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Electrospray ionization mass spectrometry of oligosaccharides derived from fucoidan of *Ascophyllum nodosum*

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Abstract—Algal fucoidan is a complex sulfated polysaccharide whose structural characterization requires powerful spectroscopic methodologies. While most of the structural investigations reported so far have been performed using NMR as the main spectroscopic method, we report herein data obtained by negative electrospray ionization mass spectrometry. MS analysis has been carried out on oligosaccharides obtained by partial hydrolysis of fucoidan from the brown algae *Ascophyllum nodosum*. Oligosaccharide mixtures were fractionated by size exclusion chromatography, which allowed the analysis of oligomers ranging from monosaccharide to pentasaccharide. Monosaccharides were detected as monosulfated as well as disulfated forms. Besides, part of the oligosaccharides exhibited a high content of sulfate, evidencing that fucoidan contains disulfated fucosyl units. Fragmentation experiments yielded characteristic fragment ions indicating that the fucose units are mainly 2-O-sulfated. This study demonstrates that highly sulfated oligosaccharides from fucoidan can be analyzed by ESIMS which gives additional information about the structure of this highly complex polysaccharide.

Keywords: Fucoidan; Sulfated fucose; Algal polysaccharide; ESIMS

1. Introduction

Algal fucoidan is a sulfated polysaccharide extracted from brown algae belonging mainly to Fucales and Laminariales orders (e.g., *Ascophyllum nodosum* and *Chorda filum*). It exhibits several biological activities in mammalian systems that are very promising for therapeutic applications.[1,2] Fucoidan is made up of α-L-fucose units linked by (1→4) and (1→3) glycosidic bonds and sulfated at positions 2 and/or 3 and/or 4 depending on the alga species.[3–5] The structural heterogeneity of fucoidan with respect to linkage and sulfate position is still a limitation to trace clear correlations between the structure and biological activities of this polysaccharide, although much effort has been made during the past years to elucidate its structure.[6] Among modern spectroscopic techniques, electrospray ionization mass spectrometry (ESIMS) is widely recognized as a powerful and highly sensitive analytical method for the characterization of sulfated oligosaccharides,[7,8] mainly because these anionic compounds readily ionize in negative ESIMS mode. Recent studies have demonstrated the usefulness of tandem mass spectrometry for the sequencing of glycosaminoglycan oligosaccharides

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and the differentiation of sulfated isomers.[9,10] Indeed, the fragmentation of sulfated oligosaccharides produces diagnostic ions that reflect the pattern of substitutions, including the sulfate groups and the glycosidic bonds. Three main fragmentation reactions were observed: (i) loss of a small molecule (SO_3, H_2O, CO_2), (ii) glycosidic cleavage, and (iii) cleavage across the glycosidic ring. It has been previously shown that the fragmentation of sulfated disaccharides produced mainly two cross-ring fragments,[9] the ions $^{0,2}A_2$ and $^{0,2}X_1$ following the Domon and Costello nomenclature.[11] Their formation results of the breaking of the C-1–O (ring) and C-2–C-3 bonds, which leads to the loss of C-1–C-2 atoms of the reducing end for the $^{0,2}A_2$ ion, and to the loss of C-6–C-3 atoms of the nonreducing residue for the $^{0,2}X_1$ ion, allowing the determination of the sulfated position on the parent molecule. We have recently reported that negative ESIMS affords an efficient method for the analysis of sulfated monosaccharides, which allows the differentiation between the sulfated L-fucose positional isomer constituents of fucoidan.[12] To date, however, MS analysis of algal fucoidan has never been described. We now report on the negative ESIMS analysis of sulfated oligosaccharides derived from algal fucoidan.

2. Results and discussion

Due to the polydispersity and the high molecular weight of fucoidan, the study was carried out in a first step on low molecular weight fractions (LMWF) obtained by partial hydrolysis of fucoidan and consisting of sulfated oligosaccharide mixtures which are consequently easier to characterize by MS (cf. Experimental section for preparation). Figure 1a shows a typical mass spectrum of this mixture (LMWF1). A MS signal of high intensity was obtained in negative mode, as expected due to the presence of sulfate groups. The very numerous peaks distributed along the wide bell-shape curve reveal the mass heterogeneity of the oligosaccharide mixture. In addition, the ability of polysulfated oligosaccharides to form multiply charged ions likely add to the complexity of the spectrum. In order to obtain simpler spectra, the oligosaccharide mixture was filtered through a membrane (cut-off 1000). The resulting filtrate (LMWF2) led to a better-resolved spectrum (Fig. 1b) showing discrete peaks that could be assigned to sulfated fucose oligomers (Table 1). The size of the oligosaccharides ranged from mono- to tetrafucose, carrying most often one sulfate group per fucosyl unit. However the detection of trisulfated disaccharide ions at *m*/*z* 274 $[M-2H]^{2-}$, 285 $[M-3H+Na]^{2-}$ and 593 $[M-3H+2Na]^{-}$ indicated that some fucosyl units in fucoidan are also disulfated.

