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ISOLATION, PURIFICATION, AND CHARACTERIZATION OF FUCOSE-CONTAINING SULFATED POLYSACCHARIDES FROM THE BROWN SEAWEED *Ecklonia kurome* AND THEIR BLOOD-ANTICOAGULANT ACTIVITIES

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ABSTRACT

A sulfated polysaccharide fraction, obtained from the hot-water extract of the brown seaweed, *Ecklonia kurome* by removing laminaran and the major part of alginic acid, gave sulfated polysaccharides (B-I, B-II, C-I, and C-II) by both anion-exchange chromatography on a column of Ecteola-cellulose and by fractional precipitation with ethanol containing 0.3% calcium acetate, and then by gel-filtration chromatography on a Sepharose 4B column. B-I and B-II are composed of fucose, galactose, mannose, xylose, glucuronic acid, and ester sulfate in the approximate molar ratios of 1.00:0.36:0.48:1.08:1.85:2.35 and 1.00:0.81:0.18:0.45:0.61:2.00, respectively. C-I and C-II are composed of fucose, galactose, glucuronic acid, and ester sulfate in approximate molar ratios of 1.00:0.03:0.03:1.61 and 1.00:0.19:0.07:1.48, respectively. Blood-anticoagulant activities with respect to activated partial thromboplastin time (APTT) were approximately 24, 19, 81, and 85% of that of heparin for B-I, B-II, C-I, and C-II, respectively. All the polysaccharides showed slight antithrombin activity. No anti-factor Xa activity was observed for any of the polysaccharides.

INTRODUCTION

Sulfated, fucose-containing polysaccharides from brown algae have been studied for blood-anticoagulant activities. Recently it has been reported that highly purified fucans from *Fucus vesiculosus*[1] and *Eisenia bicyclis*[2], fucogalactan sulfate from *Undaria pinnatifida*[3], sargassan from *Sargassum linifolium*[4], and heteropolysaccharide (sulfated galactofucoglucuronan) from *Dictyota dichotoma*[5] showed relatively high activities as compared with heparin. However, the possible relationship between the structure and the blood-anticoagulant activity of each sulfated polysaccharide remains to be established.

