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Anticoagulant and antithrombin activities of oversulfated fucans

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ABSTRACT

Three species of oversulfated fucans having different sulfate contents (the ratio of sulfate/total sugar residues, 1.38–1.98) were prepared by chemical sulfation of a fucan sulfate (sulfate/sugar ratio, 1.28) isolated from the brown seaweed *Ecklonia kurome*. The anticoagulant activities of the oversulfated fucans were compared with that of a parent fucan with respect to activated partial thromboplastin time (APTT) and thrombin time (TT) in plasma. The respective activities (for APTT and TT) of the oversulfated fucans increased to 110–119% and 108–140% of the original values with increase in their sulfate content. The anticoagulant activity with respect to APTT (173 units/mg) of an oversulfated fucan (sulfate/sugar ratio, 1.98) was higher than that (167 units/mg) of heparin used as a standard. The heparin cofactor II-mediated antithrombin activity of the oversulfated fucans also increased significantly with increase in sulfate content. The maximum activity was higher than those of the parent fucan and heparin. However, the increment of the anticoagulant and the antithrombin effects gradually decreased with increase in the sulfate content of the fucans. These results indicate that the effects of the fucan sulfate are dependent on its sulfate content until a plateau is reached.

INTRODUCTION

Since the report[1] that the anticoagulant activity of heparin is dependent on its sulfate content, the activity of various fucan sulfates, isolated from brown seaweeds, has been studied[2–4]. However, a possible relationship between the structures and the anticoagulant activities of the fucan sulfates remains to be fully established.

Previously, we isolated[5] a fucan sulfate (C-II) of potent anticoagulant activity from the brown seaweed *Ecklonia kurome*, and reported[6] that C-II consisted of a brackbone of (1 → 3)-linked α-L-Fuc with sulfate groups mainly attached to position 4. Recently, we prepared[7] various fucans having different proportions of sulfate by solvolytic desulfation from C-II, and found that both the anticoagulant

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activity[7] and the heparin cofactor II (HC II)-mediated antithrombin activity[8,9] of C-II decreased significantly with decrease in the sulfate content, suggesting that the activities may be dependent on the sulfate content of the polysaccharide.

In the present study, to investigate how the anticoagulant and antithrombin activities of the fucan sulfate are enhanced with increase in sulfate content, we have prepared oversulfated fucans having different proportions of sulfate by chemical modification from C-II and have then examined the chemical and physical properties and the activities of the fucan derivatives obtained.

EXPERIMENTAL

Materials. — A fucan sulfate (C-II) was obtained from the brown seaweed *Ecklonia kurome* as described previously[5]. Heparin (167 units/mg) from porcine intestinal mucosa was purchased from Wako Pure Chemical Industries. Pyridine–sulfur trioxide was obtained from Tokyo Kasei Industries. Normal human plasma was obtained from Kohgin Bio, human thrombin (500 NIH units) from Green Cross, human heparin cofactor II (1 PEU) from Diagnostical Stago, and chromogenic substrate S-2238 (H-D-Phe-Pip-Arg-NA) from KabiVitrum AB.

General. — Neutral sugars, 6-deoxy-sugars, and uronic acids were determined as described previously[5]. Sulfates in polysaccharides were determined by the turbidimetric method of Dodgson and Price[10] after the polysaccharides had been hydrolyzed with M HCl for 5 h at 100°. Molecular weights of fucan sulfates were estimated by gel filtration on a column (1.2 × 99 cm) of Sepharose CL-6B with 0.2 M NaCl as the eluent. For the estimation, blue dextran (type 2000) and pullulans (P-400, 200, 100, 50, 20, 10, and 5, Shodex standard P-82) were used as the molecular-weight standards. Electrophoresis was performed on a membrane (6 × 11 cm) of cellulose acetate using 0.1 M HCl (conditions; 16.5 V, 150 min) for examination of the difference in the sulfate content alone of the polysaccharides tested.

Preliminary sulfation studies. — *(a) Direct sulfation of C-II.* A solution of C-II (8.2 mg) in *N*,*N*-dimethylformamide (DMF, 1 mL) was stirred for 1 h at room temperature and then pyridine–sulfur trioxide (10.7 mg, 3 mol/mol equiv of available hydroxyl groups of Fuc in C-II) was added. After 1 h of additional stirring, the reaction was stopped by adding water (1 mL) and the pH of the solution was adjusted to 9.0 with 0.1 M NaOH. The mixture was then dialyzed against distilled water and lyophilized to give the sodium salt of the sulfated product (10 mg). The sulfation was also performed by the same procedure under various modified conditions, as shown in Table I.

