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Note

Isolation and preliminary characterization of fucose-containing sulfated polysaccharides with blood-anticoagulant activity from the brown seaweed *Hizikia fusiforme*

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The sulfated polysaccharides of marine brown algae have been extensively studied for blood-anticoagulant activity. Abdel-Fattah *et al.*[1] isolated a sulfated heteropolysaccharide, "sargassan", from *Sargassum linifolium*, which was composed of fucose, galactose, mannose, xylose, glucuronic acid, and a protein component, and demonstrated that the heteropolysaccharide possessed higher anticoagulant activity than heparin. Similar sulfated heteropolysaccharides having anticoagulant activity were also isolated from *Dictyota dichotoma*[2], *Padina pavonia*[3], and *P. tetrastromatica*[4]. Bernardi and Springer[5] obtained, from *Fucus vesiculosus*, a highly purified, sulfated fucan containing almost entirely fucose as the sugar constituent and showed the fucan to have significant anticoagulant activity. Similar fucan-like polysaccharides having anticoagulant activity were also isolated from *Eisenia bicyclis*[6] (sulfated fucan), *Undaria pinnatifida*[7] (sulfated fucogalactan), and *Ecklonia kurome*[8] (sulfated galactofucan).

Recently, we reported[9] that a crude, sulfated polysaccharide (SPS) fraction from *Hizikia fusiforme* showed considerable anticoagulant activity. However, electrophoretic studies indicated that the fraction from the alga consisted of more than four sulfated polysaccharide components. Thus, in order to identify the biologically active substances, we attempted to isolate each of the polysaccharide components from the SPS fraction by anion-exchange chromatography, fractional precipitation with ethanol, and gel-filtration chromatography. As a result, two purified, fucan-like sulfated polysaccharides having anticoagulant activity were obtained. We describe herein the isolation of the purified sulfated polysaccharides, their physical and chemical properties, and their blood-anticoagulant activities.

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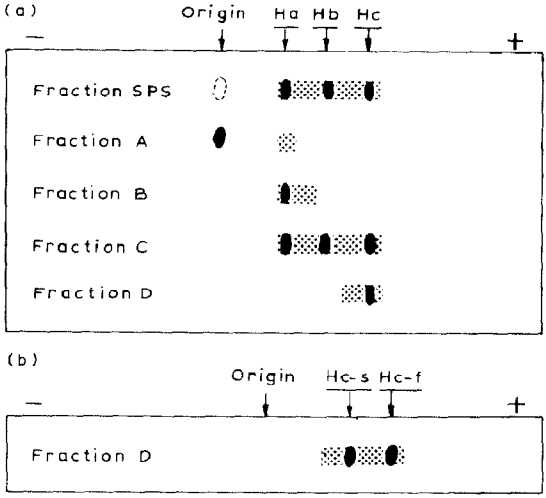

Fig. 1. Cellulose acetate electrophoresis (Toluidine Blue stain)[8]: (a) Electrophoretic patterns of Fractions SPS, A, B, C, and D, in 0.1M zinc acetate, pH 6.6 at 200 V for 60 min. (b) Electrophoretic pattern of Fraction D in 0.1M HCl at 16.5 V for 150 min.

EXPERIMENTAL AND RESULTS

Fractionation of H. fusiforme *polysaccharides.* — *Hizikia fusiforme,* which was collected from a foreshore of Kanaya in Tokyo Bay in February 1986, was kindly donated by Kimitsu Chemical Industries Co. Ltd. The crude SPS fraction (2.5 g) was prepared from freshly collected seaweed fronds (500 g) by extraction with diluted HCl (pH 2.0), after pretreatment of the fronds with methanol, and then by fractionation of the HCl extract with aqueous cetyl pyridinium chloride and $CaCl_2$ solutions according to the method described previously[9]. Electrophoresis (0.1M zinc acetate, pH 6.6) showed that the fraction consisted of three SPS components, which were named tentatively, in order of increasing mobility, Polysaccharides Ha, Hb, and Hc (Fig. 1a). The ratios of Polysaccharides Ha to Hb and Hc were densitometrically estimated at 1:3:36, indicating that Polysaccharide Hc was the major component. For separation of each component, the SPS fraction (1.00 g) was first chromatographed on a column of Ecteola-cellulose (2.64 × 41.7 cm, Cl^-) with stepwise elution with 0.5, 0.7, 1.0, and 2.0M NaCl (800 mL each). Each eluate was collected, dialyzed, and lyophilized. The respective yields of 0.5M NaCl-eluted (Fraction A), 0.7M NaCl-eluted (Fraction B), 1.0M NaCl-eluted (Fraction C), and 2.0M NaCl-eluted (Fraction D) fractions were 0.08, 0.11, 0.16, and 0.34 g. Electrophoresis (0.1M zinc acetate) indicated that the respective major components of Fractions A, B, and D were alginate, and Polysaccharides Ha and Hc, whereas Fraction C was still a mixture of Polysaccharides Ha, Hb, and Hc (Fig. 1a). Further fractional precipitation of Fractions B and C with ethanol (containing 0.3% calcium acetate) gave a fraction containing Polysaccharide Ha in moderately