The oligosaccharide mixture (LMWF2) was fractionated by size exclusion chromatography on Biogel P4 in order to obtain size-homogeneous fractions. Figure 2 shows the mass spectrum of the last eluted fraction (OF5) with a major peak at *m*/*z* 243 corresponding to the deprotonated molecule $[M-H]^{-}$ of monosulfated fucose. The low level of contaminating disaccharides observed in this spectrum (for instance at *m*/*z* 389) indicated that the fractionation by size exclusion chromatography was efficient. Some desulfation probably due to fragmentation in the MS ion source occurred as evidenced by the ion at *m*/*z* 97 corresponding to the hydrogenosulfate anion HSO_4^{-}. Fragmentation of the ion at *m*/*z* 243 led to the loss of sulfate detected at *m*/*z* 97 and to fragment ions observed at *m*/*z* 139, 183 and 225. The first two ions correspond to cross-ring cleavages, and the last one to dehydration as we have previously reported.[12] The fragment ions at *m*/*z* 183 and *m*/*z* 139 were assigned to the fragment ions $^{0,2}A$ and $^{0,2}X$, respectively. We have observed that the ion at *m*/*z* 139 was the major fragment observed upon CID-type fragmentation of the monosaccharide fractions analyzed in this study. The high abundance of this fragment (Fig. 3a) indicates that the fucosyl units of *A. nodosum* fucoidan are mainly sulfated at position 2,[12] in agreement with former NMR studies that have established the prevalence of this sulfated position in fucoidan from the brown algae *A. nodosum*.[3,4,13] In comparison, Figure 3b shows the fragmentation spectrum of a monosaccharide sample mainly containing 4-O-sulfated fucose issued from sea cucumber fucosylated chondroitin sulfate, whose regular structure has been determined by high-field NMR.[14,15] In contrast with the algal 2-O-sulfated fucose, the fragmentation of the marine invertebrate 4-O-sulfated fucose leads to the major fragment ion at *m*/*z* 183, in agreement with our previous report.[12] The intensity of this ion at *m*/*z* 183 was variable in the algal fucoidan samples and even almost absent for some fractions. Given the low abundance of this ion in the fragmentation mass spectra, the 4-O-sulfated fucose appears to be a minor component in our samples of *A. nodosum* fucoidan.

In the mass spectrum of the fraction (OF4) that was eluted just before the monosaccharide fraction, the base peak is found at *m*/*z* 389 corresponding to the singly charged anion $[M-H]^{-}$ of a monosulfated difucose (Fig. 4, inset). Interestingly, this spectrum exhibits also a small peak at *m*/*z* 161 corresponding to a doubly-charged species $[M-2H]^{2-}$ attributed to disulfated fucose. This data indicates that the disulfated monosaccharide was co-eluted from the size-exclusion column with the monosulfated disaccharide, suggesting that both may have similar hydrodynamic volumes. Besides, this result brings additional evidence that disulfated fucosyl units occur in the structure of algal fucoidan. Fragmentation of the ion at *m*/*z* 389 (Fig. 4) led to few products arising from glycosidic bond cleavage as indicated by the very minor fragment ions at *m*/*z* 243