In a previous study[6], we examined the blood-anticoagulant activity of sulfated

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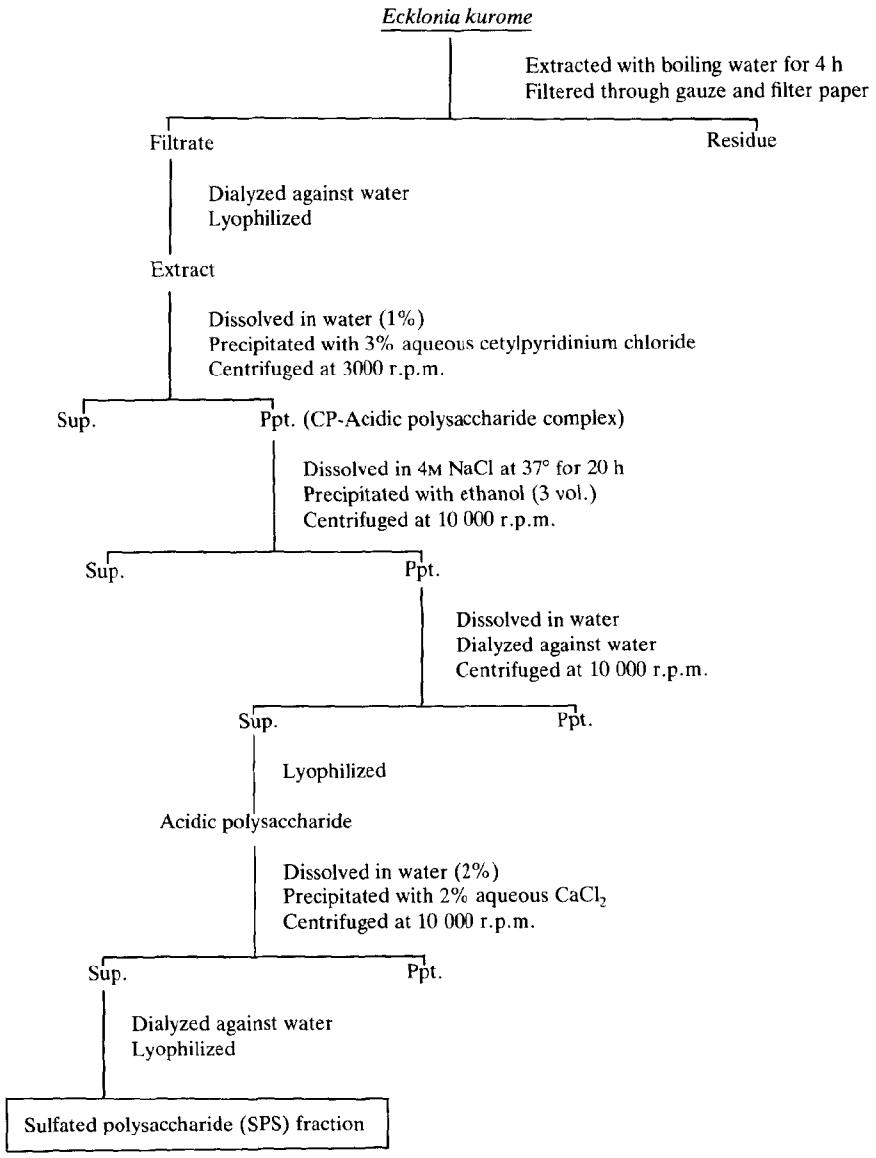
polysaccharide (SPS) fractions from nine species of brown algae, and demonstrated that the fraction from *Ecklonia kurome* showed the highest activity. However, electrophoresis indicated that this fraction consisted of more than three polymer components. Therefore, in order to identify the biologically active components, we attempted to isolate each of the polysaccharide components from the SPS fraction by anion-exchange chromatography, fractional precipitation with ethanol, and then gel-filtration chromatography. As a result, four polysaccharide components were obtained, and their blood-anticoagulant activities and physical and chemical properties were examined. We describe herein the isolation and purification of the four sulfated polysaccharides, their physical and chemical properties, and their blood-anticoagulant activities.

EXPERIMENTAL

Materials. — The brown seaweed *E. kurome* was collected in Japanese waters in February 1985. The freshly collected seaweed fronds were washed with tap water and then de-ionized, air-dried, and milled.

General methods. — Optical rotations were measured for an aqueous solution of each polysaccharide sample with a Jasco DIP-4 polarimeter at 589 nm. The i.r. spectrum was recorded for a KBr pellet of a test sample with a Perkin–Elmer 983 infrared spectrophotometer. T.l.c. was performed on silica gel (Replate 50, 0.2-mm layers, Yamato Scientific Co. Ltd.) after impregnation[7] of the plates with phosphates as follows: (*A*) 0.3M Na_2HPO_4, 2:1:1 2-propanol–acetone–0.1M phosphoric acid; (*B*) 0.3M NaH_2PO_4, 5:1:1:2 ethanol–phenol–pyridine–0.1M phosphoric acid for uronic acids; (*C*) 0.5M NaH_2PO_4, 16:1:3 2-propanol–methanol–water; and (*D*) 0.3M Na_2HPO_4, 4:5:1 butanol–acetone–water for neutral sugars. Detection was effected with diphenylamine–aniline–phosphoric acid[8]. G.l.c. was carried out with a Hitachi gas chromatograph Model 063 equipped with a flame-ionization detector and a glass column (3mm × 2m) of 2% XF-1105 on Gaschrom P at 125° for trifluoroacetylated alditols[9]. Electrophoresis was performed on a cellulose acetate paper Separax (6 × 11 cm, Fuji Film Co. Ltd.) under the following conditions: (*E*) 0.1M pyridine–acetic acid buffer (pH 3.5), 200 V, 15 min; (*F*) 0.1M HCl, 16.5 V, 2.5 h; and (*G*) 0.1M zinc acetate (pH 6.6), 200 V, 1 h. Staining was carried out with 0.5% Toluidine Blue in 3% acetic acid for *E* and *G*, and with aqueous 0.5% Toluidine Blue solution for *F*. Nitrogen content was determined with an elemental analyzer, Shimazu Analyzer Model 240, fucose content by the method of Gibbons[10] using L-fucose as a standard, uronic acid content by a modified carbazole method[11] using D-glucuronic acid as a standard, and sulfate content by the modified method[12] of Dodgson and Price[13].

