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Isolation and partial characterization of a novel amino sugar-containing fucan sulfate from commercial *Fucus vesiculosus* fucoidan †

Takashi Nishino *, Chie Nishioka, Hiroaki Ura and Terukazu Nagumo

Department of Biophysics, School of Hygienic Sciences, Kitasato University, 1-15-1 Kitasato, Sagamihara, Kanagawa 228 (Japan)

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ABSTRACT

Commercial crude fucoidan (Sigma) from the brown seaweed *Fucus vesiculosus* was fractionated into its polysaccharide components by gel filtration and anion-exchange chromatography to clarify the structure–anticoagulant activity relationship. The products comprised a wide spectrum of fucans ranging from typical fucoidans (major components) containing mainly fucose, sulfate, and no uronic acid to low sulfate-containing heteropolysaccharide-like fucans (minor components) being composed of neutral sugars other than fucose and a high content of uronic acid(s). The polysaccharide components also had a wide range of molecular weight. The typical fucoidans showed a potent anticoagulant activity, whereas the other fucans had no or only slight activity. One of the fractions found as a minor component, was a novel polysaccharide containing an appreciable amount (11.5%) of glucosamine and a small amount (5.2%) of protein in addition to fucose and sulfate, and having a low apparent molecular weight of 6800. This is the first report that a proteoglycan-like, amino sugar-containing fucan sulfate, composed of fucose, galactose, glucose, mannose, xylose, uronic acid, glucosamine, and sulfate in the molar ratio of 1.00:0.04:0.01:0.48:0.24:0.18:0.56:1.90, could be obtained from brown seaweed. However, this polysaccharide showed no anticoagulant activity.

INTRODUCTION

Since Bernardi and Springer found[1] that a fucan sulfate (fucoidan) from the brown seaweed *Fucus vesiculosus* showed a potent anticoagulant activity, various fucan sulfates showing anticoagulant activity were isolated[2–6] from several brown seaweeds. The fucan sulfates are unique polysaccharides not occurring in other seaweeds or land plants, and composed mainly of fucose and sulfate, with smaller portions of uronic acid, galactose, mannose, and xylose. A fucoidan fraction prepared from *F. vesiculosus* is available from Sigma Chemical Co. The fucoidan

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* Corresponding author.

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fraction has been studied for biological activities such as antithrombin activity[7], human T-cell mitogen activity[8], anti-allergic encephalomyelitis activity[9], and anti-human immunodeficiency virus activity[10], and many investigators are interested in the fraction as a biologically active polysaccharide. It is known[11–13] that the main structure of the fucoidan is that of a 2)-α-L-fucopyranose-(1 → polymer with sulfate groups located at C-4. However, in the present study, the commercial fucoidan was shown to contain a wide spectrum of polysaccharides (by electrophoresis and gel filtration chromatography) suggesting that the fucoidan fraction consisted of several structurally different polysaccharide components. In order to clarify the relationship between the structure and the biological activity of the polysaccharide components, we attempted to isolate each of the polysaccharide components from the crude commercial fucoidan by gel filtration and anion-exchange chromatography. We describe herein the physical and chemical properties, and anticoagulant activity of the subfractions obtained. Preliminary characterization of a novel amino sugar-containing fucan sulfate is also reported, which was obtained during the fractionation of the fucoidan fraction.

EXPERIMENTAL

Materials.—Fucoidan (Lot, 110H3854 and 11H3901) from the brown seaweed *Fucus vesiculosus* was obtained from Sigma Chemical Co. Heparin (167 units/mg) from porcine intestinal mucosa was purchased from Wako Pure Chemical Industries, Ltd., Japan. Sepharose 4B and CL-6B and Sephadex G-50 were purchased from Pharmacia Fine Chemicals, and Ecteola-cellulose from Serva FeinBiochemica. Normal human plasma was obtained from Baxter Healthcare Co., and human thrombin (500 NIH units) from Green Cross, Ltd. Japan. Actin, which contained ellagic acid and phospholipid from rabbit, was purchased as activated partial thromboplastin time (APTT) reagent from American Hospital Supply del Caribe Inc.