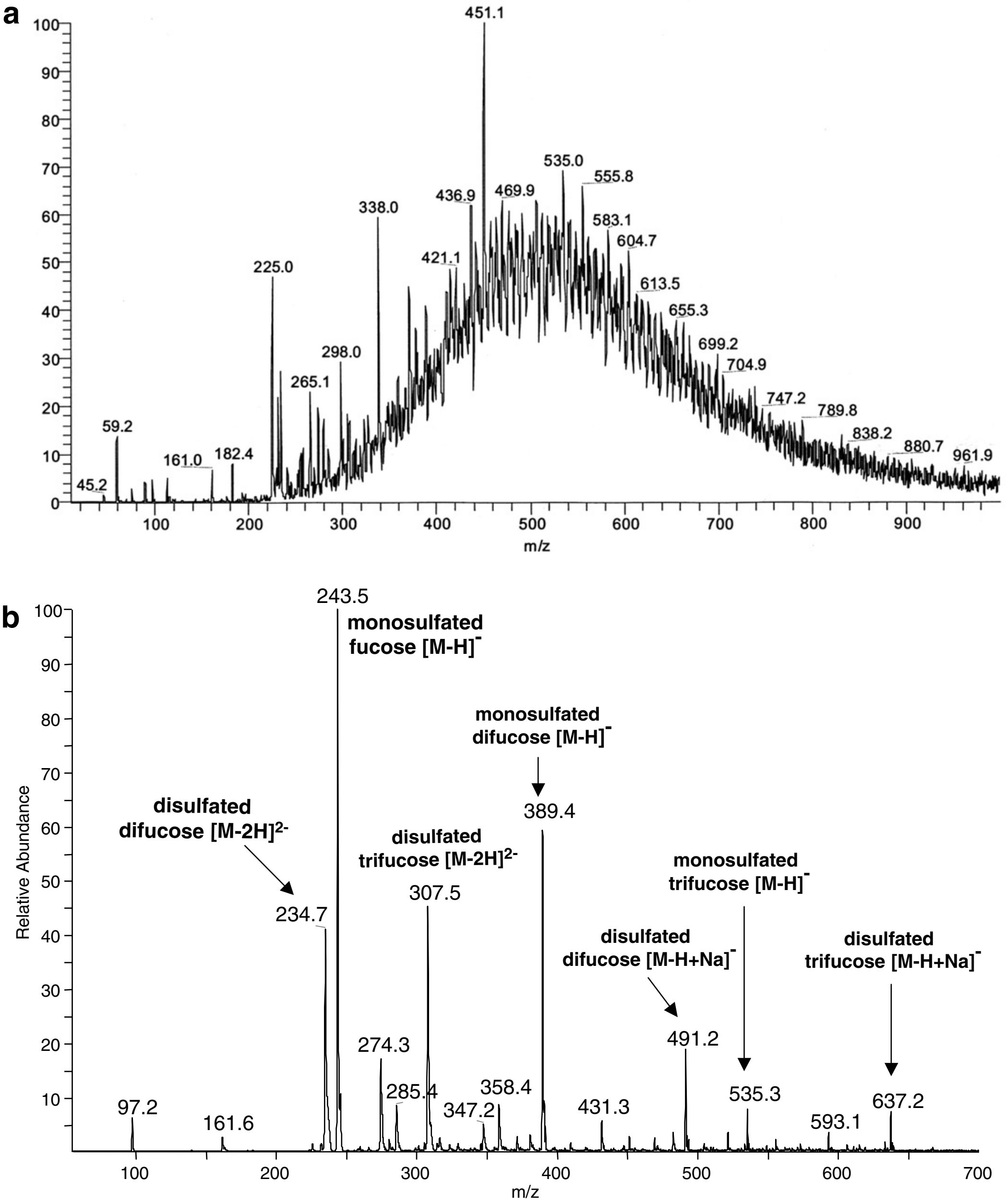

Figure 1. Negative ESIMS spectra of sulfated oligosaccharides derived from the hydrolysis of fucoidan of the brown alga *Ascophyllum nodosum*: (a) crude hydrolysis mixture (LMWF-1); (b) oligosaccharide fraction (LMWF-2) filtrated on cut-off 1000 membrane.

and 225 (monosulfated fucose and its dehydrated form, respectively). The main fragmentation observed here is cross-ring cleavage yielding a major fragment ion at *m*/*z* 329 assigned to the fragment ion $^{0,2}A_2$, and three other minor fragment ions at *m*/*z* 285 (fragment ions $^{0,2}X_1$), 315 ($^{0,3}X$) and 345 (loss of CH_3CHO). The dehydrated form of the precursor ion was also observed at *m*/*z* 371. The fragmentation of the major daughter ion $^{0,2}A_2$ at *m*/*z* 329 (MS^3 experiment, data not shown) gave only one ion at *m*/*z* 285 (loss of CH_3CHO), which was also subsequently fragmented (MS^4) leading to the formation of dehydrated sulfated fucose at *m*/*z* 225 and to the loss of hydrogenosulfate anion at *m*/*z* 97. The ion $^{0,2}A_2$ resulted from the loss of the C-1–C-2 carbons, indicating that this ion originated from a disaccharide in which the reducing fucose is not sulfated in position 2. The absence of sulfation at this position was confirmed by the lack of the fragment ion $^{0,2}X_0$ at *m*/*z* 139. We have previously shown in a NMR study of disaccharide fractions obtained by mild hydrolysis of *A. nodosum* fucoidan[16] that the nonreducing fucose of mono-, di- and trisulfated difucose was mostly sulfated at position 2. Therefore we assume that the monosulfated difucose detected at *m*/*z* 389 is likely sulfated at position 2 of

Table 1. Main sulfated oligosaccharides derived from hydrolyzed algal fucoidan observed by negative ESIMS in Biogel P4 fractions and their corresponding calculated *m*/*z* values