(b) Sulfation of the tributylammonium salt of C-II. Sulfation of the tributylammonium salt of C-II was performed by the method of Uchiyama et al.[11]. A 1% solution of the sodium salt of C-II (200 mg) in water was passed through a column (3 × 6.6 cm) of AG 50w X8 (H^+, 200–400 mesh) at 4°. The effluent was brought to pH 5.4 with 10% Bu_3N in EtOH and then the solution was lyophilized to give the tributylammonium salt of C-II (168 mg). A solution of pyridine–sulfur trioxide

TABLE I

Conditions for sulfation of C-II

Conditions	C-II (mg)	Solvent (1 mL)	Pyridine–SO_3 [a] (mg) (mol equiv [b])	Temperature (°)	Time (h)
1	8.2	DMF	10.7 (3)	r.t. [c]	1
2	8.8	DMF	38.5 (10)	r.t.	1
3	8.0	DMF	37.6 (10)	r.t.	5
4	8.0	DMF	37.6 (10)	r.t.	20
5	9.5	DMF	41.3 (10)	50	5
6	8.5	Me_2SO	11.2 (3)	r.t.	1
7	8.8	Me_2SO	38.5 (10)	r.t.	1
8	15.5	pyridine	20.4 (3)	r.t.	1
9	15.8	pyridine	20.7 (3)	r.t.	20
10	16.7	pyridine	73.3 (10)	r.t.	1
11	15.5	pyridine	66.7 (10)	r.t.	20
12	17.1	pyridine	74.7 (10)	50	5

[a] Pyridine–sulfur trioxide. [b] Mol/mol equiv of available hydroxyl groups of Fuc in C-II. [c] Room temperature.

(57.5 mg, 10 mol/mol equiv of available hydroxyl groups) in DMF (1.5 mL) was added to a solution of the tributylammonium salt (~ 15 mg) in DMF (1 mL) at −15°, and then the mixture was stirred for 1 h at room temperature or 50°. After the mixture had been cooled to −15°, the reaction was terminated by addition of cold water (2.5 mL) and the pH of the soultion was adjusted to 9.5 with 0.1 M NaOH. Ethanol (3 vol) saturated with anhyd NaOAc was added to the solution, and the precipitate was collected by centrifugation (2000*g*, 15 min, 4°) and then dissolved in water. The solution was evaporated to low volume and the concentrated solution was chromatographed on a column (2.64 × 90 cm) of Sephadex G-10. The high-molecular-weight fraction in the void volume was collected and lyophilized to give the sulfated products (~ 7 mg).

The degree of sulfation of the sulfated products was monitored by electrophoresis.

Preparation of oversulfated fucan sulfates from C-II. C-II (70.5 or 86.6 mg) was sulfated directly with pyridine–sulfur trioxide (300 or 990 mg, 10 or 26 mol/mol equiv of available hydroxyl groups) in DMF (8 or 9 mL) for 20 h at room temperature according to the procedure described in (*a*) to give two sulfated products (S-1 and S-2) having different sulfate contents. Sulfation of the tributylammonium salt of C-II (205.3 mg) was also performed with the foregoing reagent (22 mol/mol equiv of available hydroxyl groups) in DMF (20 mL) at room temperature for 20 h according to the procedure described in (*b*) to give a sulfated product (S-3). The products obtained were applied to a column (2.5 × 92 cm) of Sephadex G-10, and the respective major high-molecular-weight fractions eluted in the void volume were collected. The high-molecular-weight fractions were further fractionated by anion-exchange chromatography on a column (1.5 × 25 cm) of

ECTEOLA-cellulose (Cl^-). The column was developed with a linear gradient of $0 \rightarrow 2$ M NaCl and the major fractions were isolated by lyophilization after dialysis (S-1, 61.4 mg; S-2, 100.8 mg; and S-3, 119.4 mg).

Assay for anticoagulant activities. — *(a) Activated partial thromboplastin time (APTT).* The APTT assay was performed by the method of Andersson et al.[12] as described previously[13].

(b) Thrombin time (TT). The TT clotting assay was performed by the method of Denson and Bonnar[14] as described previously[13]. The clotting time of APTT and TT was measured with a Coagulometer KC1A (Amelung GmbH). The activities of fucan sulfates are expressed as units/mg in relation to that of heparin (167 units/mg).

