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Structural studies on fucoidans from the brown seaweed *Sargassum stenophyllum*

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Abstract

The brown seaweed *Sargassum stenophyllum* biosynthesizes two different sets of fucoidans. One of them is characterized by higher percentages of glucuronic acid and fewer sulfate groups, which are situated on different sugar units. α-L-Fucose was the major component but other sugars like β-D-galactose, β-D-mannose, α-D-glucuronic acid, α-D-glucose and β-D-xylose were also in substantial amounts. Fucoidans from the other set contain small amounts of α-D-glucuronic acid and high percentages of sulfate groups, which are concentrated on the fucose residues, with only fucose and galactose as major components. Structural studies of one fucoidan from each set suggest that these products have a general basic structure that has a formal resemblance to that of the fucosylated chondroitin sulfates from the body wall of sea cucumbers, namely, a linear core (formed mainly by (1→6)-β-D-galactose and/or (1→2)-β-D-mannose units) with branched chains of 'fucans' (formed by (1→3) and/or (1→4)-α-L-fucose, (1→4)-α-D-glucuronic acid, terminal β-D-xylose and, sometimes, (1→4)-α-D-glucose). In fucoidans from the second set, the 'core' is reduced to short galactan chains.

Keywords: Fucoidans; Fucans; Chemical structure; Sulfated heteropolysaccharides; Brown seaweed; *Sargassum stenophyllum*

1. Introduction

Brown seaweeds (Phaeophyceae) produce families of sulfated fucoidans[†] among other polysaccharides. They frequently contain other sugars besides L-fucose, namely, D-xylose, D-galactose and D-glucuronic acid; however, additional sugars, like D-mannose and D-glucose, have also appeared.

In particular circumstances, either fucose or galactose constitutes more than 95% of the component monosaccharides, as in the 'fucan' of *Fucus vesiculosus*[1] or the 'β-D-galactan sulfate' from *Laminaria angustata*.[2] Several biological activities for these polysaccharides have been examined,[1,3–8] but as the structure of fucoidans has not been elucidated, the relationship between structure and biological properties is far from being established. A study of fucoidans from the brown seaweed *Sargassum stenophyllum*, is reported herein.

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[†] 'Fucoidan' will be used as a general term for all the L-fucose-containing polysaccharides from brown seaweeds, while 'fucan' will be reserved for the polysaccharides built up mostly (>95%) with L-fucose.

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2. Experimental

Collection of specimens.—The brown seaweed, *S. stenophyllum*, was collected in Bombinhas (Santa Catarina, Brazil, February 1996). It was cleaned by hand and washed with sea water and then with tap water.

Analytical methods.—Total carbohydrates were determined by the phenol–H_2SO_4 method using fucose to construct the standard curve.[9] Uronic acids were determined by the procedure of Blumenkrantz and Asboe-Hansen with glucuronic acid as the standard.[10] Sulfates were determined by the method of Dodgson and Price,[11] and protein by the method of Lowry.[12] The standard deviation for the colorimetric determinations was estimated up to 5%.

Hydrolysis of the polysaccharides was performed with 1 M TFA (3 h, 100 °C). GLC analyses of the neutral sugars as their alditol acetates were carried with an HP-5890 gas chromatograph equipped with a flame-ionization detector (FID), using a fused silica capillary column (30 m × 0.25 mm) coated with DB-225. Chromatography was run isothermally at 220 °C. Both injector and FID temperatures were 250 °C. Nitrogen was used as the carrier gas at a flow rate of 1 mL/min and a split ratio of 100:1.

Methylated alditol acetates were analyzed with the same GLC system used for monosaccharide composition analysis except that a gradient of temperatures (180 °C for 2 min, then 180–210 °C at 1°C/min and 210–230 °C at 2 °C/min) was used with an SP2330 capillary column. Analyses on a DB-225 column were conducted isothermally at 210 °C. GLC–MS analyses were performed using a Varian 3300 chromatograph and a Finnigan Mat ITD spectrometer. The chromatograph was programmed to run at 50 °C for 1 min, then 50–220 °C at 40 °C/min. Helium was used as the carrier gas at 1 mL/min.

For NMR analysis the lyophilized sample was dissolved in D_2O (20–40 mg/mL). NMR spectra of solutions were recorded at 50 °C using a Bruker Avance DRX 400 NMR spectrometer, equipped with a 5-mm multinuclear inverse detection probe. Chemical shifts are expressed in ppm by reference to an external standard (Me_4Si). The polysaccharide sample was analyzed by 1D ^1H and ^{13}C NMR spectroscopy and also by DEPT (DEPT135) and HMQC routines that were carried out using the pulse programs supplied with the instrument. FTIR spectra of the polysaccharides were recorded using KBr pellets on a Shimadzu FTIR 8300 instrument scanning between 4000 and 400 cm^{-1}. Optical rotations of aqueous solutions of the polysaccharide samples (0.2%), were measured at 20 °C, using a 10-cm cell and the sodium D line (589.3 nm) with a Rudolph Autopol III automatic polarimeter.

Extraction.—The powdered seaweed was extracted with water under mechanical stirring at rt (7% w/v mL, 12 h, 3 ×) and centrifuged. The supernatant was concentrated, dialyzed against distilled water, and centrifuged, and the residue was discarded. The polysaccharides were precipitated with EtOH (3 vols) and sequentially treated with $CaCl_2$ and further precipitated with cetylpyridinium chloride (pH 7).[13] The pyridinium salts were solubilized with 3 M $CaCl_2$ and reprecipitated with EtOH. The ethanol-insoluble material was suspended in water and centrifuged to produce an insoluble and a soluble fraction (SF, yield 0.4% after dialysis against 0.5 M NaCl and water) (Fig. 1). The insoluble salts were solubilized after dialysis against EGTA (1%, pH 7.0), 0.5 M NaCl and water giving IF (yield 0.3%) (Fig. 1). Analysis and monosaccharide composition of SFs are given in Table 1. Fraction IF was kept for further work.