Preparation of the sulfated polysaccharide (SPS) fraction. — The pretreated seaweed fronds (200 g) were extracted with hot water (3 L) for 4 h in a boiling-water bath. The SPS fraction was prepared from the extracts with aqueous cetylpyridinum chloride (CPC) and $CaCl_2$ solutions as described previously[14] (Scheme 1). The yield of the fraction was 6.4 g.



Scheme 1. Preparation procedure of the sulfated polysaccharide (SPS) fraction from *Ecklonia kurome*.

Fractionation of SPS fraction by anion-exchange chromatography. — The SPS fraction (3 g) described above was fractionated by anion-exchange chromatography on a column (5 × 40 cm) of Ecteola-cellulose (Cl^-). After an aqueous solution (1%) of the SPS fraction had been applied to the column, it was eluted stepwise with 0.6M (1500 mL, Fraction A), 0.95M (1800 mL, Fraction B), and 2.0M NaCl (1500 mL, Fraction C) successively until the eluates were free from carbohydrates

by the phenol–H_2SO_4 test[15]. All the eluates of each fraction were then combined, dialyzed, and lyophilized. The yield of Fractions A, B, and C was 0.2, 0.4, and 1.6 g, respectively.

Fractional precipitation of Fractions B and C with ethanol containing 0.3% calcium acetate. — Each Fraction B and C was dissolved in water (1%). Ethanol containing 0.3% calcium acetate was added to the solution of Fraction B until the final concentration of ethanol became 40%. The precipitate (Fraction B-P) and supernatant (Fraction B-S) were separated by centrifugation. Both fractions were evaporated to dryness *in vacuo* for removal of ethanol, and the residues were redissolved in water, dialyzed, and lyophilized. The respective yield of Fractions B-P and B-S was 65 and 35% of Fraction B. Fractional precipitation of Fraction C was also carried out in the same manner as described above, except that the final concentration of ethanol was 46%, to give Fractions C-P (precipitate) and C-S (supernatant) in 45 and 55% yield, respectively.

Purification of Fractions B-P, B-S, C-P, and C-S by gel-filtration chromatography. — A solution of Fraction B-P (113 mg) in 0.2M NaCl (11 mL) was applied to a column (5 × 85 cm) of Sepharose 4B, and the column was eluted with 0.2M NaCl. Fractions (14 mL each) were monitored on the basis of the phenol–H_2SO_4 reaction for carbohydrates. The major fractions (fraction numbers 55–72) were combined, dialyzed, and lyophilized, to give Fraction B-I (84 mg). Chromatography of Fractions B-S (200 mg), C-P (290 mg), and C-S (329 mg) was also carried out in the same manner to give Fractions B-II (140 mg), C-I (232 mg), and C-II (297 mg), respectively.

Characterization of Fractions B-I, B-II, C-I, and C-II. — The constituent sugars of each fraction were determined by the following procedures. Each polysaccharide sample was hydrolyzed with 90% formic acid by the method of Mian and Percival[16]. After formic acid had been removed completely, the dried hydrolyzate was dissolved in water. A portion of the solution was examined by t.l.c. for neutral sugars and uronic acids. The residual solution was adjusted to pH 8.0 with 0.1M NaOH, and kept at that pH level for 30 min or more; it was then chromatographed on a column of Dowex 1-X2 (1.2 × 20 cm; Cl^-) with water as an eluent, until the eluate was free from carbohydrates, in order to remove uronic acids. All the eluates were combined and evaporated to dryness. The dried sample was then reduced with $NaBH_4$, and trifluoroacetylated with trifluoroacetic anhydride according to the method of Imanari *et al.*[9], and analyzed by g.l.c. Neutral sugars were identified by referring to the retention times of authentic sugars, and their approximate proportion calculated from the ratio of the g.l.c. peak area to that of galactose in the chromatograms by use of calibration curves for respective authentic sugars.

