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Carbohydrate Research 336 (2001) 155–159

CARBOHYDRATE RESEARCH

Note

A fucoidan fraction from *Ascophyllum nodosum*

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Received 13 July 2001; accepted 21 September 2001

Abstract

A fucoidan fraction was purified from the brown alga *Ascophyllum nodosum*. The polysaccharide contained L-fucose and sulfate as the only constituents. Combination of methylation analysis, Smith degradation, FTIR and NMR spectroscopy on the native and the de-sulfated polymers demonstrated that the fucoidan consisted of a highly branched core region with primarily α-(1→3)-linked fucosyl residues and a few α-(1→4) linkages. Branch points were at position 2 of the →3-linked internal residues. The side chains consisted of single and multi-unit fucosyl residues. The combined analytical data suggested also a complex sulfation pattern with substitution principally at position 2 and/or position 4. Such diversity in the structural features of this fucoidan may be of importance for its various biological properties. © 2001 Published by Elsevier Science Ltd.

Keywords: Fucoidan; *Ascophyllum nodosum*; Brown alga

1. Introduction

Like heparin, the sulfated fucans (fucoidans) from brown marine algae have potent anticoagulant and other antiviral and anti-inflammatory activities.[1,2] Although fucans and fucoidans have been described for some time,[4] their precise structure is still debated. A reason for that originates from the difficulty of their extraction and isolation in a pure form.[5] Also, their heterogeneity and polydispersity[6] limit their structural study. However, the fucan isolated from *Fucus vesiculosus* has been partially characterized[3] as a complex mixture of fucose-rich polysaccharides ranging from typical fucoidans containing fucose, sulfate and no uronic acid, to low-sulfated heteropolysaccharide with high content of uronic acids. Recently, a fucoidan isolated from the brown seaweed *Cladosiphon okamuranus* was shown to consist of an α-(1→3)-linked fucosyl backbone carrying a half sulfate substitution at position 4 and α-glucuronic acid residues at position 2, with one *O*-acetyl ester present in every six fucose residues.[7] Highly sulfated fucans from *Ascophyllum nodosum* have been extracted[8] and their polyanionic characteristics were analyzed together with their cation binding capacity and viscoelastic properties. The chemical composition of most of these preparations suggested that they contained a significant proportion of ascophyllan-like (xylofucoglycuronans) macromolecules.[9] In fact, the exact structure of pure fucoidan from *A. nodosum*

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0008-6215/01/$ - see front matter © 2001 Published by Elsevier Science Ltd.

PII: S0008-6215(01)00257-9

Table 1
Carbohydrate analysis of the Fucoidan fractions

Fraction	Composition (mol%)				
	Fuc	Xyl	Gal	Glc	Sulfate
Cetavlon extract	31	49	t	20	
NaCl extract	63	28	3	6	
EtOH insoluble (= fucoidan)	66	3	0	0	31

t, trace; n.d., not determined.

and its sulfation pattern is still largely uncertain,[6,10] and only parts of the structure have been clearly established.[11]

Because of the anti-tumor and anti-proliferative effects reported for a fucoidan extracted from *A. nodosum*,[12] it was of interest to investigate the chemical structure of a pure fucoidan fraction extracted from this brown alga.

2. Results and discussion

Four polysaccharide fractions were isolated from *A. nodosum* by HCl extraction following Mabeau et al.[5] Carbohydrate analysis of the four fractions (Table 1) showed that they all contained significant proportions of L-fucose. The final fraction isolated at the end of the purification process was mainly composed of L-fucose (95%) with only minor proportions of xylose (5%). No uronic acids could be detected. In addition, total sulfate estimation by the barium chloride-gelation method[13,14] indicated an average of one sulfate group for two fucosyl residues. All these data pointed to a true fucoidan.

Further analytical data were provided by the FTIR spectrum which showed typical absorption bands of fucoidans.[15,16] Of particular interest for structural investigation were the bands for sulfate groups. The strong absorption band at 1240–1255 cm^{-1} (S=O stretching) confirmed the significant amount of sulfate in the polysaccharides. The sharp band at 840 cm^{-1} and the shoulder at 820 cm^{-1} (C–S–O) suggested a complex pattern of substitution, primarily at C-4 position (axial C-4 substitution of α-linked L-fucopyranose)[17,18] with other substitution at C-2 or/and C-3 (equatorial positions) in lower amount.