Fractionation of the SF fucoidan fraction.—A 10% solution of cetyltrimethylammonium bromide (34.5 mL) was slowly added, under magnetic stirring, to SF (3.32 g/335 mL of water). After 12 h stirring, the cetyltrimethylammonium salts were centrifuged off and suspended in water (345 mL). Stepwise addition of powdered solid NaCl until 0.5 M concentration produced a supernatant that was extracted with 1-pentanol (3 × 60 mL) dialyzed using membranes with molecular weight cut-offs (MWCO) of 12,000–14,000 Da, and freeze-dried (F1). The remaining precipitate was sequentially submitted to similar procedures with NaCl at concentrations of 1.0, 1.5, 2.0, 3.0, and 4.0 M, producing the fucoidan

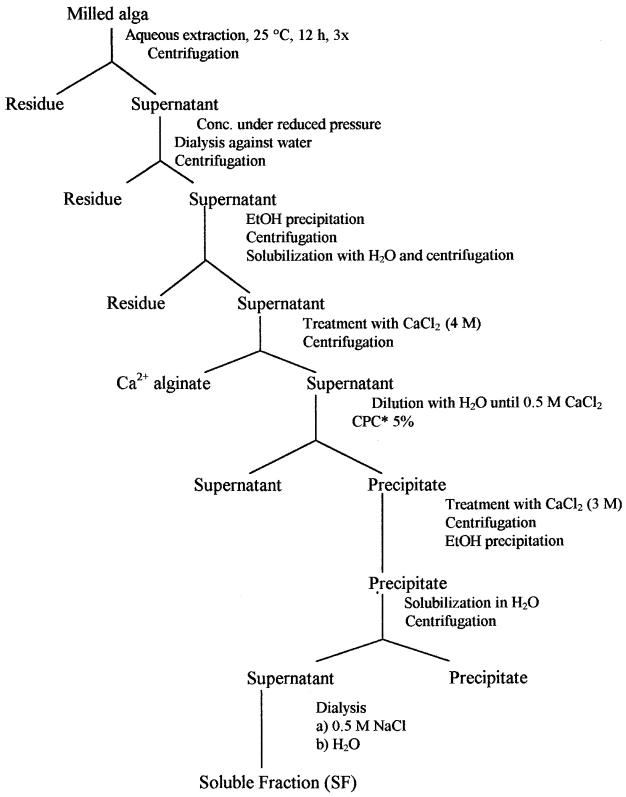
Table 1
Yield, analysis, optical rotation and monosaccharide composition of the extract SF and the fractions obtained, through redissolution in sodium chloride, of the cetyltrimethylammonium salts of SF

Fraction	Range of redissolution (1 M NaCl)	Yield [a] (%)	Carbohydrate (%)	Uronic acid (%)	Sulfate ($NaSO_3$) (%)	Protein (%)	$[\alpha]_D^{25}$ (%)	FTIR (cm^{-1})	Fuc (mol%)	Xyl (mol%)	Man (mol%)	Gal (mol%)	Glc (mol%)
SF		0.4	56.6	3.5	19.0	2.0	n.d.		67.8	16.1	1.2	13.6	tr. [b]
F1	0.5–1	8.5 (5.1)	66.3	17.3	17.7	9.6	−15.0	819.0	35.5	28.0	5.0	20.7	10.8
F2	1.0–1.5	19.7 (11.7)	78.3	11.0	18.8	11.1	−27.9	817.8	60.0	9.0	9.9	21.0	tr.
F3	1.5–2	19.7 (11.7)	68.0	10.2	20.7	11.8	−31.8	821.6	52.4	6.6	16.6	23.4	tr.
F4	2–2.5	12.7 (7.6)	59.6	10.1	22.4	12.5	−21.8	820.0	46.4	13.6	6.2	29.6	4.3
F5	2.5–3.0	24.2 (14.4)	73.8	2.5	28.5	7.5	−50.3	837.0	59.6	4.8	1.9	31.5	2.1
F6	3.0–4.0	3.6 (2.1)	54.0	1.7	28.3	6.7	−21.4	837.0	65.0	3.0	2.0	28.6	tr.

[a] In parenthesis, percentages of the recovered material. Yield of the fractionation 60%.
[b] Percentages less than 1.5% are considered as traces.

fractions F2–F6 (Table 1). The final insoluble material was suspended in water and freeze-dried (F7).

Gel-permeation chromatography on Sepharose 2B.—Fractions F3 (375 mg) and F5 (458 mg) were chromatographed through Sepharose 2B using 0.1 M NaCl as eluent. The elution was monitored with the phenol–H_2SO_4 reaction, and the void volume was marked using Blue Dextran. F3B and F5B were, respectively, selected (Fig. 2) for further work.



* CPC: Cetyl pyridinium chloride

Fig. 1. Extraction and purification scheme of the fucoidan fraction (SF) from *S. stenophyllum*.