Gel-filtration chromatography was performed on a column (1.2 × 99 cm) of Sepharose 4B for Fraction B-I, and a column (1.2 × 99–100 cm) of Sepharose CL-6B for Fractions B-II, C-I, and C-II with 0.2M NaCl as an eluent. Fractions (~2 mL each) were monitored by the phenol–H_2SO_4 reaction for carbohydrates. In

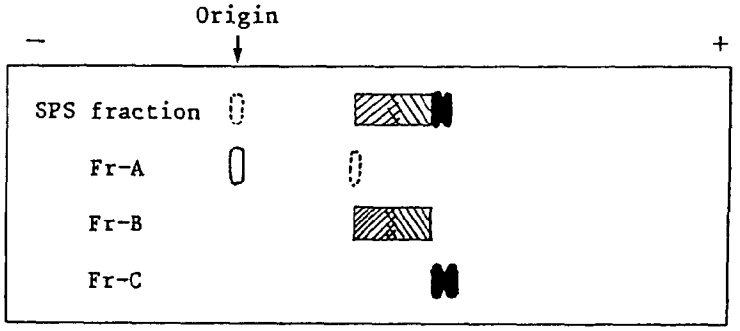


Fig. 1. Electrophoretic patterns of Fractions SPS, A, B, and C. Condition: *G*, 0.1M zinc acetate (pH 6.6), 200 V, 60 min.

order to estimate the molecular weight of polysaccharide samples, Blue Dextran (type 2000) and pullulan (Shodex standard P-82) were used as the mol. wt. standards.

Blood-anticoagulant activities. — Blood-anticoagulant activity of polysaccharide samples was determined for human plasma (Ci-Trol I, Dade), with respect to activated partial thromboplastin time (APTT)[17], with an actin reagent (Dade), thrombin time (TT)[18] with bovine thrombin (Mochida Pharmaceutical Co. Ltd., Tokyo), and anti-factor Xa activity[19] with a Testzyme-Heparin kit using Bz-Ileu-Glu-Gly-Arg-*p*NA (S-2222) as the substrate (Dai-ichi Kagaku Yakuhin Co., Tokyo). These activities were expressed as units/mg in relation to that of porcine intestinal mucosa heparin (167 units/mg, Wako Pure Chemical Industries, Ltd., Tokyo) as a standard.

RESULTS AND DISCUSSION

Isolation and purification of polysaccharide components from SPS fraction. — The SPS fraction obtained from *E. kurome* by the procedures shown in Scheme 1 was shown by electrophoresis (*G*) to contain more than three major sulfated polysaccharide components, and alginate as the contaminant (Fig. 1). In order to isolate each component, the SPS fraction was first chromatographed on a column of Ecteola-cellulose (Cl^-) stepwise with 0.6, 0.95, and 2.0M NaCl, successively, as the eluents. The fractions were examined by electrophoresis (*G*) which indicated that the fraction eluted with 0.6M NaCl (Fraction A) was composed of alginic acid (major component) and a small proportion of sulfated polysaccharides (minor components). The alginate was contained in that fraction alone, whereas the same sulfated components were also found in the fraction eluted successively. Thus, in further experiments, the SPS fraction was first chromatographed on the aforementioned column with 0.6M NaCl as the eluent for complete removal of alginic acid contaminating the fraction, even though the fractionation resulted in a loss of a small amount of sulfated polysaccharides. On the other hand, the fraction eluted