General.—The carbohydrates in column eluates were monitored by the phenol–H_2SO_4 method[14]. Nitrogen content was determined with an elemental analyzer, Shimadzu Analyzer Model 240, 6-deoxysugar content by the method of Gibbons[15] using L-fucose as a standard, uronic acid content by a modified carbazole method[16] using D-glucuronic acid as a standard, and sulfate content by the modified method[17] of Dodgson and Price[18]. Amino sugar content was determined by the method of Gardell[19] after the polysaccharide was hydrolyzed with 3 M HCl at 100°C for 15 h. Protein was estimated by the method of Lowry et al.[20] with bovine serum albumin as a standard. Sulfated polysaccharides were hydrolyzed[21] with 90% formic acid for 6 h at 100°C, followed by addition of water (5 vol) and heating for 2 h at 100°C. The acid hydrolyzates were analyzed[22] by TLC on silica gel (Replate 50, 0.2-mm layers, Yamato Scientific Co. Ltd., Japan) after impregnation of the plates with phosphates as follows: impregnant 0.5 M NaH_2PO_4, developing solvent 16:1:3 2-propanol–MeOH–H_2O for neutral sugars; impregnant 0.3 M

NaH_2PO_4, developing solvent 5:1:1:2 EtOH–phenol–pyridine–0.1 M phosphoric acid for uronic acids. Detection was effected with diphenylamine–aniline–phosphoric acid[23]. The hydrolyzate containing neutral and amino sugars and uronic acids was converted into alditol acetates by the method of Jones and Albersheim[24]. The components were reduced to alditols (from neutral and amino sugars) and aldonic acids (from uronic acids) by treatment with $NaBH_4$ in 0.25 M NH_4OH. The alditols and the aldonic acids were separated by the addition of Dowex 1-X8 (AcO^- form) resin after removal of sulfate with barium carbonate. Unbound alditols were then acetylated with acetic anhydride at 121°C for 3 h. The aldonic acids were eluted from the resin with 1 M HCl, and the HCl-eluate was evaporated to dryness at 40°C, converting the aldonic acids to aldonolactones. The aldonolactones were reduced with $NaBH_4$ to the corresponding alditols, dried and acetylated. Alditol acetates were analyzed by GLC on a Hitachi G-3000 gas chromatograph equipped with a Neutra Bond-1 (0.4-μm film thickness, 25 m × 0.25 mm i.d., GL Sciences Inc., Japan) in a splitless mode. The flow rate of nitrogen as the carrier gas was 0.9 mL/min. The oven temperature was programmed at 60°C for 1 min, 30°C/min to 180°C and then 3°C/min to 250°C. Molar ratios of sugars were calculated from the peak areas of the corresponding alditol acetates by using their molecular weights.

Electrophoresis was performed on a cellulose acetate membrane (6 × 11 cm and 9 × 12 cm, Fuji Film Co. Ltd., Japan). HPLC was performed on a Jasco Tri Rotar-V equipped with a Shodex RI SE-31 detector. The molecular weight of each polysaccharide was estimated by HPLC on Asahipak GS-510 (7.6 mm i.d. × 500 mm) + GS-310 (7.6 mm i.d. × 500 mm) columns (Asahi Chemical Industry Co. Ltd., Japan) equilibrated with 0.4 M NaCl by using a calibration curve obtained with pullulans (Shodex standard P-82, Showa Denko Ltd., Japan) and laminara-heptaose and -tetraose (Seikagaku Kogyo Co., Ltd., Japan) as molecular weight standards. The anticoagulant activities of polysaccharides were determined for human plasma, with respect to activated partial thromboplastin time (APTT)[25], with an actin reagent, to prothrombin time (PT)[26] with a thromboplastin, and to thrombin time (TT)[27] with human thrombin as described previously[28]. The clotting time was recorded with a Coag-Stat Super BC-2230 (International Reagents Co., Japan) for APTT and PT assays and an Amelung Coagulometer KC 1A for TT assay. The activity was expressed as units/mg in relation to that of heparin (167 units/mg) as a standard.