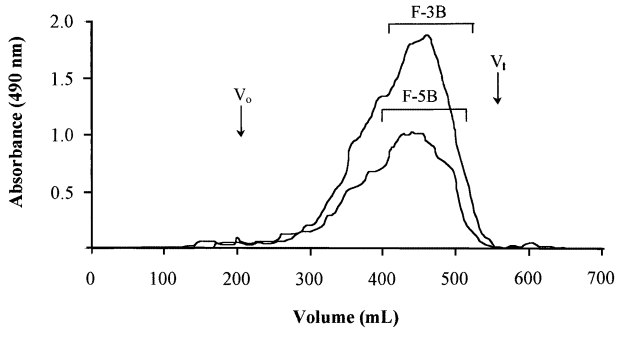


Fig. 2. Elution profile on Sepharose 2B of fractions F3 and F5.

Desulfation.—Fraction F3B was submitted to solvolysis in 80:10:1 Me_2SO–MeOH–pyridine[14] at 105 °C, for 30 min producing F3B-30 (yield 95%) and 3 h (F3B-3, yield 56%). When the reaction was carried out with F5B, partially desulfated products were obtained after 20 min (F5B-20, yield 73%) and after 4 h (F5B-4, yield 20%). The partially desulfated fractions were dialyzed against distilled water using membranes of MWCO 3,500 Da.

Carboxyreduction.—Fucoidan F3B and its products of solvolysis (F3B-30 and F3B-3) were reduced using $NaBD_4$ and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulfonate[15] producing F3B-R, F3B-30R and F3B-3R. Buffers MES and TES[16] were used to maintain the pH.

Methylation analysis.—Fucoidan fractions F3B-R, F3B-30R, F3B-3R, F5B, F5B-20 and F5B-4 as triethylammonium salts[17] were methylated according to the method of Ciucanu and Kerek.[18] Three additions of NaOH and CH_3I were carried out in each step, and two–three steps were used in each case. The permethylated fucoidans were hydrolyzed with 45% formic acid (16 h, 100 °C), and the mixture of partially methylated sugars was reduced with $NaBH_4$ or $NaBD_4$ and derivatized to the acetylated alditols.

Assay for anticoagulant activity.—Anticoagulant activities of the fucoidans were determined using the activated partial thromboplastin time (APTT)[19] and thombin time (TT)[20] assays, using heparin (150 units/mg) as standard and fucoidans in various concentrations (25–200 μg/mL). Data are provided in Table 2.

3. Results

Extraction.—A fucoidan was extracted from *S. stenophylum* with water at room temperature and purified through ethanol precipitation and further elimination of the alginic acid as its calcium salt. The sulfated polysaccharides were precipitated with cetylpyridinium chloride, and centrifuged, and the

pyridinium salts were redissolved in 3 M $CaCl_2$ (Fig. 1). The salt solution was reprecipitated with ethanol, and the ethanol-insoluble material was treated with water at room temperature to produce a solution, which after dialysis and lyophilization yielded SF (Fig. 1), whose analysis and monosaccharide composition are given in Table 1. The insolubles from the precipitation with cetylpyridinium chloride and redissolution with 3 M $CaCl_2$, after treatment with 0.5 M NaCl and EGTA, gave a solution that yielded IF. This last fraction was kept for further work.

Fractionation of SF.—Fractionation of SF was carried out by precipitation with cetyltrimethylammonium bromide and stepwise redissolution of the salts with increasing concentrations of NaCl. Table 1 shows the ranges of sodium chloride concentrations that solubilized fractions F1–F6, while F7 remained insoluble at 4 M sodium chloride and was kept for further work. Table 1 also shows yields, analyses and monosaccharide compositions of fractions F1–F6. There is a direct relationship between the percentages of sulfate and the concentrations of NaCl necessary to solubilize the fractions. It is also seen that higher amounts of uronic acid improved the dissolution, suggesting that these units have not reacted with the cetyltrimethylammonium bromide. All the soluble fractions show negative rotations, indicating that most of the sugar constituents have a β-D- and/or α-L-anomeric configuration. This is in agreement with the major amounts of fucose in all the fractions (Table 1), indicating that it is possible that the preponderance of that sugar is still higher considering its decomposition during the acid hydrolysis.[21] The other major sugar was galactose, and in some cases (fractions 1, 3 and 4), xylose. Small amounts of mannose and glucose were found in all the fractions (Table 1). The FTIR spectra of the fractions show, in fractions 1–4, an ill-defined shoulder at 817.8–821.6 cm^{-1}, suggesting the presence of equatorial sulfate groups on the C-2 and C-3 positions or sulfate groups linked to the primary C-6 position.[22] A well-defined peak at 837 cm^{-1} appeared in F5 and F6, in the middle between 830 and 845 cm^{-1} (Table 1). ^{13}C NMR spectra of F1–F6 fractions show absorptions corresponding to methoxyl groups possibly linked to C-4 (57.4 ppm) and to acetyl groups (173.0 and 20.6–20.7 ppm) (data not shown).

Gel-permeation chromatography of F3 and F5.—Fractions F3 and F5 were submitted to chromatography on Sepharose 2B. Both elution patterns were similar showing broad asymmetric peaks (Fig. 2). These were fractionated, assuming an unimodal distribution (Fig. 2), into a major subfraction F3B (F5B) and subsidiary ones [F3A (F5A), F3I (F5I) and F3II (F5II), not shown in Fig. 2 (F5II)] that were reserved for further work. Table 3 shows yields, analysis and their monosaccharide compositions. Subfraction F3B contained similar amounts of uronic acids and sulfate groups. Fucose was the major sugar, together with significant percentages of xylose, mannose, and galactose. Subfraction F5B contained high amounts of sulfate and minor quantities of uronic acids, with only fucose and galactose being noteworthy.