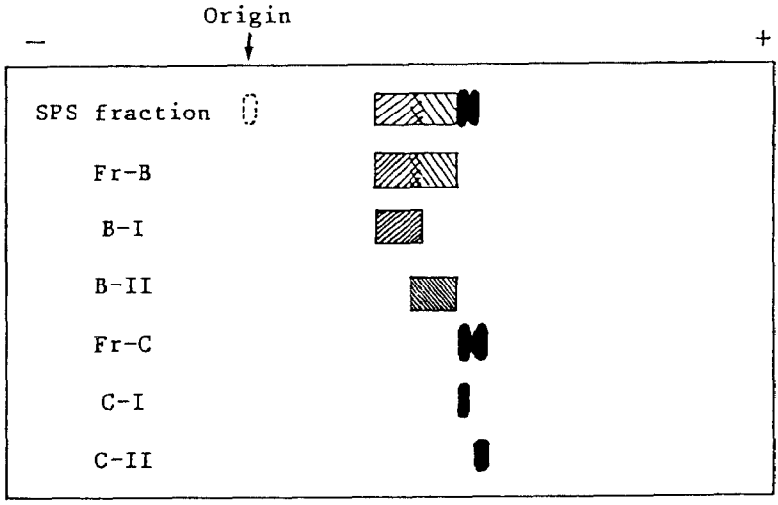


Fig. 2. Electrophoretic patterns of Fractions SPS, B, B-I, B-II, C, C-I, and C-II. Condition: *G*, 0.1M zinc acetate (pH 6.6), 200 V, 60 min.

with 0.95M NaCl (Fraction B) was found to contain a sulfated polysaccharide component (lower electrophoretic mobility) giving a broad band, and the fraction eluted with 2.0M NaCl (Fraction C) to be a mixture of two sulfated polysaccharide components having higher electrophoretic mobilities (Fig. 1). Thus, Fractions B and C were further fractionated with ethanol containing 0.3% calcium acetate. Fractional precipitation of Fractions B and C was effected with a final concentration of 40 and 46% of ethanol, respectively, to give Fractions B-P (precipitate) and B-S (supernatant) from Fraction B, and Fractions C-P and C-S from Fraction C. The four fractions, B-P, B-S, C-P and C-S, were then treated by chromatography on Sepharose 4B gel to give B-I, B-II, C-I and C-II, respectively.

The four fractions (B-I, B-II, C-I and C-II) were first examined for homogeneity by electrophoresis. As shown in Fig. 2, all the fractions behaved as a single band (*G*), although the electrophoretic pattern of Fractions B-I and B-II was shown to be somewhat broad. Their homogeneity was also confirmed by electrophoresis (*E* and *F*). Among the fractions, Fraction C-I was considerably different from the others when stained with Toluidine Blue (C-I: reddish, and the others: blue). The four fractions, B-I, B-II, C-I, and C-II, were then examined for homogeneity by gel-filtration chromatography on Sepharose 4B (for Fraction B-I) and Sepharose CL-6B (for the others) gels. The chromatography of each fraction gave a single, symmetrical elution curve, as shown in Fig. 3, indicating that each fraction was homogeneous. The average mol. wts. of Fractions B-I, B-II, C-I, and C-II were estimated at 270 000, 51 000, 32 000, and 21 000, respectively. Electrophoresis and gel-filtration chromatography indicated that the four fractions were highly purified, sulfated polysaccharide components.