Fractionation of a commercial fucoidan.—Gel filtration chromatography. The commercial fucoidan (Sigma) from the brown seaweed *F. vesiculosus* (~ 1 g in 50 mL of 0.2 M NaCl) was applied to a Sepharose 4B column (5 × 83 cm) equilibrated with 0.2 M NaCl and eluted with the same solution at room temperature at a flow rate of ~ 1.2 mL/min. Fractions (14.5 mL) were collected. As shown in Fig. 1A, the eluates were collected as separate subfractions, dialyzed, and then lyophilized to give subfractions I (~ 138 mg), II (~ 183 mg), and III (~ 582 mg). Fractions I and II were rechromatographed (0.2 M NaCl, 6.0 mL/fr., 0.4 mL/min) on a

Sepharose CL-4B column (2.64 × 95 cm), and both fractions were recovered in yields of 80%. Fraction III was further fractionated (0.2 M NaCl, 14 mL/fr., 1.3 mL/min) on a column (5 × 86 cm) of Sepharose CL-6B to give a high molecular weight fraction III-1 (~ 484 mg) and a low molecular weight fraction III-2 (~ 53 mg) as shown in Fig. 1B. Fractions III-1 and III-2 were rechromatographed on a Sepharose CL-6B column (5 × 86.5 cm) and a Sephadex G-50 column (2.64 × 94.5 cm), respectively. The respective major fractions were recovered in yields of 86% for III-1 and 76% for III-2.

Anion-exchange chromatography. As a preliminary experiment, fractions I, II, and III-1 were each first chromatographed on an Ecteola-cellulose (Cl^-) column by eluting with a linear gradient of 0 → 2 M NaCl. On the basis of these results, fractions I, II, and III-1 were fractionated by chromatography on Ecteola-cellulose (Cl^-) with stepwise elution as follows. Fraction I (109 mg) was applied to a column 1.9 × 41 cm which was then eluted stepwise with 0.1 M (250 mL, Fraction $I_{0.1}$), 0.45 M (250 mL, Fraction $I_{0.45}$), 0.8 M (250 mL, Fraction $I_{0.8}$), 1.8 M (250 mL, Fraction $I_{1.8}$), and 2 M NaCl (300 mL, Fraction I_2) successively until the eluates were free from carbohydrates. All the eluates of each fraction were combined, dialyzed, and lyophilized. The yield of fractions $I_{0.1}$, $I_{0.45}$, $I_{0.8}$, $I_{1.8}$, and I_2 was 1, 7, 4, 67, and 4%, respectively. Fraction II (145.4 mg) was fractionated in a similar way on a column (1.9 × 42 cm) by stepwise elution with 0.1 M, 0.5 M, 0.8 M, 1.15 M, 1.35 M, and 2 M NaCl to give the respective fractions $II_{0.1}$ (1.2 mg), $II_{0.5}$ (5.2 mg), $II_{0.8}$ (5.6 mg), $II_{1.15}$ (25.81 mg), $II_{1.35}$ (50.8 mg), and II_2 (52.3 mg). Fraction III-1 (481.1 mg) was also fractionated on the same column (2.64 × 39 cm) by eluting stepwise with 0.5 M (Fraction III-$1_{0.5}$, 50.7 mg), 0.7 M (Fraction III-$1_{0.7}$, 26.1 mg), 0.9 M (Fraction III-$1_{0.9}$, 41.2 mg), 1.5 M (Fraction III-$1_{1.5}$, 232.8 mg), and 2 M NaCl (Fraction III-1_2, 36.3 mg). On the other hand, fraction III-2 (37.9 mg) was applied to a column (1.5 × 28 cm) of Ecteola-cellulose (Cl^-). The column was first washed with water to give a nonadsorbed fraction (Fraction III-2N, 1.3 mg) and then developed with a linear gradient of 0 → 1.4 M NaCl (440 mL) to elute the adsorbed acidic fraction (Fraction III-2A, 18.1 mg).

