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Note

The sulfate-content dependence of the anticoagulant activity of a fucan sulfate from the brown seaweed *Ecklonia kurome*[*],[†]

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As it has been shown that the anticoagulant activity of heparin is dependent[1,2] on its sulfate content, many heparinoids, prepared by sulfation of polysaccharides from plant or animal tissue, have been studied[3–7]. Bernardi and Springer found[8] that a fucan sulfate (fucoidan) from the brown seaweed *Fucus vesiculosus* showed potent anticoagulant activity. Thereafter, various fucan sulfates showing anticoagulant activity were isolated[9–15] from several brown seaweeds. However, a possible relationship between the physical and chemical properties, the structures, and the anticoagulant activities of these fucan sulfates remains to be firmly established, because most of the fucan sulfates obtained have not been fully purified.

Recently, we have isolated[14,16] a highly purified fucan sulfate (C-II) having potent anticoagulant activity (130–148 units/mg) from the brown seaweed *Ecklonia kurome*. It consists of Fuc, Gal, GlcA, and sulfate in the molar ratio of $1.00:0.11 \sim 0.19:0.02 \sim 0.07:1.22 \sim 1.48$. We also reported[17] that C-II has a $(1\rightarrow3)$-linked α-L-Fuc backbone with sulfate groups attached mainly at O-4. In a previous paper[16], we demonstrated the relationship between the molecular weight and the anticoagulant activity of C-II.

Here, we have prepared various fucans having different sulfate content by solvolytic desulfation of C-II and have compared the chemical and physical properties, as well as the anticoagulant activities, of the product.

EXPERIMENTAL

Materials and methods. — Fucan sulfate (C-II) was isolated from *E. kurome* and then purified as already described[14,16].

General methods used were as described previously[14]. The constituents, i.r.

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spectra, and the molecular weights were determined as already described[14]. Electrophoresis (200 V, 15 min) was performed on a cellulose acetate membrane (Separax, 6 × 11 cm, Fuji Film Co. Ltd., Japan) using 0.1M pyridine–AcOH buffer (pH 3.5). Anticoagulant activities of fucan sulfates were examined by the APTT, PT, and TT assays according to the methods of Andersson *et al.*[19], Quick[20], and Denson and Bonnar[21], respectively, as described previously[16]. The activities of the fucans are expressed as units/mg in relation to that of heparin (167 units/mg) as a standard.

Partial desulfation of C-II by solvolysis. — This was performed by the method of Nagasawa *et al.*[18]. About 750 mg of C-II (1%, w/v) in water was applied to a column (5 × 9 cm) of AG 50W × 8 (H^+, 200–400 mesh) for decationization, and the combined eluate and water washings (4°) were made neutral with pyridine at 4° and lyophilized. The resulting pyridinium salt of C-II (200 mg) was dissolved in 20 mL of 9:1 (v/v) Me_2SO–MeOH. The solution was heated for 3 h at 80° and then adjusted to pH 9.0 with 0.1M NaOH. The product was applied to a column (2.5 × 92 cm) of Sephadex G-10, and a major fraction (60 mg) of high molecular weight eluted in the void volume was obtained. It was further fractionated by anion-exchange chromatography on a column (1.5 × 25 cm) of ECTEOLA-cellulose (Cl^-). The column was first washed with water to give a non-adsorbed fraction (DS-80-3, 26 mg) and then developed with a linear gradient of 0→2M NaCl (400 mL) to elute the adsorbed acidic fraction. The fractions were obtained after dialysis by lyophilization. Other batches of the pyridinium salt of C-II (200 mg each) were partially desulfated by solvolysis under various reaction conditions, namely for 1 h at 80° or for 1, 3 and 6 h at 50°. The respective partial desulfated fucans, DS-80-1 (34 mg), DS-50-1 (61 mg), DS-50-3 (66 mg), and DS-50-6 (81 mg) were obtained by treatment as just described.