Desulfation and carboxyreduction.—Solvolysis of F3-B (uronic acids: 12.6%) for 30 min (yield > 95%) produced no desulfation and a small loss of some fucose and xylose (F3B-30, uronic acids: 11.8%). Longer treatment (3 h, yield 56%) eliminated nearly all the sulfate (remaining 5.2%), reducing further the amount of fucose and concentrating the uronic acids (F3B-3, uronic acids: 22.0%) (Table 3). Fraction F3B and its solvolyzed derivatives (F3B-30 and F3B-3) were submitted to carboxyreduction (F3B-R, F3B-30R, F3B-3R). The three samples showed net increases of glucose (Table 3), indicating that the uronic acid was glucuronic acid.

Solvolysis of F5-B for 20 min (yield 73%) changed only slightly the sugar composition but eliminated 60.2% of the sulfates, while the same treatment for 4 h eliminated nearly all the sulfate groups, degrading most of the sample (yield 20%) (Table 3).

Methylation analysis of carboxyl-reduced F3B and desulfated derivatives.—The results of methylation analysis for F3B-R, F3B-30R and F3B-3R are shown in Table 4. The monosaccharide composition of methylated F3B-3R was determined to be similar to that of the parent compound (F3B-3), showing

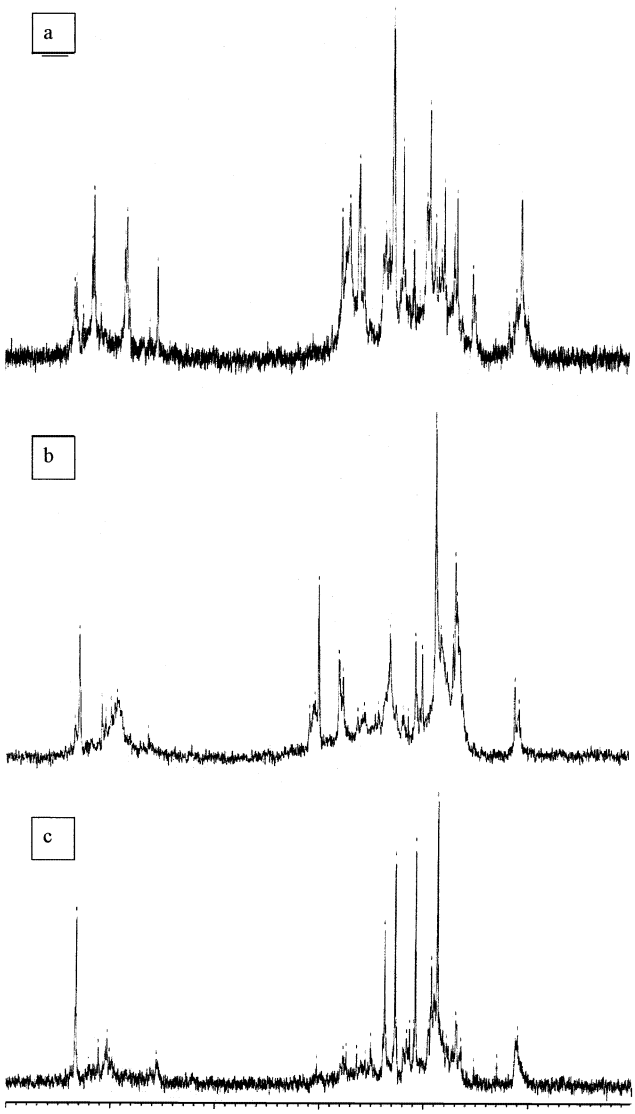


Fig. 3. ^{13}C NMR spectrum of fractions F3B-3 (a), F5B (b) and F5B-4 (c).

major amounts of fucose (ca. 50%). This 'fucan' shows a backbone with major amounts of 4-linked fucoses and minor quantities of 2- and 3-linked fucoses, and 2,4- and 3,4-disubstituted units as possible branching points and/or sulfate groups at C-2 and C-3, and single stubs of α-L-fucoses. The sudden increase of terminal fucoses in F3B-3R is in agreement with the degradation produced in this solvolysis. The pattern of methylation of the 'galactan' is highly dispersed and shows nine partially methylated galactoses (Table 4), five of which have percentages of 1.7% or less. Table 4 shows a 6-linked galactose chain with smaller amounts of 3- and 4-linked units. Disubstituted residues in the original fraction (F3B-R) which disappear in the desulfated derivatives suggest that most of them are monosulfated units. Table 4 also shows a 2-linked mannose backbone with disubstituted (2,3- and 2,4-) and trisubstituted (2,3,4-) units that were maintained after solvolysis, suggesting branching points at C-3 or C-4, together with both C-3, C-4 ramifications. Glucosyl and glucosyluronic acid units are linked through C-4 with branches at C-3, and the xylosyl residues are always terminal (Table 4).

^{13}C NMR spectra of F3B and desulfated derivatives.—The ^{13}C NMR spectrum of F3B-3 (Fig. 3(a)) showed sharp resonances at (ppm) 103.4 (C-1), 70.8 (C-2), 72.8 (C-3), 73.8 (C-5), 68.7 (C-4) and 61.0 (C-6) together with 69.3 (C-6-linked), 77.7 (C-4-linked) and 80.5 (C-3-linked), corresponding to a (1→6) (1→4) (1→3)-β-D-linked galactan in agreement with the methylation analysis. The same spectrum also showed peaks at 101.8–101.7 and 98.8–98.2 ppm corresponding to β-D-mannose and 2-substituted β-D-mannose, and at 100.9–100.8 ppm corresponding to α-D-glucuronic acid, α-D-glucose and β-D-xylose linked to C-3 of L-fucose.[42] Absorptions at 99.5–99.3 and 95.6–95.2 ppm would correspond to 4-linked and 3,4-disubstituted α-L-fucose. Resonances at 174.9–174.7 ppm confirmed the presence of a uronic acid. The ^{13}C NMR spectra of F3B and F3B-30 (not shown) are consistent with the above results.