pure form (Fraction B-II-2) and a fraction containing almost only Polysaccharide Hb (Fraction C-II-1) (procedures not described).

Subfractionation of Fraction D. — As Fraction D was found to be the only one showing anticoagulant activity (see later), it was subjected to further purification. Electrophoretic examination (0.1M HCl) indicated that the fraction consisted of two major SPS components, which were named tentatively, in order of increasing mobility, Polysaccharides Hc-s and Hc-f in the ratio of 13:5 by densitometry (Fig. 1b). Fraction D (150 mg) was further fractionated by addition of 86% ethanol (containing 0.3% calcium acetate) to a 1% aqueous solution until the final ethanol concentration was 50%, followed by centrifugation. The supernatant solution and the precipitate were dialyzed, and then lyophilized to give Fractions D-I (104 mg) and D-II (46 mg), respectively. Electrophoresis (0.1M HCl) showed that the respective major components of Fractions D-I and D-II were Polysaccharides Hc-s and Hc-f.

In a preliminary test for blood-anticoagulant activity (thrombin time) with human plasma by the method of Denson and Bonnar[10], the respective activities of Fractions B-II-2, D-I, and D-II were 1, 4, and 27 $IU.mg^{-1}$, relative to a standard of heparin (167 $IU.mg^{-1}$). Fraction C-II-1 was inactive.

Purification of Fractions D-I and D-II. — A solution of Fraction D-I (275 mg) in 0.7M NaCl (1% w/v) was chromatographed on a column of DEAE-cellulose (DE-23, 1.9 × 38.5 cm, Cl^-) with stepwise elution with 0.7, 0.8, 0.9 (800 mL each), and 2.0M NaCl (400 mL). Each eluate was collected, dialyzed, and lyophilized to give 69.5, 63.3, 95.4, and 45.9 mg, respectively. Electrophoresis (0.1M HCl) showed that the fraction eluted with 0.9M NaCl contained the most homogeneous Poly-

TABLE I

CHEMICAL AND PHYSICAL PROPERTIES OF POLYSACCHARIDES pD-I AND pD-II[a]

Properties	*Polysaccharide*	
	pD-I	*pD-II*
$[\alpha]_D$ (degree)	−119	−103
N (%)	3.3	2.1
Fucose (%)	44.9	29.1
Uronic acid (%)	2.3	1.0
Sulfate (%)	26.2	31.6
Neutral sugars[b]		
Fuc	67	77
Xyl	trace	2
Man	2	11
Gal	31	11
Mol. wt.[c]	42 000	95 000

[a]Not corrected for ash content. [b]By g.l.c. [glass column (3 mm × 2 m) of 2% of XF-1105 coated on Gaschrom P at 125°] considering the total area under the four peaks as 100%. [c]Estimated by gel-filtration chromatography on a Sepharose CL-4B column (1.2 × 96.0 cm).

TABLE II

BLOOD-ANTICOAGULANT ACTIVITY OF POLYSACCHARIDES pD-I AND pD-II

Polysaccharide	*Activity test*[a]		
	APTT	*TT*	*Anti-factor Xa*
pD-I	16	14	<1
pD-II	16	36 (≦213 μg/mL) >36 (>213 μg/mL)	<1

[a]Expressed as units mg^{-1} in relation to that of heparin (167 units mg^{-1}) as a standard.