The ^{13}C NMR spectrum of the native fucoidan fraction exhibited several major signals, most of them showing a certain degree of multiplicity. The multiplicity of signals with slightly different chemical shifts suggested either diversity in the positions of inter-glycosidic linkages, or/and multiplicity of the patterns of sulfation of the fucosyl residues. Such diversity of substitution is a typical structural feature of sulfated α-L-fucoidans that broadens the signals in their NMR spectra. In the anomeric region, two major signal centered at 99.4 and 95.5 ppm were indicative of (1→3)-linked residues[19] and of pluri-substituted fucosyl residues, respectively. Carbon atoms carrying a substitution undergo a downfield shift effect that isolates these resonances from the rest of the ring carbon atoms. This was the case for the signals at 80.2 ppm and 75.8 ppm indicative of C-4 and C-3 substitutions in sulfated fucoidans (Table 2).[19,20] In contrast, the resonance of the methyl group (C-6) appeared as a strong signal centered at 16.5 ppm. On sulfate group substitution, the

Table 2
^{13}C NMR shifts for native and desulfated fucoidan

Assignment [a]		Chemical shift (δ ppm)	
		Native fucoidan	Desulfated fucoidan
C-1	(1→3-linked α-L-Fuc)	99.4	102.2–99.6
	(1→4-linked α-L-Fuc)	95.5	96.2
C-4	(1→3-linked)	80.2	81
C-3	(1→3-linked)	75.8	77.2
C-6	(CH_3 α-L-Fuc)	17.3–16.5	16.4

[a] Main signals assigned by analogy with literature.[10,11,16,20,24]

Table 3
Methylation analysis of the Fucoidan fraction

Position of *O*-methyl groups [a]	Deduced position of substitution	Composition (mol%) [b]		
		Original fraction	Desulfated fraction	Smith-degraded desulfated fraction
2,3,4-Fuc	terminal	25	31	15
2,4-Fuc	→3	23	36	65
2,3-Fuc	→4	11	5	
3,4-Fuc	→2	11	4	
2-Fuc	→3,4	10		
4-Fuc	→2,3	18	24	21

[a] As alditol acetate derivatives.
[b] Calculated from peak areas (uncorrected for molecular response factors).

resonance of C-6 is expected to shift from about 16.5 to 19 ppm.[3,7] The appearance of a strong signal at 16.5 ppm in the spectrum of *A. nodosum* fucan suggested that the polysaccharide might have a less complex pattern of substitution than anticipated. Solvolytic removal of sulfate substituents of the pyridinium salt of the polymer with dimethyl sulfoxide containing methanol[21] led to some modification of the NMR spectrum. The anomeric region remained complex with signals centered at 102.2, 99.6 and 96.7 ppm. The relative intensity of the down-field shifted signal at 77.2 ppm of the substituted C-3 was enhanced. Finally, the resonance of C-6 at 16.4 ppm was sharper than in the sulfated polysaccharide.

The fucoidan was fully methylated and the analysis by GC-EIMS of the derived methylated alditol acetates obtained after hydrolysis revealed a highly substituted structure, as shown by the number of the different partially methylated products (Table 3). The exact structural features of the fucoidan were difficult to deduce from the methylation pattern because of the presence of the sulfate groups substituting the polysaccharide. After desulfation of the fucoidan fraction, methylation analysis gave the results shown in Table 3. It is clear that the increase in the proportion of non-reducing terminal fucosyl residues after desulfation followed by methylation indicated that some of the terminal fucosyl residues were originally sulfated. Therefore, any of the di-*O*-methyl-L-fucose derivatives identified in the sulfated polymer could have been in part converted into tri-*O*-methyl-L-fucose in the methylated de-sulfated fraction. Similarly, mono-*O*-methyl derivatives could be partially replaced by di-*O*-methyl derivatives in the case of mono-sulfate substitution, and by tri-*O*-methyl derivative in the case of di-sulfate substitution on a terminal residue.[11]

The main structural linkages and sulfate-substitution pattern could be deduced from the combined data of the original and the desulfated polymers (Table 3). It could thus be concluded that the core region of the fucoidan was composed primarily of α-(1→3)-linked fucose with a lower proportion of α-(1→4)-linked residues. The large proportion of non-reducing terminal fucosyl residues indicated that about every second fucosyl residue was a side chain made of a single-fucosyl residue on a short side chain. The results also suggested that residues of the main chain and terminal residues carried sulfate groups at position 2 or 4.

Further characterization of structural features were provided by the analysis of the products obtained by the sequence of periodate oxidation and partial mild-acid hydrolysis (Smith degradation) followed by permethylation of the polymeric material recovered after dialysis of the Smith-degraded desulfated fucoidan.

Smith-degradation afforded non-dialyzable material in about 36% yield. This was lower than the prediction made on the basis of methylation analysis, unless some of the ter-

minal residues would be doubly substituted by sulfate groups. Fucosyl residues bearing two sulfates had recently been characterized in *A. nodosum*.[10,11] The results in Table 3 showed that the Smith sequence significantly reduced the number of non-reducing groups. This is the indication that a large proportion of the terminal fucosyl residues that had been removed by the periodate-mild-acid hydrolysis treatment corresponded to side chains containing more than a single fucose residue.