Methylation analyses of F5B and desulfated derivatives.—This fraction contains major amounts of fucose and galactose, which is reflected in the methylated derivatives in which mannoses and xyloses are nearly absent (Table 4). Methylation analysis of the desulfated F5B suggests that the molecule is a block copolymer made up of a (1→6)-galactan backbone with minor amounts of 2-, 3-, and 4-linked units branched with a linear (1→3)-linked 'fucan' with some 4-linked residues. Sulfate groups on the galactan should be at the C-3 of a (1→6)-linked unit and at the C-2 of a (1→4)-linked residue [alternatively C-4 of a (1→2)-linked galactose]. The 'fucan' branching point could be at any position of the galactose units. Sulfate groups on the 'fucan' would be at C-4 of a (1→3)-linked unit or at C-2 of a (1→4)-linked residue. It is

noteworthy that there are major amounts of 2,4-disulfated units. The degradation of the galactose and fucose chains during the solvolysis is evident from the increasing of terminal units in F5B-40 and F5B-4 (Table 4).

NMR spectra of F5B and its desulfated derivatives.—The ^{13}C NMR spectrum of the desulfated F5B (F5B-4, Fig. 3(c)) showed sharp absorptions corresponding to a (1→6)-β-D-linked galactan at (ppm) 103.4 (C-1), 73.8 (C-5), 72.8 (C-3), 70.8 (C-2), 69.3 (C-6-linked, DEPT inverted), 68.7 (C-4). The HMQC spectrum showed the correlation of these signals with (ppm) 4.47 (H-1), 3.91 (H-5), 3.67 (H-3), 3.56 (H-2), 4.04/3.95 (H-6/H-6′) and 3.98 (H-4), respectively (spectrum not shown). Minor signals were observed at 80.3 (C-3-linked), 77.8 (C-4-linked) and 61.0 ppm (C-6) corresponding to galactose units (1→3) and (1→4)-linked, in agreement with the methylation analysis. Absorptions at 100.4, 99.4 and 95.6 ppm correspond to terminal α-L-fucose, 3-linked α-L-fucose, and 3,4-disubstituted α-L-fucose and are consistent with the methylation analysis, which suggested the presence of 3-sulfated 4-linked and 4-sulfated 3-linked α-L-fucose. The partially desulfated derivative (F5B-20) and the native fraction F5B also showed the aforementioned peaks together with another at 103.0 ppm (3,6-disubstituted β-D-galactose). In the spectrum of F5B (Fig. 3(b)), the resonance at 95.6 ppm is replaced by a peak at 96.4 ppm (possibly the same unit with a more sulfated surrounding). Acetate absorptions were found at 173.0 (carbonyl carbon) and 20.6 ppm (methyl carbon).

Anticoagulant activity of fucoidan fractions.—The activated partial thromboplastin time (APTT) and thrombin time (TT) were measured to evaluate the anticoagulant activity of the fucoidans (Table 2). The APTT and TT values of the blood treated with saline were 31.1 and 19.7 s, respectively. Fucoidans F5 and F6 showed APTT and TT times higher than those of the F1–F4, although these activities are low when compared to heparin, which at concentrations of 4 and 5 μg/mL showed APTT and TT times higher than 120 and 100 s, respectively.

Table 2
Anticoagulant activity of the fucoidans from *Sargassum stenophylum* [a]

Fraction	APTT[b]/TT[c] (μg/mL)			
	25	50	100	200
F1	33.0/19.7	36.6/20.2	39.5/20.7	42.0/24.2
F2	37.1/23.9	38.6/26.2	50.0/32.2	70.0/42.9
F3	41.5/24.3	54.0/34.9	70.6/47.9	98.3/53.7
F4	46.7/33.0	60.6/39.7	90.0/65.2	>120/66.2
F5	68.2/68.8	80.1/>100	>120/>100	>120/>100
F6	83.8/70.0	>120/>100	>120/>100	>120/>100

[a] The data are the mean values of two experiments.
[b] APTT for control sample without fucoidan: 31.1 s.
[c] TT for control sample without fucoidan: 19.7 s

4. Discussion

The brown seaweed *S. stenophyllum* biosynthesizes a complex system of fucoidans from which, after an elaborate procedure of extraction and fractionation, were obtained six fucoidan fractions (F1–F6). The way they were obtained, as well as their analyses and monosaccharide compositions, suggest that they were not mixtures of different types of polysaccharides but members of the same polysaccharide family, and that the quantitative variation of its components and distribution patterns, as well as the differences of structural details, were not due to heterogeneity of the samples but to extreme compositional and structural dispersion,[23] which is larger than that normally found in plant polysaccharides. The six fucoidan fractions were composed of L-fucose, D-galactose, D-mannose, D-xylose, D-glucose and D-glucuronic acid. L-Fucose was the major sugar, but in all the cases the other sugars were in important or, at least, in significant amounts. Fucoidans containing significant percentages of non-fucose sugar components have been previously isolated,[24–33] while in other cases the sample contained L-fucose only with small or trace amounts of the others sugars.[1,31,34] Galactose is a constituent of the 'fucans' extracted from *Macrocystis pyrifera*,[35] and it is found in trace quantities in the 'fucans' from *Himanthalia lorea* and *Bifurcaria bifurcata*[25,36] and *Desmarestia aculeata*.[23] In some cases galactose has been reported as a major con-

stituent, but the polysaccharides also had substantial amounts of fucose, xylose and glucuronic acid.[23,37,38] In only one case, to the best of our knowledge, a sulfated 'β-D-galactan', was isolated from the brown seaweed *L. angustata* in trace amounts, which could be considered a 'fucoidan' with β-D-galactose as the major sugar and L-fucose and D-glucuronic acid as trace components.[2]