saccharide Hc-s. Thus, a solution of the fraction (95 mg) in 0.2M NaCl (10 mL) was further chromatographed on a column of Sepharose 4B (5.0 × 87.3 cm) with 0.2M NaCl as the eluent. Fractions (15 mL each) were monitored for carbohydrates by the phenol–H_2SO_4 reaction[11]. Eluates of fraction Nos. 70–91 were combined, dialyzed, and lyophilized to give Polysaccharide pD-I (75 mg). A solution of Fraction D-II (92.4 mg) in 0.7M NaCl (1% w/v) was chromatographed on the same column of DEAE-cellulose, as described above, with stepwise elution with 0.7, 0.95, 1.1 (800 mL each), and 2.0M NaCl (400 mL). Each eluate was collected, dialyzed, and lyophilized to give 14.0, 22.4, 49.7, and 6.3 mg, respectively. Electrophoresis (0.1M HCl) indicated that the fraction eluted with 1.1M NaCl contained the most homogeneous Polysaccharide Hc-f. A solution of this fraction (56 mg) was chromatographed on a Sepharose 4B column with 0.2M NaCl as the eluent in the same way as described above. Eluates of fraction Nos. 77–92 (15 mL each) were combined, dialyzed, and lyophilized to give Polysaccharide pD-II (48 mg). Rechromatography of fractions containing Polysaccharides pD-I and pD-II each (1–3 mg) gave a symmetrical peak by gel filtration on a Sepharose CL-4B column (1.2 × 96.0 cm) with 0.2M NaCl as the eluent. The homogeneity of the two fractions were further examined by electrophoresis. Under each condition of electrophoresis [0.1M pyridine–acetic acid buffer, pH 3.5 (ref. 8), 0.1M HCl, and 0.1M zinc acetate, pH 6.6], Polysaccharides pD-I and pD-II each behaved as a single component. These results indicated that the two fractions contained purified polysaccharides.

Characterization of polysaccharides pD-I and pD-II. — Analysis of the constituents showed that these two polysaccharides differ considerably from each other in their content of fucose[12], sulfate[13], uronic acid[14], nitrogen, and neutral sugar (Table I). The neutral sugar composition was determined by g.l.c. analysis of the alditol trifluoroacetate derivatives[15] after acid hydrolysis[16]. The uronic acid was identified by t.l.c.[17] as glucuronic acid after acid hydrolysis in the same way as described above. As shown in Table I, the glucuronic acid content was found to be very low in both polysaccharides. The strongly negative rotation of the two polysaccharides suggested that their anomeric linkages are predominantly α-L. The molar ratios of Fuc, Gal, Man, Xyl, GlcUA, and ester sulfate are 100:42:3:trace:4:100 and 100:6:6:1:3:186 for Polysaccharides pD-I and pD-II, respectively.

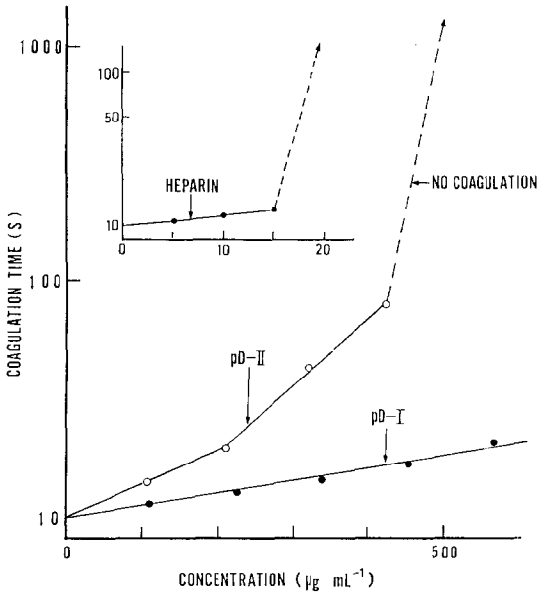

Fig. 2. Effect of Polysaccharides pD-I, pD-II, and heparin on human plasma coagulation (TT).

Anticoagulant activity of Polysaccharides pD-I and pD-II. — The anticoagulant activity was examined for human plasma by use of activated partial thromboplastin time (APTT) (ref. 18), thrombin time (TT) (ref. 10), and anti-factor Xa (ref. 19) assays (Table II). Both polysaccharides showed the same activity with respect to APTT. However, in the TT test, the activity of Polysaccharide pD-II (22% that of heparin) was higher than that of Polysaccharide pD-I (8% that of heparin), indicating that the former was the most active polysaccharide of the sulfated polysaccharides of *H. fusiforme*. It showed markedly prolonged clotting times in the TT test in the concentration range of more than 213 $\mu g.mL^{-1}$, about six times that in the range of below 213 $\mu g.mL^{-1}$. Furthermore, at concentrations over 532 $\mu g.mL^{-1}$, no coagulation occurred until after 24 h (Fig. 2). These results suggest that the activity of Polysaccharide pD-II in the TT test may be dependent upon its concentration. Heparin showed a similar pattern, but coagulation of plasma occurred after 6 h in the presence of heparin (40 $\mu g.mL^{-1}$). Similar results have been reported for the SPSs of marine brown algae, but only for fucoidin from *F. vesiculosus* in the whole blood coagulation inhibition test[20]. On the other hand, the activity (TT) of Polysaccharide pD-I was unchanged at all the concentrations tested (<567 $\mu g.mL^{-1}$).