RESULTS AND DISCUSSION

Fractionation of a commercial fucoidan.—A commercial crude fucoidan, which was prepared from the brown seaweed *F. vesiculosus* by the modified method of Black[29], was purchased from Sigma Chemical Co. as a brownish powder. The patterns of electrophoresis (Fig. 1) and of HPLC (gel filtration, Fig. 2) of the crude commercial fucoidan showed a long broad band and broad peaks with at least more than three different apparent molecular weight (M_{app} > 680 000, 190 000, and 6 600) suggesting that the fucoidan consists of different polysaccharide components with respect to charge density and molecular weight. The first peak (M_{app} > 680 000) was assumed to be a void volume peak typically observed in gel filtration chromatograms of polydisperse materials. The crude fucoidan consisted of fucose,

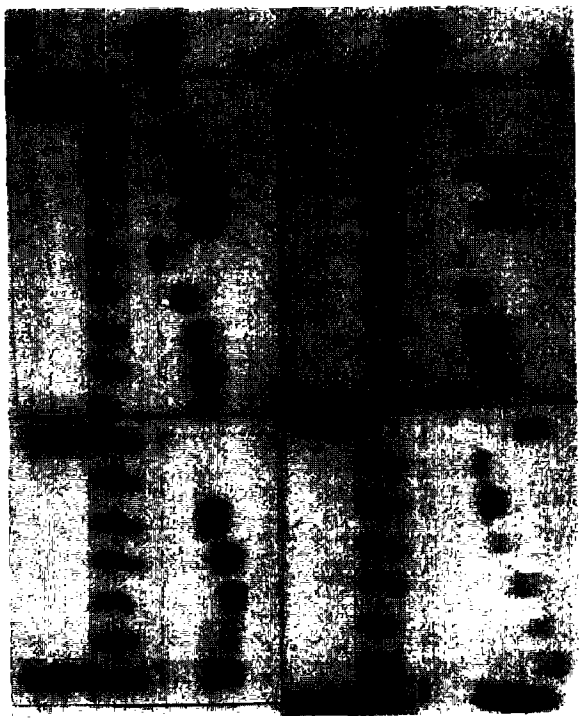

Fig. 1. Cellulose acetate membrane electrophoresis of a commercial crude fucoidan and its subfractions. A, 0.1 M HCl, 16.5 V, 2.5 h. B, 0.1 M zinc acetate (pH 6.6), 200 V, 1 h. Designation of the fractions is as explained in the text.

galactose, glucose, mannose, xylose, uronic acid, and sulfate in the molar ratio of 100:3:trace:2:4:20:120 (Table I). Proteins and amino sugars were scarcely detectable in the fucoidan by colorimetric methods. In order to isolate each polysaccharide component, the crude fucoidan was first fractionated by gel filtration. Fractions I, II, and III-1 (white powders), and III-2 (a yellowish powder) were obtained as shown in Fig. 3. They were further fractionated into the respective subfractions by anion-exchange chromatography through stepwise elution for fractions I, II, and III-1 (see Experimental section), and with a linear gradient of NaCl for III-2 (Fig. 3D). The subfractions $I_{0.1}$–I_2, $II_{0.1}$–II_2, III-$1_{0.5}$–III-1_2, and III-2N (a minor neutral polysaccharide fraction) and III-2A (a major acidic polysaccharide fraction) were obtained from fractions I, II, III-1, and III-2, respectively. Fraction III-2A was separated from the brownish material in this procedure. All the subfractions showed narrow bands compared with the crude fucoidan on electrophoresis (Fig. 1). Their mobilities differed from each other. Fractions $I_{0.45}$, $II_{0.5}$, and III-$1_{0.5}$ did not migrate from the origin on electrophoresis in 1 M HCl (Fig. 1A). However, fractions $I_{0.45}$, $II_{0.5}$, and III-$1_{0.5}$ migrated toward the anode on electrophoresis in 0.1 M zinc acetate buffer (pH 6.6) (Fig. 1B), suggesting that they may have a low sulfate and high uronic acid content. Fractions eluted with higher