The six fucoidan fractions can be grouped into two sets (F1–F4) and (F5–F6).[32] The first set is characterized by high percentages of uronic acids and lower sulfate content. The sulfate groups are mainly equatorial in the fucose units[38] and equatorial or primary sulfates in the galactose[2] and mannose residues (FTIR spectra). They contain major amounts of L-fucose, D-xylose and D-galactose. Fractions F1 and F4 show methoxyl substitution. This set showed very low anticoagulant activity as described for others fucoidans with similar chemical characteristics[32] (Table 2).

The second set (F5–F6), with structures which usually have potent anticoagulant activity, showed positive but low activity (Table 2), showing that the sulfate content is only one of the requirements needed for this activity. This set is characterized by a lower uronic acid content and a higher number of sulfate groups (Table 1). D-Galactose was the only non-fucose major sugar, and all the fractions show methoxyl and acetyl substitutions. ^{13}C NMR signals corresponding to C-6 and C-4 methoxyls on the fucose and/or galactose units were found in the fucan from *L. brasiliensis*.[40] Acetyl resonances were detected in the ^{13}C NMR spectrum of *L. brasiliensis*[40] and *O*-acetyl groups attached to O-2 in two different (1→3)-α-L-linked fucose residues in the *Chorda filum* fucan.[41] The FTIR spectra show a significant peak at 837 cm^{-1}. According to the well-known generalization that equatorial sulfates absorbed at ca. 830 cm^{-1} and axial ones at ca. 845 cm^{-1}, this peak would be unassignable. The possibility that axial and equatorial sulfate groups could be distinguished by C–O–S bending vibration bands has been questioned in the fucoidan family,[34] when disulfated fucose residues are present, on the basis of the fact that the exact position of the C–O–S bending band would depend on the overall substitution pattern and that these crowded molecules could exist as a mixture of various conformers.[42] The 837 cm^{-1} band could be explained as the result of a equilibrium between the chair conformers of an axial/equatorial disulfated fucose unit.

One fraction from each set (F3 and F5) was selected for structural studies. Gel-permeation chromatography on Sepharose 2B showed similar elution curves — broad asymmetric peaks from which subfractions F3B and F5B, were selected following the Gaussian curves corresponding to an unimodal distribution (Fig. 2). Both products maintain the characteristics of the sets from which they derived (Table 3). Mild solvolysis of F3B gave a product (F3B-30) with slight degradation of the L-fucose chains and no loss of sulfate. Harsher conditions (F3B-3) nearly eliminated the sulfate content and produced further degradation of the 'fucan' moiety. This and the increased concentration of glucuronic acid (Tables 3 and 4) in the degraded derivative are consistent with separated unsulfated-, sulfated- and glucuronic acid-rich domains in the 'fucan'. This relative resistance to degradation, compared to that of F5B (see latter), could be related to the high percentages of glucuronic acid in the 'fucan'.[24] This is consistent with the isolation, from a 'glucuronoxylofucan' from *Ascophyllum nodosum*, of oligosaccharides containing fucose, glucuronic acid and xylose[25,30,34] and with the isolation of α-D-glucuronosyl-(1→2)-L-fucose from the partial hydrolysis of *Cladosiphon okamuranus*.[39] In F5B the degradation of the fucose and galactose chains was concomitant with the loss of the sulfate groups. Without this shielding most of the sample was depolymerized and lost, and the small remainder showed the presence of short chains of fucose and galactose (Table 4).

The methylation patterns (Table 4) are very complex, as they are in the few cases reported of methylations of 'fucoidans' or 'fucans'.[2,29,39] These are characterized by the great number and diversity of structural units, most of them in small amounts. These patterns, which have not been found in other plant polysaccharides, are characteristic for these products.[1,2,25,29,31,39,43]

Table 3
Yield, sulfate content and monosaccharide composition of the fucoidans F3B and F5B and those of the partially desulfated fucoidans obtained by solvolytic treatment and reduction of the carboxyl-groups

Fractions	Yield [d] (%)	Sulfate ($NaSO_3$) (%)	Fuc (mol%)	Xyl (mol%)	Man (mol%)	Gal (mol%)	Glc (mol%)
F3B	41.4 (45.8)	18.0	44.2	11.1	20.1	19.0	5.6
F3B-R [a]			39.0	9.9	19.2	17.8	14.1
F3B-30 [b]	95	18.0	34.4	6.6	25.1	20.6	13.1
F3B-30R [a]			34.7	9.8	20.1	17.7	17.6
F3B-3 [b]	56	5.2	25.5	5.8	34.3	24.5	9.9
F3B-3R [a]			22.7	9.4	26.8	18.0	23.1
F5B	50.2 (56.5)	30.8	58.4	2.9	2.3	34.1	2.4
F5B-20 [c]	73	11.7	53.0	3.8	3.0	36.8	3.4
F5B-4 [c]	20	4.5	39.7	6.8	4.6	43.8	5.0

[a] Fractions carboxyl-reduced.
[b] Desulfated for 30 min and 3 h, respectively.
[c] Desulfated for 20 min and 4 h, respectively.
[d] In parenthesis, percentages of the recovered material. Uronic acid content: F3B, 12.6%; F3B-30, 11.8%; F3B-3, 22.0% and F5B, 2.1%.