Altogether, the structural analysis of the purified fucoidan fraction from the brown alga *A. nodosum* shows that the sulfated polysaccharide consists of a highly branched core region with primarily α-(1→3)-linked fucosyl residues and a few α-(1→4) linkages. Branch points are on position 2 of →3-linked internal residues. The side chains consist of single and multi-unit fucosyl residues. A great variability in the structure of this brown seaweed fucoidan resides in its complex sulfation pattern with various locations for mono- and disulfation on internal and/or terminal residues.[10,11] Fucoidans from *F. vesiculosus* and from *A. nodosum* are characterized by their heterogeneous and branched structures,[11,15] randomly organized.[10] The polydiversity of these algal polysaccharides is another difficulty that prevents from describing them under a single structure. These features, and particularly the variability in side chains may be of importance for the various biological properties exhibited by the series of fucans and fucoidans from algal origin.

3. Experimental

General methods.—GC-MS was performed using a capillary column (SP 2380) at 200 °C. The ionizing potential was 70 e V and the temperature of the ionizing chamber was 200 °C. For NMR analysis, samples were dissolved in D_2O (15–30 mg/mL). The deuterium resonance was used as the field-frequency lock. The spectra were recorded at 75–46 MHz using 5 mm diameter tubes on a Bruker AM spectrometer. Chemical shifts are expressed in δ values. FTIR were performed in KBr pellets (1 mg polysaccharide in 100 mg KBr). The spectra were recorded on a Perkin–Elmer 1600 FTIR spectrometer from 600 to 4000 cm^{-1}.

Isolation and purification of fucoidan.—Fresh thalus of *A. nodosum* (43 g) were ground and successively extracted at rt, then at 70 °C with 0.01 NaCl containing 1% $CaCl_2$. The extraction steps were repeated twice, and the extracts were pooled. The mixture was dialyzed against distilled water and concentrated to 100 mL. The crude polysaccharide extract was precipitated with EtOH and collected by centrifugation. The insoluble material was redissolved in 0.1 N HCl and the soluble product purified by centrifugation. The crude extract was then purified by a procedure adapted from Ref. 5. Addition of 3% CETAVLON have led to a precipitate that was centrifuged off, resulting in the soluble CETAVLON extract. The bottom of centrifugation was further suspended in 2 M NaCl and after centrifugation the insoluble material was washed with EtOH and a solution of 1 M $CaCl_2$. The soluble polysaccharide fraction was collected by centrifugation and precipitated in EtOH (3 vols) to yield the purified fucoidan.

Carbohydrate analysis.—The polysaccharide fractions were hydrolyzed with 2.5 M trifluoroacetic acid. The resulting monosaccharides were converted to their alditol acetate derivatives and analyzed by gas chromatography on Hewlett–Packard 3380 A integrator gas chromatograph equipped with a macrobore column (30 m × 0.53 mm) of 3% SP 2380. Uronic acid content was estimated by the *O*-hydroxydiphenyl reagent method.[22]

Sulfate group determination.—This was carried out using the $BaCl_2$ gelatin method[13,14] by turbidimetry of the released barium sulfate suspension measured a 360 nm.

Desulfation.—This was performed by the solvotic desulfation method, according to Nagasawa et al.[21] The fucoidan (18.5 mg) was deionized using an IR 77 ion-exchange resin column to acidic pH. Addition of a few drops of Me_2SO gave the pyridinium salt of the polysaccharide which was dialyzed and recovered by lyophilization. The pyridinium salt was treated with a mixture of Me_2SO–5% MeOH for 3 days at 50 °C. After dialysis and

lyophilization, the desulfated product was obtained as a white powder (12 mg).

Periodate oxidation–Smith degradation.—The native and desulfated fucoidans (ca. 10 mg) were dissolved in water and oxidized with 0.1 M sodium metaperiodate (10 mL) for 18 h at rt in the dark. The oxidized polymers were reduced conventionally with $NaBH_4$. After dialysis, the products were hydrolyzed overnight with 0.5 M trifluoroacetic acid. The mixture was neutralized with 50% NH_4OH and dialyzed against distilled water. Samples of the fractions retained in the dialysis tube and dialyzing out were respectively hydrolyzed and analyzed for fucose. No fucose could be identified in the latter.

Methylation analysis.—Native, desulfated and Smith-degraded polysaccharides were methylated by the Hakomori procedure.[23] The methylated products were hydrolyzed with formic acid (aq 90%, 1 h, 100 °C) and then with trifluoroacetic acid (2 M, 3 h, 100 °C), and the partially methylated sugars were analyzed in gas-chromatography and characterized by GC-MS.

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