(c) Antithrombin activity. This assay was performed by a method similar to that of Kim and Linhardt[15], as described previously[8]. A 340-μL sample (0–5 μg/mL of 50 mM Tris-HCl buffer (pH 8.4) containing 7.5 mM Na_2-EDTA and 150 mM NaCl) was incubated for 1 min at 37° together with 30 μL of HC II (1 PEU/mL of the buffer) and 50 μL of thrombin (6.7 units/mL of the buffer). S-2238 (200 μL, 1 mM in the buffer) prewarmed at 37° was then added to the mixture. After 1 min, the reaction was stopped by adding 1.9 mL of 10% AcOH. The absorbance was measured at 405 nm. In order to correct the absorbance, a blank test was also performed using the foregoing buffer instead of thrombin in the same manner as already described. A calibration curve was obtained by this procedure using thrombin in various concentrations in the absence of samples.

RESULTS AND DISCUSSION

Preparation and properties of oversulfated fucans. — In preliminary studies, C-II was sulfated directly with two different amounts (3 or 10 mol/mol equiv of available hydroxyl groups of Fuc in C-II) of pyridine–sulfur trioxide in DMF, dimethyl sulfoxide (Me_2SO), or pyridine at room temperature or 50° for 1–20 h, as shown in Table I. Electrophoresis of the products obtained under different conditions indicated that direct sulfation of C-II progressed only when DMF as the reaction solvent and 10 mol equiv of the reagent were used. The failure to sulfate C-II in pyridine or Me_2SO may be caused by the heterogeneity of the reaction; the polysaccharide was insoluble in these solvents. However, the reaction was not affected by the reaction times and temperatures tested. We also sulfated the tributylammonium salt of C-II by a method similar to that of Uchiyama et al.[10]. Electrophoresis showed that the products obtained had higher mobilities than C-II, indicating that C-II was sulfated under the conditions used.

On the preparative scale, three oversulfated fucans (S-1, S-2, and S-3) were prepared from C-II on the basis of the results of the foregoing preliminary studies. Chromatography on ECTEOLA-cellulose showed that C-II, S-1, S-2, and S-3 were eluted in the range of 0.90–1.43 M (elution peak, 1.24 M), 0.95–1.49 M (1.26 M), 0.98–1.54 M (1.28 M) and 1.00–1.56 M (1.30 M) NaCl, respectively (chromato-

TABLE II

Properties of C-II and its oversulfated fucans

Fucans	C-II	S-1	S-2	S-3
Molecular weight [a]	26 000	23 500	26 500	24 500
Components (molar ratio)				
Fuc	1.00	1.00	1.00	1.00
Gal	0.14	0.17	0.16	0.12
GlcA	0.02	0.03	0.02	0.02
Sulfate	1.49	1.65	1.94	2.26
Anticoagulant activity (units/mg) [b]				
APTT	145	159	166	173
TT	24	26	31	33

[a] Estimated by gel-filtration chromatography on a column of Sepharose CL-6B. [b] Expressed as units/mg in relation to activity of heparin (167 units/mg).

grams not shown). These results suggest that the sulfate content of these polysaccharides is in the increasing order: C-II, S-1, S-2, and S-3. Electrophoresis and gel-filtration chromatography on a column of Sepharose CL-6B of all the oversulfated fucans gave for each a single, sharp band and a single, symmetrical elution-curve (data not shown).

The properties of C-II, S-1, S-2, and S-3 are summarized in Table II. The respective molar ratios of components of the fucans were very similar to each other, except for their sulfate content. The molar ratios of sulfate to total sugar residues of C-II, S-1, S-2, and S-3 were 1.28, 1.38, 1.64, and 1.98, respectively. This result indicated that sulfation of the tributylammonium salt of C-II was more effective than direct sulfation of the polysaccharide. Position 2 of L-Fuc in C-II has mainly free hydroxyl groups, because C-II consists[6] of a backbone of (1 → 3)-linked α-L-Fuc having sulfate groups attached mainly to position 4. Therefore, this result also suggests that free hydroxyl groups of L-Fuc in S-3 were almost completely sulfated. The molecular weights of the fucans were similar to each other despite the fact that the sulfate content increased in the order of C-II to S-3, suggesting that slight cleavage of the glycosidic linkages in the polysaccharides occurred during the chemical sulfation. These results indicated that each fucan was different from the others only in its sulfate content. Thus, it is suggested that a relationship between the sulfate content and the anticoagulant and the antithrombin activities of the fucan sulfate may be established.