The structural units of the fucoidan F3B and of its desulfated derivatives (Table 5) suggest a highly branched molecule with short 'fucan' chains and ca. 19% of terminal units, ca. 36% of unsubstituted chain units and ca. 44% of substituted chain residues (Table 5). If the substituted chain residues of the highly desulfated derivative (F3B-3R) are considered as branching points, they agree with the terminal residues in F3B-R. The backbone is formed by (1→2)-linked mannose units together with (1→6), (1→4) and (1→3)-linked galactose residues, while the 'fucan' chains are formed by major amounts of (1→4)-linked fucose and glucuronic acid units, together with lesser quantities of (1→3) and (1→2)-linked fucoses, (1→4)-linked glucoses and terminal β-D-xyloses linked to C-3 of an L-fucose.[45] The major branching points would be on C-3 and C-4 of a (1→2)-linked mannose (in the backbone) and on C-3 of a (1→4)-linked α-D-glucuronic acid and/or α-D-glucose (in the 'fucan' domain). Most of the sulfate groups are on C-3 of a (1→4)-linked fucose, on different carbons of the galactose units (Table 4), and on C-3 and C-6 of (1→2)-linked disulfated mannose units.

The structural units of the fucoidan F5B and its desulfated derivatives suggest a less branched molecule with larger 'fucan' chains. The original sample (F5B) contains ca. 7.8% of terminal units, ca. 20% of unsubstituted chain residues and ca. 73% of substituted chain units (Table 6). If, as above, the substituted chain units of the desulfated derivative (F5B-4) can be mainly considered as branching points, they agree with the terminal residues of the undegraded F5B (Table 6). The backbone is made up of (1→6)-linked galactose with minor amounts of (1→4), (1→3) and (1→2)-linked galactoses units, together with (1→4)-linked mannose residues. The 'fucan' chains are formed by (1→3)-linked fucoses with minor amounts of 4- and 2-linked fucoses and 4-linked glucoses and glucuronic acid. The branching points would be at C-4 of a 3-linked fucose and at C-4 of a 2-linked fucose and, possibly, at C-2 and C-3 of a 6-linked disubstituted galactose unit. Most of the sulfate groups in the 'fucan' domain are at C-2 and C-4 of a 3-linked disulfated fucose unit, at C-4 of a 3-linked fucose, and at C-2 of a 4-linked fucose (alternatively on C-4 of a 2-linked fucose). Disulfated units in the galactose backbone contain sulfates at C-2, C-3 and C-3, C-4 of a 6-linked residue. Monosulfated galactose units could have sulfate groups on any carbon atom of the units.

Several fucans have been investigated; however, they usually do not represent the polymer biosynthesized by the seaweed, but samples selected for their high content of fucose and/or anticoagulant activity. In most of the cases[1,2,39–41,44] they have a (1→3)-linked

Table 4
Methylation analyses of the fucoidans F3B-R [a] and F5B and of their partially desulfated subfractions

Glycosyl residue	Position of *O*-methyl group	Deduced position of substitution	F3B-R (mol%)	F3B-30R [b] (mol%)	F3B-3R [b] (mol%)	F5B (mol%)	F5B-20 [c] (mol%)	F5B4 [c] (mol%)
Fucosyl	2,3,4	terminal	10.2	10.8	17.1	5.8	11.2	13.4
	2,4	3	1.0	3.4	4.1	4.4	12.2	15.8
	2,3	4	12.9	13.3	2.0	4.9	9.8	5.5
	3,4	2	4.3	5.4		2.9	3.4	
	2	3,4	5.4	4.3	1.5	21.3	11.5	3.7
	3	2,4	9.3	2.0		12.2	5.2	4.1
		2,3,4				11.8		
Galactosyl	2,3,4,6	terminal	4.6	4.6	7.1	2.0	3.7	11.6
	2,3,6	4	2.0	2.3	1.4		7.8	4.2
	2,3,4	6	4.9	5.6	6.9	4.8	15.4	26.7
	2,4,6	3	1.2	4.8	2.2		1.9	2.9
	3,4,6	2						3.6
	2,3	4,6	1.3			2.1		
	2,4	3,6	2.6			8.5	5.6	
	2,6	3,4	1.7	1.6		1.4		
	3,6	2,4				7.8	6.6	
	4,6	2,3				1.4	1.2	
	2	3,4,6	1.2			1.9		
	3+4	2,4,6+2,3,6	1.5		1.4	4.3	2.1	1.2
Mannosyl	3,4,6	2		1.5	12.1			
	2,3,6	4						3.6
	4,6	2,3	7.6	9.8	8.4			
	4	2,3,6	4.6	3.9				
	6	2,3,4	1.5	2.5	1.4			
	3,6	2,4	3.2	3.6	4.4			
Glucosyl	2,3,6	4	2.3	3.0	4.1	2.5	2.4	1.8
	2,6	3,4	1.3	0.8	1.6			
Glucosyl	2,3,6	4	7.8	10.2	14.7	n.d.	n.d.	n.d.
GlcA	2,6	3,4	3.0	2.0	3.7	n.d.	n.d.	n.d.
Xylosyl	2,3,4	terminal	4.6	4.6	5.9			1.9

[a] F3B-R, fraction F3B carboxyl-reduced.
[b] Desulfated for 30 min and 3 h, respectively.
[c] Desulfated for 20 min and 4 h, respectively.