RESULTS AND DISCUSSION

Preparation of desulfated fucans from C-II. — Partially and completely desulfated fucans were prepared from C-II by solvolysis[18] under various conditions. The products obtained by treatment at 80° were fractionated into a large proportion of high molecular weight (HMW) and a small proportion of depolymerized fractions by column chromatography on Sephadex G-10, suggesting that solvolysis of C-II under these conditions may be accompanied by some cleavage of glycosidic linkages. Under all of the procedures conducted at 50°, little depolymerization of C-II occurred. The respective HMW fractions were further chromatographed on a column of ECTEOLA-cellulose. Their elution profiles are summarized in Fig. 1. The HMW fraction (80°, 3 h) gave only one non-adosorbed fraction (DS-80-3), whereas the corresponding fraction (80°, 1 h) gave a large amount of acidic fraction (DS-80-1) along with a small amount of a non-adosorbed one. The other HMW fractions (50°, 1, 3, and 6 h) each consisted of an acidic fraction only (DS-50-1, DS-50-3, and DS-50-6). Electrophoresis indicated that DS-50-1, DS-50-3, and DS-50-6 each behaved as a single band, and showed a descreasing order of mobilities, as follows: C-II > DS-50-1 > DS-50-3 > DS-50-6 (Fig. 2). The DS-80-1 and DS-80-3 fractions were not stained with Toluidine Blue.

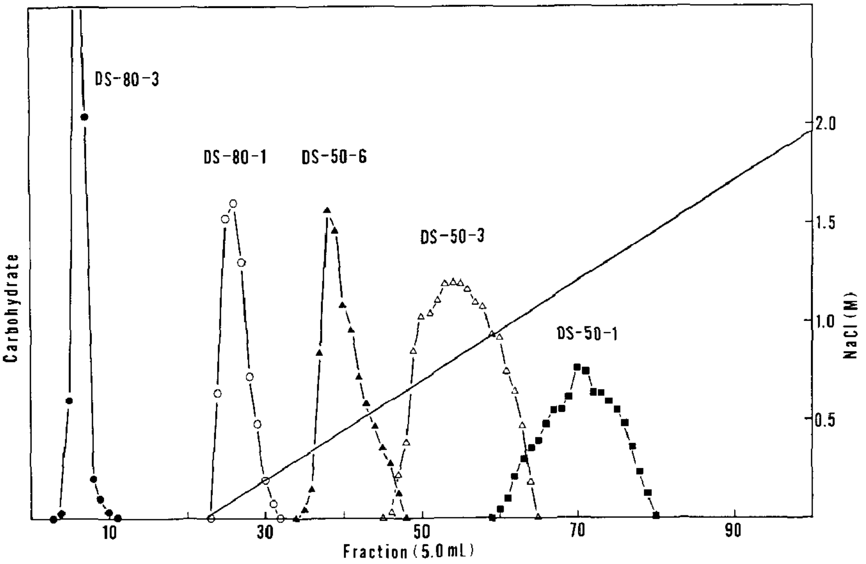

Fig. 1. The elution profiles of the partially desulfated fucans by ECTEOLA-cellulose column chromatography. [Carbohydrates in the column eluates were monitored by the phenol–sulfuric acid method[25].]

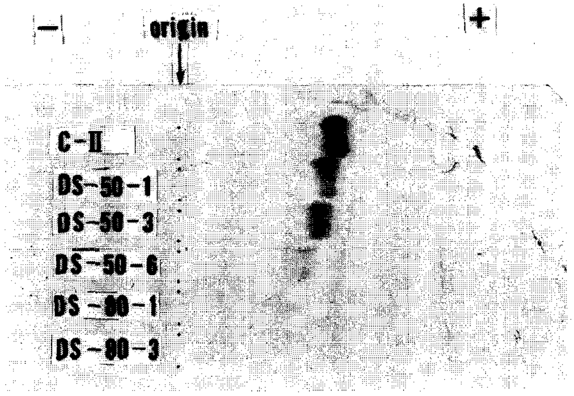

Fig. 2. Electrophoretic patterns of C-II and the partially desulfated fucans.

