, clearly methylation analysis of the native and desulfated subfraction S2 showed that the content of 2,3-di-*O*-methylfucose was distinctively increased when methylated fucose derivatives obtained from the desulfated polysaccharide were compared to those from the native polysaccharide (not shown), which suggests the presence of 2,3-disulfated, 4-linked fucose units. We found a wide variety of methyl derivatives from galactose, as already observed for similar polysaccharides from other species of brown algae.[3]

In order to elucidate the anticoagulation mechanism of the sulfated polysaccharide from *L. cichorioides*, we investigated the influence of the purified subfraction S2 on thrombin and factor Xa inactivation by antithrombin and heparin cofactor II. The sulfated polysaccharide inhibited the thrombin amidolytic activity in the presence of antithrombin (Fig. 2a) but had no effect in the absence of antithrombin, as expected. The IC_{50} for sulfated polysaccharide-mediate inhibition of thrombin was approximately 10-fold higher than for heparin-induced inhibition. The sulfated polysaccharide also inactivated thrombin in the presence of heparin cofactor II (Fig. 2b), in which the inhibitory effect occurred at lower concentrations than the one required for antithrombin. Finally, the effect of the sulfated polysaccharide was essentially the same when factor Xa, instead of thrombin, was the target proteinase for antithrombin-mediated inactivation (Fig. 2c) even though slight differences were observed in the concentration of sulfated polysaccharide for thrombin and factor Xa inhibition in the presence of antithrombin.

In conclusion, the anticoagulant activity of a sulfated polysaccharide extracted from the brown alga *L. cichorioides* was investigated in this study. The preponderant structure of the polysaccharide is repeating units of [4-α-L-Fuc-2,3(diSO_4)-1]. The sulfated fucan enhances thrombin inhibition by heparin cofactor II in almost the same range of concentration as heparin. The polysaccharide also accelerates thrombin and factor Xa inhibition by antithrombin but it is less effective than heparin.

1. Experimental

1.1. Isolation and fractionation of acidic polysaccharides

The brown alga *L. cichorioides* was collected in the East Sea (Korea). The dried tissue was cut into small pieces and immersed in MeOH, and kept for 24 h at room temperature. The mixture was then filtered to remove MeOH-soluble material and the residual material was extracted with 0.4% HCl at room temperature for 4 h. The extraction mixture was filtered and the supernatant was saved. The residue was submitted to a second extraction procedure with 0.4% HCl at 50 °C for 5 h, filtered, and combined. The precipitate was saved and the supernatant containing water-soluble crude polysaccharide was precipitated with 3 volumes of MeOH/1-butanol (3:1, v/v) for 24 h and centrifuged at 6500 rpm for 20 min. After drying, the precipitates were dissolved in