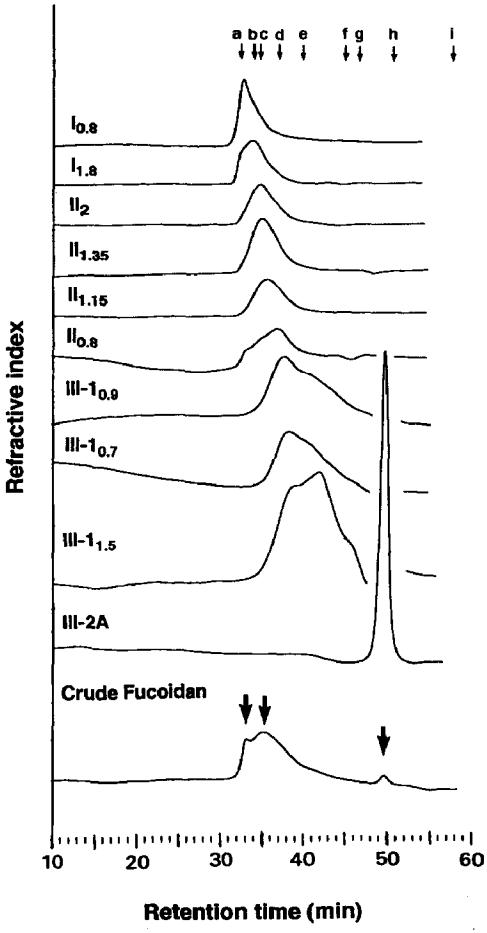

Fig. 2. HPLC of a commercial crude fucoidan and its subfractions on Asahipak GS-510+GS-310 (see text for details). The small arrows indicate retention times of the standards: a, pullulan P-800 (MW 758000); b, pullulan P-400 (338000); c, pullulan P-200 (194000); d, pullulan P-100 (95400); e, pullulan P-50 (46700); f, pullulan P-20 (20800); g, pullulan P-10 (12000); h, pullulan P-5 (5300); i, laminaraheptaose (1153).

concentrations of NaCl by anion-exchange chromatography migrated more than the others on a membrane in 0.1 M HCl, suggesting that they may contain larger amounts of sulfate than the others. Fractions $I_{1.8}$ and I_2 showed broad bands as shown in Fig. 1B. Fraction III-2A migrated faster than the other fractions (Fig. 1), suggesting that it may have a low molecular weight and/or high sulfate content.

Characterization of the fucans obtained from the crude fucoidan.—Sixteen polysaccharide fractions were obtained from the commercial crude fucoidan as described above. Total yield of fractions was only 58% of the starting fucoidan. The low yield may be caused by repeated chromatography for purification of the polysaccharide fractions and by removal of brownish materials in the fractions,

because the yields at each fractionation step were more than 80% of the starting materials. Since the yields of fractions $I_{0.1}$, $I_{0.45}$, $II_{0.1}$, and III-2N were very low (0.1–0.8%), their properties were not further determined except for the nitrogen contents (for $I_{0.1}$, $I_{0.45}$, and III-$1_{0.5}$) by elemental analysis. Further study of fraction III-$1_{0.5}$ was also impossible, because it became insoluble in water after it was dried by evaporation. Fractions $I_{0.1}$, $I_{0.45}$, and III-$1_{0.5}$ contained nitrogen at 1.8, 0.5, and 0.9%, respectively, indicating that they may be proteoglycan-like materials. The yields, and physical and chemical properties of the polysaccharide fractions obtained in the relatively high yields are shown in Table I. All the polysaccharides contained fucose as the major component sugar in addition to galactose and xylose as minor ones, indicating that they are fucans (sulfated), but the proportions of these sugar components vary from fraction to fraction. Fraction $II_{0.5}$ contained the largest amount of uronic acid but little sulfate. Fractions $II_{0.8}$, III-$1_{0.7}$, and III-$1_{0.9}$, which were eluted in lower concentrations of NaCl by anion-exchange chromatography, were richer in uronic acid and poorer in sulfate than the others. They also contained relatively large proportions of mannose and xylose. Furthermore, fraction III-$1_{0.9}$ contained 2.8% protein (0.45 N% × 6.25). On the other hand, fractions $I_{1.8}$, $II_{1.15}$, $II_{1.35}$, II_2, III-$1_{1.5}$, and III-1_2, which were eluted in higher concentrations of NaCl by chromatography, had a high sulfate content and low or no uronic acid content. These results indicated that the commercial crude fucoidan was comprised of a wide spectrum of fucose-containing polysaccharides (fucans) from typical fucoidans containing fucose, sulfate, and no uronic acid to low sulfate-containing heteropolysaccharide-like fucans being composed of neutral sugars other than fucose and high content of uronic acid(s). Similar results have been reported for fucan sulfates from several brown seaweeds[5,21,30]. The typical fucoidans were major components in the crude fucoidan. Uronic acids were identified by GLC of the corresponding alditol acetates. Fraction $I_{1.8}$ contained glucuronic acid only, whereas the others were composed mainly of glucuronic acid and also contained small proportions of mannuronic acid as shown in the other brown seaweeds[31,32]. However, galacturonic acid was not detected in any of the polysaccharide fractions. The molar ratios of sulfate to total sugar residues varied from almost nil in $II_{0.5}$ to 1.16 of II_2. In general, the ratios were higher in typical fucoidans than in heteropolysaccharides and also increased with increase in fucose content. The subfractions from fraction I had higher M_{app} (260 000–680 000) than fractions II (96 000–510 000) and III-1 (19 000–140 000), as expected from their chromatography on Sepharose 4B (Fig. 3A). The respective HPLC of fractions $I_{1.8}$, III-$1_{0.7}$, and III-$1_{0.9}$ showed a major peak at M_{app} 260 000 ($I_{1.8}$), 68 000 (III-$1_{0.7}$), and 79 000 (II-$1_{0.9}$) in addition to a shoulder corresponding to M_{app} 560 000, 45 000, and 49 000 (Fig. 2). Fraction $II_{0.8}$ contained a major peak at M_{app} 96 000 and a minor peak at M_{app} 510 000. The major fraction III-$1_{1.5}$ showed a broad peak on HPLC, indicating that it contains three polysaccharide components with different M_{app} (62 000 as medium, 33 000 as major, and 19 000 as minor). These results suggested that the two or three polysaccharide components in fractions $I_{1.8}$, $II_{0.8}$,