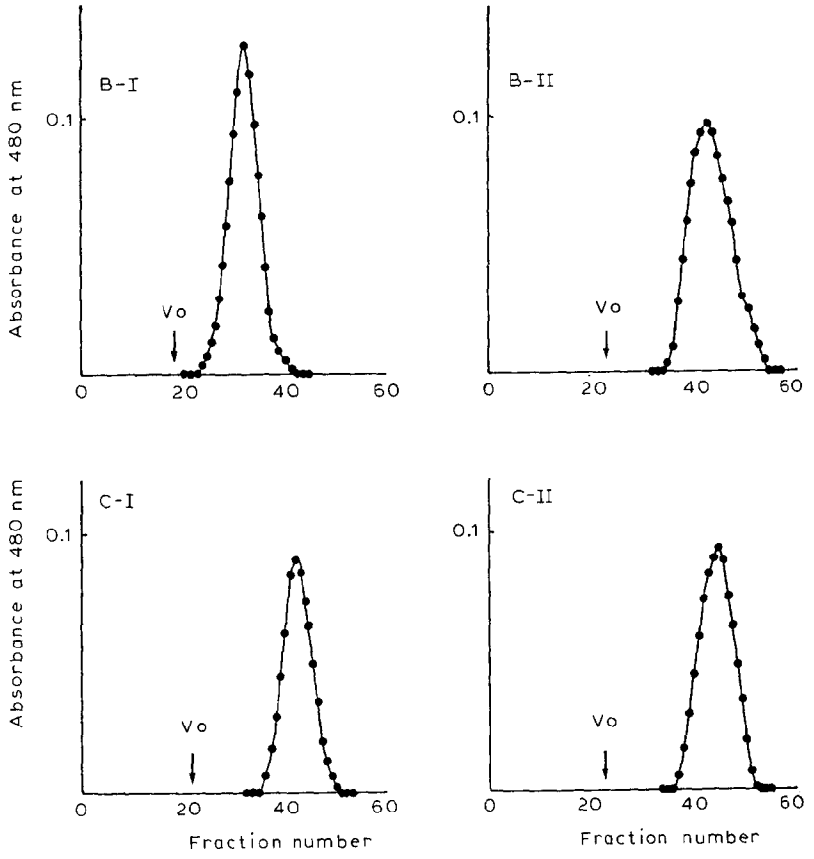


Fig. 3. Gel-filtration chromatograms of Polysaccharides B-I, B-II, C-I, and C-II. Conditions: Column (1.2 × 99 cm) of Sepharose 4B for B-I. and Sepharose CL-6B (1.2 × 99–100 cm) for B-II, C-I, and C-II. Samples: 1–3 mg/1.0 mL of 0.2M NaCl. Fraction volume; 2.10, 2.01, 2.15, and 2.14 mL for Polysaccharides B-I, B-II, C-I and C-II, respectively. Flow rate, 4–5 mL/h.

Characterization of the purified, sulfated polysaccharides. — Four homogeneous sulfated polysaccharides (B-I, B-II, C-I, and C-II) were isolated from *E. kurome* as described above. Their yields, and physical and chemical properties are shown in Table I. Each polysaccharide was found to be composed mainly of varying proportions of fucose, galactose, uronic acid, and ester sulfate. Fucose and sulfate were the major constituents. Uronic acid was identified as glucuronic acid by t.l.c. However, minor Polysaccharides B-I and B-II were fairly different from the other major polysaccharides in composition. As for constituents, Polysaccharides B-I and B-II contained also xylose, mannose, and nitrogen as constituents, and a small proportion of glucose was also detected in Polysaccharide B-I. As regards uronic acid and ester sulfate contents, Polysaccharides B-I and B-II were fairly higher in the uronic acid content and lower in ester sulfate than the others, whereas Poly-