In the present study, it was found that the anticoagulant-active substances of *H. fusiforme*, pD-I and pD-II, are both polysaccharides composed of Fuc, Gal, Man, GlcUA, and ester sulfate. For Polysaccharide pD-I, the major constituents are Fuc, Gal, and ester sulfate, and the molar ratio of Gal to Fuc is very low, indicating that the polysaccharide may be considered a galactofucan sulfate, though

it contains very small proportions of Man and GlcUA. The major constituents of Polysaccharide pD-II are Fuc and ester sulfate indicating that it is a fucan sulfate, though the polysaccharide contains very small proportions of Gal, Man, and GlcUA.

The activity of Polysaccharide pD-II was found to be much higher than that of pD-I, but the relationship between composition and anticoagulant activity remains to be clarified. In Polysaccharide pD-I, the content of Gal is much higher than in Polysaccharide pD-II, and the reverse for the sulfate content, suggesting that the difference in Gal and sulfate contents between the two polysaccharides may be partly related to their anticoagulant activities. Mori *et al.*[7] suggested a similar relationship for the sulfated polysaccharides from *Undaria pinnatifida*.

REFERENCES

1 A. F. ABDEL-FATTAH, M. M.-D. HUSSEIN, AND H. M. SALEM, *Carbohydr. Res.*, 33 (1974) 9–17.
2 A. F. ABDEL-FATTAH, M. M.-D. HUSSEIN, AND T. FOUAD, *Phytochemistry*, 17 (1978) 741–743.
3 M. M.-D. HUSSEIN, A. ABDEL-AZIZ, AND H. M. SALEM, *Phytochemistry*, 19 (1980) 2131–2132.
4 N. V. S. A. P. RAO, K. V. SASTRY, AND E. V. RAO, *Phytochemistry*, 23 (1984) 2531–2533.
5 G. BERNARDI AND G. F. SPRINGER, *J. Biol. Chem.*, 237 (1962) 75–80.
6 T. USUI, K. ASARI, AND T. MIZUNO, *Agric. Biol. Chem.*, 44 (1980) 1965–1966.
7 H. MORI, H. KAMEI, E. NISHIDE, AND K. NISIZAWA, *Mar. Algae Pharm. Sci.*, 2 (1982) 109–121.
8 T. NISHINO, G. YOKOYAMA, K. DOBASHI, M. FUJIHARA, AND T. NAGUMO, *Carbohydr. Res.*, 186 (1989) 119–129.
9 T. NISHINO AND T. NAGUMO, *Nippon Nogeikagaku Kaishi*, 61 (1987) 361–363.
10 K. W. E. DENSON AND J. BONNAR, *Thromb. Diath. Haemorrh.*, 30 (1973) 471–479.
11 J. E. HODGE AND B. T. HOFREITER, *Methods Carbohydr. Chem.*, 1 (1962) 380–394.
12 H. M. GIBBONS, *Analyst.*, 80 (1955) 268–276.
13 Y. KAWAI, N. SENO, AND K. ANNO, *Anal. Biochem.*, 32 (1969) 314–321.
14 T. BITTER AND H. M. MUIR, *Anal. Biochem.*, 4 (1962) 330–334.
15 T. IMANARI, Y. ARAKAWA, AND Z. TAMURA, *Chem. Pharm. Bull.*, 17 (1969) 1967–1969.
16 A. J. MIAN AND E. PERCIVAL, *Carbohydr. Res.*, 26 (1973) 133–146.
17 N. IIZIMA, M. FUJIHARA, AND T. NAGUMO, *J. Chromatogr.*, 193 (1980) 464–469.
18 L.-O. ANDERSON, T. W. BARROWCLIFFE, E. HOLMER, E. A. JOHNSON, AND G. E. C. SIMS, *Thromb. Res.*, 9 (1976) 575–583.
19 A. N. TEIEN AND M. LIE, *Thromb. Res.*, 10 (1977) 339–410.
20 G. F. SPRINGER, H. A. WURZEI, G. M. MCNEAL, JR., N. T. ANSELL, AND M. F. DOUGHTY, *Proc. Soc. Exp. Biol. Med.*, 94 (1957) 404–409.