III-$1_{0.7}$, III-$1_{0.9}$, and III-$1_{1.5}$ might be different in molecular weight and the composition from one another. However, as fractions $I_{1.8}$ and III-$1_{1.5}$ are almost homopolymers composed of fucose and sulfate, it is suggested that they are comprised of polysaccharide components differing only in molecular weight. These results indicated that commercial crude fucoidan was heterogeneous with respect not only to molecular weight and sulfate contents but also sugar constituents.

Preliminary characterization of a novel amino sugar-containing fucan sulfate.— Fraction III-2A had the lowest M_{app} (6800) by HPLC compared with those of the other fucans described above and its HPLC pattern was a single, sharp symmetrical curve (Fig. 2). In chemical composition this fraction was also significantly different from the others (Table I). Fraction III-2A was composed mainly of fucose and sulfate, and contained, in addition, an appreciable amount of an amino sugar. The amino sugar was identified as glucosamine but not galactosamine by GLC (data not shown). However, in the present study, it could not be determined whether the glucosamine is *N*-acetylated. The fraction also contained large proportions of mannose and xylose compared with galactose and uronic acid. In addition, the fraction contained a small amount of protein. The molar ratio of sulfate-to-sugar residues was ~ 0.75. This is the first report on the presence of an amino sugar-containing sulfated polysaccharide in brown seaweeds, although three fucose-containing glycosaminoglycans and several hexosamine-containing sulfated galactan were isolated from the squid ink (*Illex argentinus*[33]) and from the tunic of ascidians[34], respectively. This result suggests the presence of a proteoglycan-like material in brown seaweeds as assumed by Percival and McDowell[35], because fraction III-2A contained a small amount of protein in addition to uronic acid and glucosamine. Abdel-Fattah and Edrees[36] also reported that a neutral glycoprotein containing mannose was obtained from the brown seaweed *Padina pavonia*. Since fraction III-2A also contained a relatively large amount of mannose compared with other fucans, the fraction may be a complex in which a fucan is linked to glycoprotein. The biochemical organization of fucans and the relationship between the fucans and the other components in cell walls have not been established yet although some data[35–38] have been published. The study on the structure of fraction III-2A is now in progress. Thereby, new information of the mode of existence of fucans in the brown seaweeds will be provided. Nevertheless, these results suggest that fraction III-2A may be a new type of fucan sulfate incorporating amino sugar residues in its structure.