α-L-fucose backbone with (1→2)-α-L-linked fucosyl branches and sulfate groups at C-4. 'Fucoidans' from *Ecklonia kurome* have this structure together with small amounts of galactopyranosyl residues with various glycosidic linkages.[29,31] This structure was found in the *C. okamuranus* fucan in which part of the fucose is substituted with (1→2)-α-linked glucuronic acid;[39] in the fucan from *Laminaria saccharina*,[44] which has also sulfate groups at C-2, and in that from *C. filum*,[41] with branching at C-4 and sulfation at C-2 and C-4.

The 'fucan' from *A. nodosum*, which contains also ca. 50% galactose, has a structure of (1→4)-α-linked fucoses with sulfate groups at C-2 and C-3 and C-2, C-3-disulfated residues. A (1→4)-linked galactose backbone with fucose ramifications at C-2 was suggested for this product.[38] A (1→6)-linked 4-sulfated galactose backbone was suggested in 'sargassan'.[27]

Table 5
Structural units of the fucoidan F3B-R and the desulfated subfractions F3B-30R and F3B-3R

Type of units	Linkage	F3B-R (%)	F3B-30R (%)	F3B-3R (%)
Terminal units				
Fucosyl		10.2	10.8	17.1
Galactosyl		4.6	4.6	7.1
Xylosyl		4.6	4.6	5.9
Unsubstituted chain units				
Fucosyl	3	1.0	3.4	4.1
	4	12.9	13.3	2.0
	2	4.3	5.4	
Galactosyl	6	4.9	5.6	6.9
	3	1.2	4.8	2.2
	4	2.0	2.3	1.4
Mannosyl	2		1.5	12.1
Glucosyl	4	2.3	3.0	4.1
GlcA	4	7.8	10.2	14.7
Substituted units				
Fucosyl	3,4	5.4	4.3	1.5
	2,4	9.3	2.0	
Galactosyl	2,4,6+2,3,6	1.5		1.4
	3,4,6	1.2		
	4,6	1.3		
	3,6	2.6		
	3,4	1.7	1.6	
Mannosyl	2,3	7.6	9.8	8.4
	2,4	3.2	3.6	4.4
	2,3,4	1.5	2.5	1.4
	2,3,6	4.6	3.9	
Glucosyl	3,4	1.3	0.8	1.6
GlcA	3,4	3.0	2.0	3.7

Table 6
Structural units of the fucoidan F5B and the desulfated subfractions F5B-20 and F5B-4

Type of units	Linkages	F5B (%)	F5B-20 (%)	F5B-4 (%)
Terminal units				
Fucosyl		5.8	11.2	13.4
Galactosyl		2.0	3.7	11.6
Xylosyl				1.9
Unsubstituted chain units				
Fucosyl	3	4.4	12.2	15.8
	4	4.9	9.8	5.5
	2	2.9	3.4	
Galactosyl	6	4.8	15.4	26.7
	3		1.9	2.9
	4		7.8	4.2
	2			3.6
Mannosyl	4			3.6
Glucosyl	4	2.5	2.4	1.8
Substituted units				
Fucosyl	2,3,4	11.8		
	3,4	21.3	11.5	3.7
	2,4	12.2	5.2	4.1
Galactosyl	2,4,6+2,3,6	4.3	2.1	1.2 [a]
	3,4,6	1.9		
	4,6	2.1		
	3,6	8.5	5.6	
	3,4	1.4		
	2,4	7.8	6.6	
	2,3	1.4	1.2	

[a] Only galactosyl 2,3,6-linked units.

Our data and previous reports[2,23,24,27,38,39] suggest a general basic structural pattern for fucoidans. This pattern shows a backbone composed of (1→6)-β-D-galactose units with minor amounts of (1→2), (1→3) and (1→4)-β-D-galactose residues[2,27,38] together with (1→2) and/or (1→4)-β(possible)-D-mannose units. Previous results[2] suggest that both sugars form a block copolymer or appear in different mmolecules. The similarity between the chain linkage (through C-4) and the branching point (through C-3) in α-D-glucuronic acid and glucose suggest that these glucose units are α-D and are situated in the 'fucan domain' interspersed in the chains.

'Fucan' chains are linked to the backbone through disubstituted (C-2, C-3 or C-2, C-4) (1→6)-β-D-linked galactoses, to monosubsti-

tuted (through C-3) (1→2)-β-D-linked mannoses or through C-2 of (1→4)-β-D-linked mannoses or disubstituted (through C-3, C-4 and C-3, C-6) (1→2)-β-D-linked mannoses.

'Fucans' are built up by α-L-fucose 3- or 4-linked units. Fucose units could be branched through C-2, C-3 or C-4 or be end-chain residues. β-D-xylosyl residues end the chains linked to C-3 of fucose units.

Sulfation can be found in any position of the galactose/mannose backbone or in the fucose units. The anticoagulant properties of fucoidans are mainly determined by the fucose sulfated chains,[2,29,45] specially by the disulfated fucosyl units.[34] Removal of fucose sulfated chains in the fucosylated chondroitin sulfate of the sea cucumber *L. grisea* reduced the anticoagulant activity; nevertheless, the isolated chains do not compete with the parent product for activation of CH-II, suggesting the necessity of the fucose chain to be presented to the protein ligand in a specific spatial distribution along the backbone.[45]

It is possible that this proteoglycan-like complex pattern, formally similar to that found in the fucosylated chondroitin sulfates from the body wall of sea cucumbers, *Stichopus japonicus*[46,47] and *Ludwig-othurea grisea*,[48] gives only a simplified view of real fucoidans in which minor amounts of new and non-predictable structural units or substituents would make the above scheme much more complex.

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