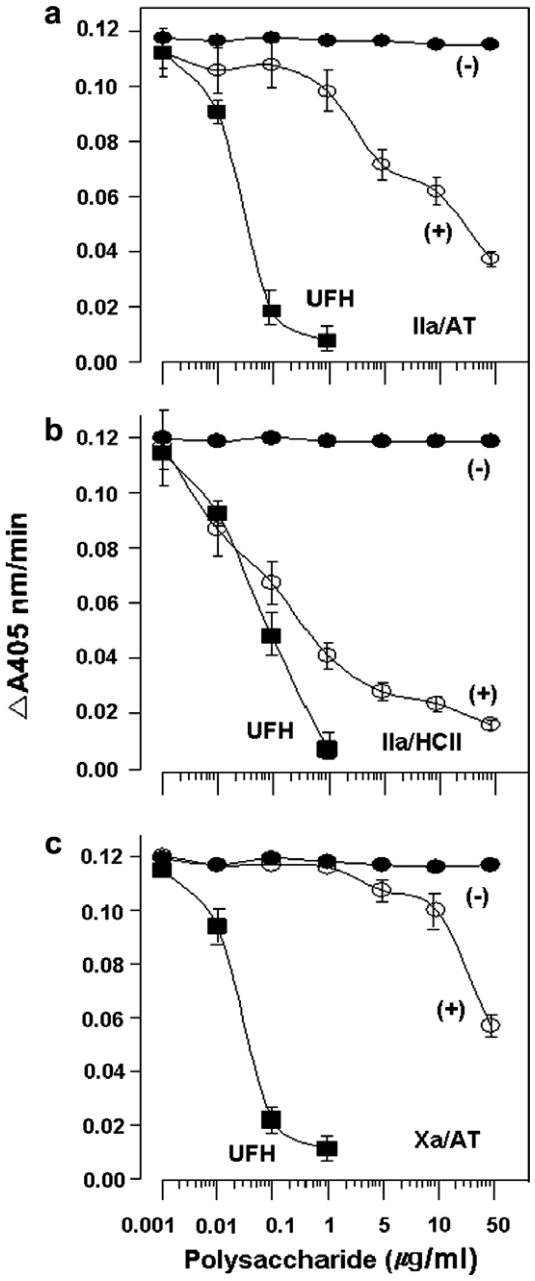

Figure 2. Dependence on the concentration of subfraction S2 for inactivation of thrombin (a and b) or factor Xa (c) by heparin cofactor II (b) or antithrombin (a and c). Heparin cofactor II (68 nM) or antithrombin (50 nM) were incubated with thrombin (15 nM) or factor Xa (15 nM) in the presence of various concentrations of polysaccharide purified from the brown alga *L. cichorioides* (○) and heparin (■). After 60 s, the remaining thrombin or factor Xa activity was determined with a chromogenic substrate (ΔA_{405}/min). The data indicated by ● (–) refers to incubation of the brown alga polysaccharide with thrombin or factor Xa in the absence of the cofactors (heparin cofactor II or antithrombin). The panels show representative results obtained from three different experiments. HCII, heparin cofactor II; AT: antithrombin.

H_2O and the acidic polysaccharides were precipitated with 0.5 M cetyltrimethylammonium bromide. The resulting precipitate was collected by centrifugation and re-dissolved in 3 M $CaCl_2$ at 37 °C for 48 h. Four volumes of absolute EtOH were then added to the solution, and the resulting precipitate was dissolved in H_2O, followed by dialysis (M_w 2000) against running water for 2 days. The non-dialyzable portion was centrifuged to remove water-insoluble material and the supernatant was lyophilized giving rise to the crude polysaccharides.

1.2. Anion-exchange chromatography of the polysaccharides from *L. cichorioides* on Mono Q-FPLC column

The crude acidic polysaccharides from *L. cichorioides* (60 mg) were applied to a Mono Q-FPLC column (HR 5/5, 0.5 × 20 cm, Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with 20 mM Tris/HCl (pH 8.0) and eluted with a linear gradient of 0–3.0 M NaCl in the same buffer. The flow rate of the column was 0.5 mL/min and fractions of 0.5 mL were collected. Fractions were assayed by the phenol–H_2SO_4 for hexose[10] by metachromasia[11] and by the carbazole reaction.[12]

1.3. Chemical analysis and molecular weight determination

Chemical analysis of the sulfated polysaccharides was performed using the same methodologies described in our previous publications.[3,8] Desulfation and methylation analysis of the sulfated fucan was performed as described.[3,8] The molecular weights of the sulfated polysaccharides were estimated by polyacrylamide gel electrophoresis.[3] Sulfated polysaccharides were also analyzed by agarose gel electrophoresis.[8]

1.4. NMR spectroscopy

NMR spectra (^1H and ^{13}C) were recorded at 400 MHz using a Bruker Avance DRX spectrometer. The sulfated polysaccharides (5 mg) were dissolved in 0.5 mL of 99.96% D_2O (deuterium oxide, NMR grade, from ISOTEC INC, USA), and the pH was adjusted to ~7.0. All spectra were recorded at 60 °C with suppression of the HOD signal by presaturation. Two-dimensional double-quantum filtered COSY, TOCSY, and $^1H/^{13}C$ heteronuclear correlation (HMQC) spectra were recorded using pulse sequences supplied by Brucker. All chemical shifts were relative to internal or external trimethylsilylpropionic acid.

1.5. Anticoagulant activity

APTT, PT and TT clotting assays were performed using normal human plasma from consenting individuals, according to the manufacturer's specifications.[13] 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 plasma proteases (thrombin or factor Xa) by antithrombin and heparin cofactor II in the presence of sulfated polysaccharides was determined as described.[14]

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