Anticoagulant activity of the crude fucoidan and its subfractions.—The crude fucoidan and its subfractions were examined for anticoagulant activity with respect to APTT, TT, and PT (Table II). The specific activities of the fucoidan were 9 units/mg in APTT and 12 units/mg in TT, but nil in PT. As for APTT, the activity of fractions $I_{1.8}$, $II_{1.35}$, and III-$1_{1.5}$ was more potent than that of the crude fucoidan fraction, and fractions I_2, $II_{1.15}$, and III-1_2 showed activity similar to the fucoidan. On the other hand, fractions $I_{0.8}$, $II_{0.5}$, III-$1_{0.7}$, and III-2A were inactive. Similar results were obtained for TT. However, no activity was detected for any fractions

TABLE I

Analysis of a commercial crude fucoidan and its subfractions (fucan sulfates)

Component	Fucoidan	$I_{0.8}$	$I_{1.8}$	I_2	$II_{0.5}$	$II_{0.8}$	$II_{1.15}$	$II_{1.35}$	II_2	III-$1_{0.7}$	III-$1_{0.9}$	III-$1_{1.5}$	III-1_2	III-2A
Yield (%) [a]		0.4	7.3	0.5	0.5	0.6	2.6	5.1	5.3	2.2	3.6	20.0	3.1	1.0
10^{-4} M_{app}	> 68	> 63	56	> 68	29	51	14	16.5	17.5	6.8	7.9	6.2	14	0.68
	19		26			9.6				4.5	4.9	3.3		
	0.66											1.9		
6-Deoxy sugar (% as Fuc)	33.3	n.d. [b]	49.0	n.d.	24.6	35.8	52.1	62.7	49.7	28.0	45.1	60.3	47.2	18.9
Uronic acid (% as GlcA)	7.8	n.d.	~ 2.7	n.d.	38.3	18.0	3.9	[c]		22.1	10.6			4.0
Amino sugar (% as GlcN)	~ 0.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	11.5
Sulfate (%)	23.0	n.d.	32.5	n.d.	0.8	14.8	23.8	33.2	35.9	9.6	18.6	33.8	29.1	20.9
Protein (% as BSA)	~ 0.1 [d]	n.d.	[c,e]	n.d.	n.d.	[c,e]	[c,e]	[c,e]	[c,e]	n.d.	2.8 [e]	[c,e]	[c,e]	5.2 [d]
Molar ratios														
Fuc	1.00	(1.00) [f]	1.00	(1.00)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Gal	0.03	(0.02)	0.06	(0.08)	n.d.	0.01	0.03	0.05	0.05	0.03	0.03	0.05	0.05	0.04
Glc	trace	(0.02)	trace	(0.01)	n.d.	trace				0.06	0.01			0.01
Man	0.02	(0.12)	0.02	(0.03)	n.d.	0.10	0.01		trace	0.36	0.05	trace		0.48
Xyl	0.04	(0.20)	0.03	(0.03)	n.d.	0.25	0.04	0.01	0.01	0.47	0.13	0.02	trace	0.24
Uronic acid	0.20	n.d.	~ 0.05	n.d.	1.31	0.43	0.06			0.67	0.20			0.18
GlcN [g]					n.d.									0.56
Sulfate	1.20	n.d.	1.13	n.d.	0.03	0.57	0.78	0.91	1.23	0.57	0.71	0.96	0.98	1.90

[a] Percent by weight of the crude fucoidan. [b] Not determined. [c] Not detected. [d] Determined by the method of Lowry et al.[20]. [e] 6.25 × %N determined by nitrogen analysis. [f] In parentheses, calculated from GLC patterns, considering the total area under the five peaks as 100%. [g] Identified by GLC comparison with acetylated alditols from GlcN and GalN standards.