Properties of the partial desulfated fucans. — Properties of C-II and the respective partial desulfated fucans are summarized in Table I. As shown in Table I, the molecular weights decreased in relation to the decrease in sulfate content. The respective molar ratios of components of the fucans obtained were very similar to each other, except for the sulfate content. The molar ratios of sulfate to total sugar residues of C-II, DS-50-1, DS-50-3, DS-50-6, DS-80-1, and DS-80-3 were 1.08, 0.85, 0.54, 0.35, 0.23, and nil, respectively. The i.r. spectrum of DS-80-3 showed that the sulfate absorption band at 1240 cm^{-1} was absent, indicating the absence of sulfate in DS-80-3. This result showed that desulfation of C-II was complete upon solvolysis for 3 h at 80° in dimethyl sulfoxide–methanol, altough a slight amount of glycosidic cleavage occurred. These results also showed that each fucan obtained differed from the others only in its sulfate content.

Anticoagulant activities of the fucans having different sulfate content. — The parent C-II and the foregoing desulfated fucans were examined for anticoagulant activities in the APTT, PT, and TT assays, which are related to intrinsic and extrinsic coagulation pathways and antithrombin activity in plasma, respectively (Table I). In all of these assays, the desulfated fucans showed significantly lower activities as compared to that of C-II (the parent fucan), and DS-80-1 and DS-80-3 were inactive. DS-50-1, DS-50-3, and DS-50-6 retained 28, 16, and 11% of the original activity (C-II taken as 100%) with respect to APTT, and 42, 29, and 4% of the original with respect to PT, respectively. In a previous study[16], we demonstrated with respect to APTT, that the fucan sulfate having molecular weight 10 000–30 000 showed the highest activity and above this range the activity decreased, whereas for PT, the activity of the polysaccharide was independent of its molecular weight. In the present study, C-II and the desulfated fucans were shown to have molecular weights ranging from 8 000 to 18 000. It was thus concluded that only the sulfate content of the polysaccharides affects their anticoagulant activities with regard to APTT and PT. As for the TT assay, DS-50-1 and DS-50-3 retained respectively 21 and 11% of the original activity. We previously reported[16] that the antithrombin activity of C-II increases with increase in the molecular weight up to ∼ 50 000. In the present study, the molecular weights of the desulfated fucans obtained were shown to be lower that of C-II. However, the difference in molecular weight between C-II and each of the desulfated fucans obtained was approximately equal to the amount

TABLE I

Properties of C-II and the partially desulfated fucans

Fucans	*C-II*[a]	*DS-50-1*	*DS-50-3*	*DS-50-6*	*DS-80-1*	*DS-80-3*
Molecular weight[b]	17 800	16 500	12 500	10 500	9400	7700
Components (molar ratio)						
Fuc	1.00	1.00	1.00	1.00	1.00	1.00
Gal	0.11	0.14	0.16	0.12	0.14	0.16
Xyl	Trace	N.D.[c]	N.D.	N.D.	N.D.	N.D.
GlcA	0.02	0.05	0.05	0.03	0.02	0.02
Sulfate	1.22	1.02	0.65	0.41	0.27	N.D.
Anticoagulant activity (units/mg)[d]						
APTT	130	36	21	14		—[e]
PT	24	10	7	1	—	—
TT	19	4	2	—	—	—

[a] Ref. 16. [b] Estimated by gel filtration on a column (1.2 × 95.3 cm) of Sepharose CL-6B with 0.2M NaCl as eluent. [c] Not detected. [d] Expressed as units/mg in relation to activity of heparin (167 units/mg). [e] No activity.

of sulfate removed from C-II (although a slight decrease in molecular weight by depolymerization was also observed), indicating that decrease in the molecular weight may be mainly due to the decrease in sulfate content. These results thus show that the antithrombin activity of the fucans may be dependent on their sulfate content. Other workers have reported very similar relationships between the anticoagulant activity and sulfate content of several sulfated polysaccharides, such as heparin[1,2], dextran sulfate[22], heparan sulfate dermatan sulfate[23], and the fucan sulfates[24] from the brown seaweeds *F. vesiculosus* and *Pelvetia canaliculata*, which resemble that obtained in the present study.

This work suggests that, in order to develop potent anticoagulant activity, the sulfate/total sugar residues ratio may be required to be more than unity in the fucan sulfate, and also indicates that the fucan sulfates having this ratio <0.3 had no anticoagulant activity. Whether or not the anticoagulant activity of the fucan sulfate can be improved by chemically increasing its degree of sulfation is yet to be established.

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