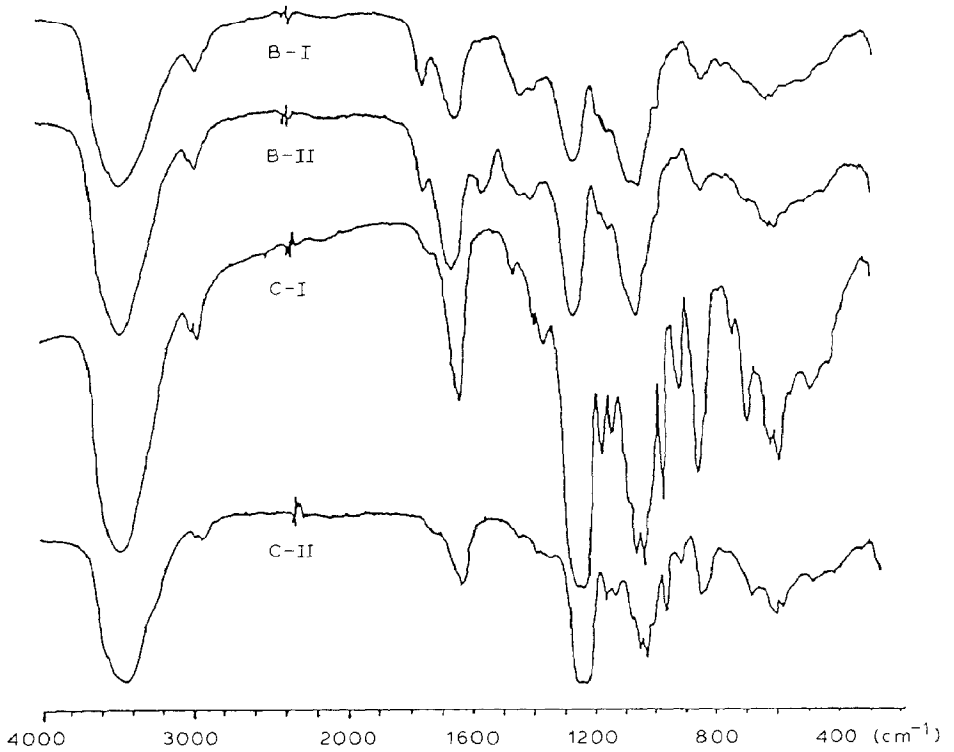


Fig. 4. Infrared spectra of Polysaccharides B-I, B-II, C-I, and C-II.

saccharides C-I and C-II were the reverse. These results indicated that Polysaccharides B-I and B-II were uronic acid-richer polysaccharides, whereas Polysaccharides C-I and C-II were ester sulfate-richer polysaccharides. The sulfated polysaccharides synthesized by *E. kurome* seem to be composed of the two species of polysaccharide components described above. Similar results have been reported for sulfated polysaccharides from several brown seaweeds[16,20].

Molar ratios of the major constituents in Polysaccharides B-I, B-II, C-I, and C-II, where fucose content is taken as unity, are shown in Table II. Polysaccharides B-I and B-II were found to differ in molar ratio of the constituent sugars except for glucose. Their molar ratios indicated that Polysaccharide B-I may be a glucuronoxylofucan sulfate containing about equal proportions of fucose and xylose, a high uronic acid content, and a small proportion of galactose, whereas Polysaccharide B-II may be a glucuronogalactofucan sulfate containing about equal proportions of fucose and galactose, and small proportions of xylose and uronic acid. In addition to these results, Polysaccharide B-I was also found to be very similar in composition to sargassan[4], except that its galactose content was fairly lower than that of sargassan, and Polysaccharide B-II was found to be an asco-

TABLE I

PHYSICAL AND CHEMICAL PROPERTIES OF POLYSACCHARIDES B-I, B-II, C-I, AND C-II

Poly-saccharide	*Yield*[a] *(%)*	$[\alpha]_D$ *(degree)*	*N (%)*	*Fucose (%)*	*Uronic acid*[b] *(%)*	*Sulfate (%)*	*Distribution of neutral sugars (%)*[c]					*Mol. wt.*[d] *(× 10⁴)*
							Fuc	*Gal*	*Glc*	*Man*	*Xyl*	
B-I	11	−34	0.5	13.6	29.8	18.7	34	13	[e]	18	34	27
B-II	6	−57	3.4	19.4	14.0	22.7	39	34	4	7	16	5.1
C-I	35	−142	[e]	50.1	1.5	47.1	97	3	[e]	[e]	[e]	3.2
C-II	48	−129	[e]	49.5	3.9	43.0	83	17	[e]	[e]	[e]	2.1

[a]Based on recovered materials only. [b]Determined as glucuronic acid. [c]Calculated from g.l.c. patterns,considering the total area under the five peaks as 100%. [d]Estimated by gel-filtration chromatography. [e]Not detected.

phyllan-like polysaccharide, because its composition was similar to that of ascophyllan[21]. On the other hand, Polysaccharides C-I and C-II were shown to be very similar in composition to each other, except for the galactose content, indicating that both polysaccharides may be highly purified fucoidans. Their compositions were similar to those of the purified fucoidans from *E. bicyclis*[2] and *Pelvetia wrightti*[22].

In Polysaccharides B-I and B-II, the respective molar ratios of sulfate groups to total sugars (including uronic acid) were ~1:2 and 2:3, indicating that one mole of the sulfate group may be attached to two sugar residues in Polysaccharide B-I and two moles of the group to three sugar residues in Polysaccharide B-II. However, the distribution of the sulfate groups in the polysaccharides remains to be clarified. On the other hand, in Polysaccharides C-I and C-II, the molar ratio in question was ~3:2, suggesting that three moles of sulfate may be attached mainly to two fucose residues in the polysaccharides, because the other constituent sugars are so minor. However, it cannot be excluded that a part of galactose or uronic acid residues might be sulfated.