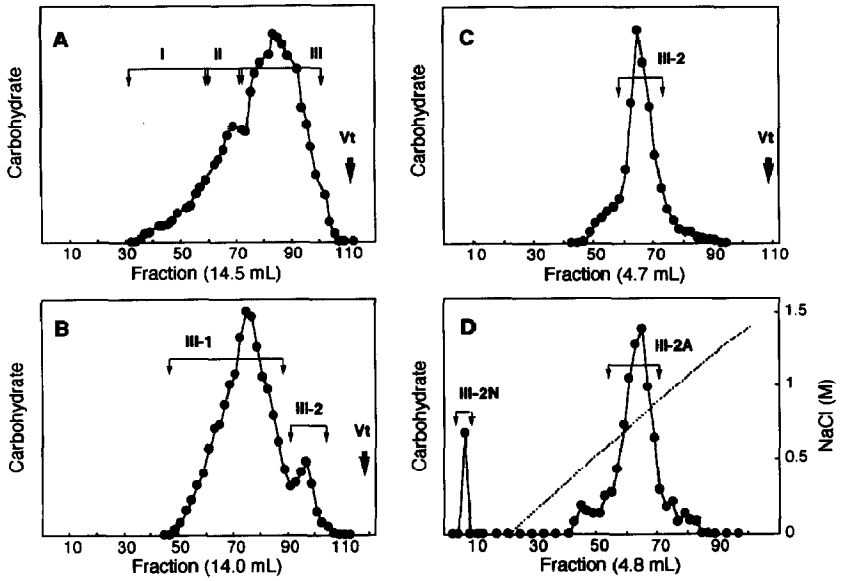

Fig. 3. (A) Gel filtration on Sepharose 4B of a commercial crude fucoidan; (B) gel filtration on Sepharose CL-6B of fraction III obtained by the chromatography shown in panel A; (C) gel filtration on Sephadex G-50 of fraction III-2 obtained by the chromatography shown in panel B; (D) anion-exchange chromatography on Ecteola-cellulose (Cl^-) of fraction III-2 obtained by gel filtration shown in panel C.

with respect to PT. These results indicate that the anticoagulant-active components in the crude fucoidan are fractions $I_{1.8}$, I_2, $II_{1.15}$, $II_{1.35}II_2$, III-$1_{1.5}$, and III-1_2. These results also indicate that the fucan sulfates (typical fucoidans), which were

TABLE II

Anticoagulant activity of a commercial fucoidan and its subfractions

	Anticoagulant activity (units/mg) [a]	
	APTT	TT
Fucoidan	9	12
$I_{0.8}$	[b]	2
$I_{1.8}$	13	10
I_2	7	12
$II_{0.5}$		
$II_{0.8}$	6	1
$II_{1.15}$	8	12
$II_{1.35}$	11	19
II_2	6	16
III-$1_{0.7}$		
III-$1_{0.9}$	6	6
III-$1_{1.5}$	12	18
III-1_2	9	15
III-2A		

[a] In relation to the activity of heparin (167 units/mg). [b] No activity.

composed of higher contents of fucose and sulfate, and lower contents of uronic acid and other neutral sugars, have a potent anticoagulant activity, and that the fucan sulfates (heteropolysaccharide types), which contained large amounts of uronic acid and neutral sugars other than fucose, and small amount of sulfate, have little activity. This result agrees with the results by Bernardi and Springer[1] that the highly purified fucan sulfates (typical fucoidans) from *F. vesiculosus* showed a potent anticoagulant activity. We have reported[39,40] that for a fucan sulfate with the same structure, the anticoagulant activity was dependent on the ratio of sulfate group to total sugar residues of the polysaccharide. Church et al.[7] reported that the antithrombin activity of a commercial fucoidan used in the present study arose by activation of heparin cofactor II. Sié et al.[41] also reported that highly charged polysaccharides may interact with the protease inhibitor in a poorly specific manner. However, the ratio (0.75) of sulfate to sugars of fraction III-2A was higher than those (0.31–0.68) of fractions $II_{0.8}$, $II_{1.15}$, and $III\text{-}1_{0.9}$, but the polysaccharide was nevertheless inactive. The ineffectiveness in the activity may be caused by low molecular weight and/or the chemical properties of the fraction, which is a proteoglycan-like, amino sugar-containing fucan sulfate. This suggests that the anticoagulant activity of fucan sulfates may correlate not only with sulfate contents of the polysaccharides but also with their structural conformation related to molecular weights and sugar constituents.

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