Anticoagulant and antithrombin activities of oversulfated fucans. — C-II and the oversulfated fucans were examined for anticoagulant activity with respect to APTT and TT, which are related to the intrinsic coagulation pathway and the third coagulation phase in plasma, respectively. As shown in Table II, the activities of the oversulfated fucans, S-1, S-2, and S-3 increased to 110, 114 and 119% over the original activity (C-II taken as 100%) with respect to APTT, and to 108, 129, and 140% of the original value with respect to TT. S-2 and S-3 exhibited equal or

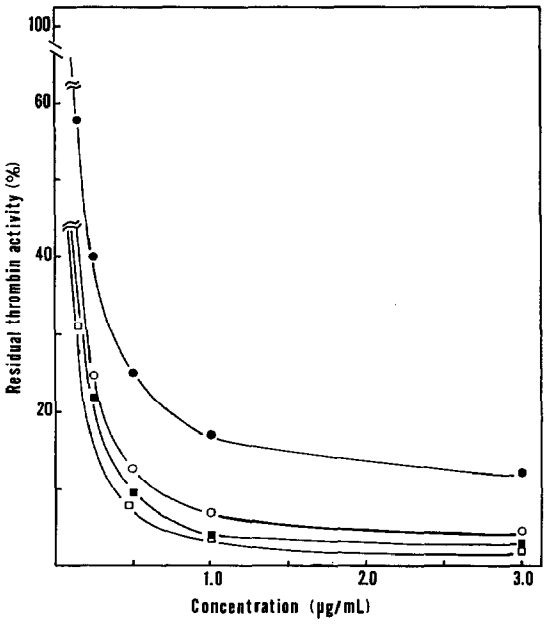

Fig. 1. Effects of fucans of different sulfate content on the amidolytic activity of thrombin in the presence of heparin cofactor II. Symbols: ●–●, C-II; ○–○, S-1; ■–■, S-2; □–□, S-3.

slightly greater inhibitory effects on APTT as compared with heparin used as standard. This result indicates that the anticoagulant activities of C-II increased with increase in its sulfate content. Our previous results indicated[7] that the anticoagulant activity of C-II in both APTT and TT was remarkably decreased when the ratio of sulfate/total sugar residues of C-II was < 1. However, in the present result, the increment of the anticoagulant activities of C-II gradually decreased with increase in the sulfate content of the polysaccharide. These results suggest that the anticoagulant effect of the fucan sulfate is strongly dependent on its sulfate content until the sulfate/sugar ratio of the polysaccharide reached 1, after which its activity tends to a plateau. In general, it is known[16–18] that the anticoagulant activity of polysaccharides is improved by chemically increasing the degree of sulfation. However, Uchiyama et al.[11] reported that the anticoagulant activity of oversulfated heparin was notably decreased as compared with that of the parent heparin owing to the probable occurrence of sulfation at unnatural positions along with natural positions of the polysaccharide. These results suggest that the anticoagulant activity of polysaccharides is dependent not only their sulfate content but also on the position of these sulfate groups.

Our previous study showed[8,9] that the HC II-mediated antithrombin action of C-II significantly diminished with decrease in the sulfate content and the molecular weight of the polysaccharide. The inhibitory effects of the oversulfated fucans on the amidolytic activity of thrombin were examined in the presence of HC II. As

shown in Fig. 1, all of the fucans strongly enhanced the inhibition of thrombin by HC II. Their inhibitory effects significantly increased with increase in the sulfate content of the fucans, suggesting that, for the fucans of almost the same molecular weight, the higher the sulfate content is, the more effective is the HC II-mediated antithrombin action, although the increment of the antithrombin effect gradually decreased with increase in the sulfate content of the fucans. Church et al.[19] reported that the antithrombin action of a fucoidan from *Fucus vesiculosus* was caused by activating HC II through forming a ternary complex by binding of the polysaccharides to the lysine residues in the serine protease inhibitor and protease. The present results also indicate that the negative charge-density of the fucan sulfate was required for expression of its HC II-mediated antithrombin activity, suggesting that the sulfate groups of the polysaccharide are important for binding to and activating HC II, but only up to a certain degree of sulfation. The fact that the ability of HC II-mediated antithrombin of C-II gradually plateaued with increase in its sulfate content may suggest that sulfate groups of C-II required for binding to HC II and/or thrombin are saturated by sulfation of the polysaccharide.

Oversulfation of heparin[16], dextran sulfate[16], chondroitin sulfate[20], and dermatan sulfate[20,21] enhances the abilities of the respective parent polysaccharides to activate HC II as well as C-II. However, as the ability of activating HC II differed with different sulfated polysaccharides in spite of the fact that highly charged polysaccharides interact with HC II in a poorly specific manner[22], the ability of the polysaccharides may be dependent not only on their sulfate content but also on their steric conformation related to sugar components, and the positions of the glycosidic linkage and the sulfate groups.

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