In the i.r. spectra of Polysaccharides B-I, B-II, C-I, and C-II (Fig. 4), the absorption patterns of Polysaccharides B-I and B-II were somewhat different from those of the others but were similar to each other, and Polysaccharides C-I and C-II

TABLE II

MOLAR RATIOS OF THE MAJOR CONSTITUENTS IN POLYSACCHARIDES B-I, B-II, C-I, AND C-II

Polysaccharide	*Molar ratio*						
	Fuc	*Gal*	*Glc*	*Man*	*Xyl*	*GlcA*	SO_4^{-2}
B-I	1.00	0.36		0.48	1.08	1.85	2.35
B-II	1.00	0.81	0.09	0.18	0.45	0.61	2.00
C-I	1.00	0.03				0.03	1.61
C-II	1.00	0.19				0.07	1.48

TABLE III

BLOOD-ANTICOAGULANT ACTIVITY OF POLYSACCHARIDES B-I, B-II, C-I, AND C-II

Polysaccharide	*Activity[a] in*		
	APTT	*TT*	*Anti-Xa*
B-I	40	13	[b]
B-II	32	2	[b]
C-I	136	13	<3.8
C-II	142	11	[b]

[a]Expressed as units/mg in relation to the activity of heparin (167 I.U./mg) used as the standard. [b]Not detected.

gave almost the same spectra. A strong absorption band of S=O stretching vibration at 1240 cm^{-1} was found in all polysaccharides, indicating the presence of ester sulfate. In addition, in the spectra of Polysaccharides B-I and B-II, a moderate band at ~820 cm^{-1} was observed, which showed that the sulfate groups were in equatorial position[23–25]. A very weak band at ~850 cm^{-1} was also found as a shoulder in the spectra, indicating the presence of a small number of sulfate groups attached in axial position[23–25]. On the other hand, in the spectra of Polysaccharides C-I and C-II, a strong band at 850 cm^{-1} and a weak one (a shoulder) at 820 cm^{-1} indicated that most sulfate groups were in axial position and the remainder in equatorial position[23–25]; thus, the sulfate groups were mainly at C-4 of the fucose residues.

All the polysaccharides showed a negative specific rotation (Table I), indicating that the fucose residues were predominantly in the α-L form.

Blood-anticoagulant activities. — Polysaccharides B-I, B-II, C-I, and C-II were examined for blood-anticoagulant activity (Table III). Polysaccharides C-I and C-II showed the most remarkable APTT activity, corresponding respectively to ~81 and 85% of that of heparin, whereas Polysaccharides B-I and B-II showed moderate activity (~20% of that of heparin). With regard to TT, Polysaccharides B-I, C-I, and C-II all were found to show similar moderate activity (~8% of that of heparin), whereas Polysaccharide B-II showed very slight activity. No anti-factor Xa activity was detected for any polysaccharide. From these results, we concluded that the anticoagulant-active components are Polysaccharides C-I and C-II.

In the present study, Polysaccharides C-I and C-II were found to be highly purified fucoidans (containing mainly fucose and sulfate groups) which possess remarkably high anticoagulant activities, whereas Polysaccharides B-I and B-II were found to be heteropolysaccharides composed of lower contents of fucose and sulfate, and a higher content of uronic acid, in addition to the constituents, galactose, mannose, and xylose (and glucose), and to have low anticoagulant activities. These results suggest that the higher contents of fucose and sulfate groups, and the lower

content of uronic acid may be closely related to the higher biological activity. Results similar to these have been reported for the highly purified fucan sulfates from *E. bicyclis*[2] and *F. vesiculosus*[1]. However, the similarities in the structures of Polysaccharides C-I and C-II, and the fucan sulfates remain to be investigated. On the other hand, it has been also reported that sargassan[4], sulfated heteropolysaccharides from *D. dichotoma*[5], *Padina tetrastomatica*[26], and *P. pavonia*[27], and fucogalactan sulfate from *U. pinnatifida*[3] have high anticoagulant activities. The results also indicate that the sugar composition, structure, sulfate content, and location of the sulfate groups of a polysaccharide may be related to its anticoagulant activity. Thus, the relationship between structure and anticoagulant activity appears to be very complex.

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