Oligosaccharides	$[M-H]^-$	$[M-2H]^{2-}$	$[M-3H]^{3-}$	Sodium adduct
Monosulfated fucose	243.01			
Disulfated fucose		160.98		
Monosulfated difucose	389.07			
Disulfated difucose	469.03	234.01		
Trisulfated difucose	548.90	273.99	182.32	284.98 $[M-3H+Na]^{2-}$ 592.95 $[M-3H+2Na]^-$
Monosulfated trifucose	535.13			
Disulfated trifucose	615.09	307.04		637.07 $[M-H+Na]^-$
Trisulfated trifucose				358.01 $[M-3H+Na]^{2-}$
Trisulfated tetrafucose	841.10			431.03 $[M-3H+Na]^{2-}$
Tetrasulfated tetrafucose	921.06	460.02		
Pentasulfated tetrafucose		500.00	333.00	
Tetrasulfated pentafucose		533.05	355.03	
Pentasulfated pentafucose		573.03	381.68	

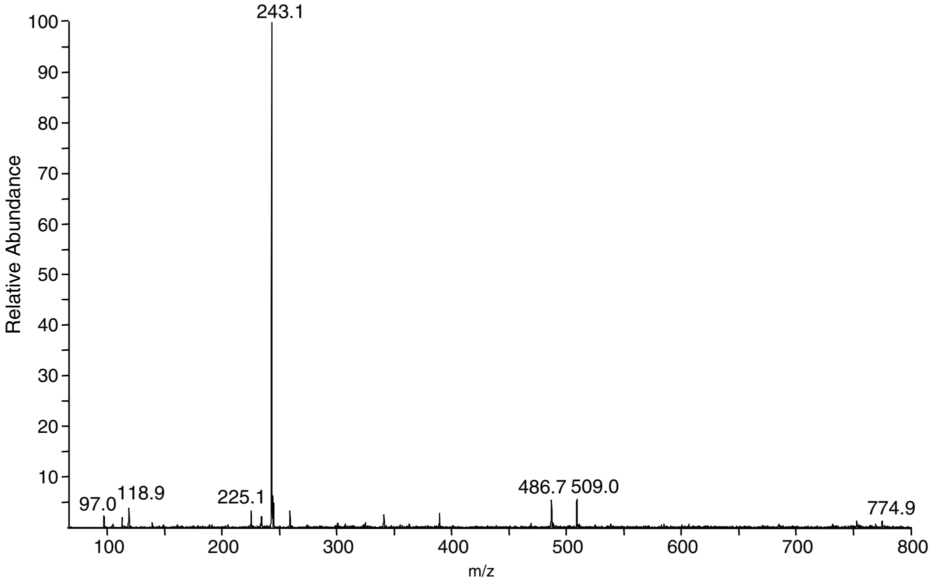

Figure 2. Negative ESIMS spectrum of the monosulfated fucose fraction (OF5) purified from LMWF-2 by size exclusion chromatography on Biogel P4.

the nonreducing fucose residue. As regard to the glycosidic linkage, a previous study of the fragmentation of heparin disaccharides showed that the mechanism of formation of the $^{0,2}A_2$ ion required an available hydrogen on the C-3 hydroxyl group, in order to assist the breaking of the C-2–C-3 bonds.[9] This condition is not fulfilled with a (1→3) linkage, and accordingly the $^{0,2}A_2$ fragment was not observed upon fragmentation of (1→3)-linked chondroitin disaccharides.[10] The essential role of the hydrogen of the hydroxyl group at C-3 in this fragmentation was also confirmed previously for sulfated L-fucose isomers which showed a lack of cross-ring cleavage at the C-2–C-3 bond for the 3-O-sulfated fucose.[12] All these gathered data suggested that the disaccharide fraction (OF4) contained (1→4)-linked species with the following structure [α-L-Fuc*p*-2-OSO_3^--(1→4)-L-Fuc*p*] (Fig. 4). Fucoidan extracted from the brow algae *A. nodosum* and other fucales has been shown to contain both (1→4) and (1→3) glycosidic bonds.[3,4,17,18] Besides, both linkages were shown to be present in the disaccharide fractions characterized in the above-mentioned NMR study.[16] Therefore, despite the lack of informative fragmentation ions issued from (1→3)-linked difucose, the presence of (1→3) glycosidic bond cannot be excluded from the disaccharide fraction (OF4). MS analysis of the deuterated monosulfated disaccharide (data not shown) showed an ion at *m*/*z* 394, that is, with an increment of 5 mass units as expected due to the presence of the five hydroxyl groups accessible to the solvent. It is worthy to point out that the dehydrated form of the disaccharide (*m*/*z* 374) resulted from the loss of D_2O and not of HOD, suggesting

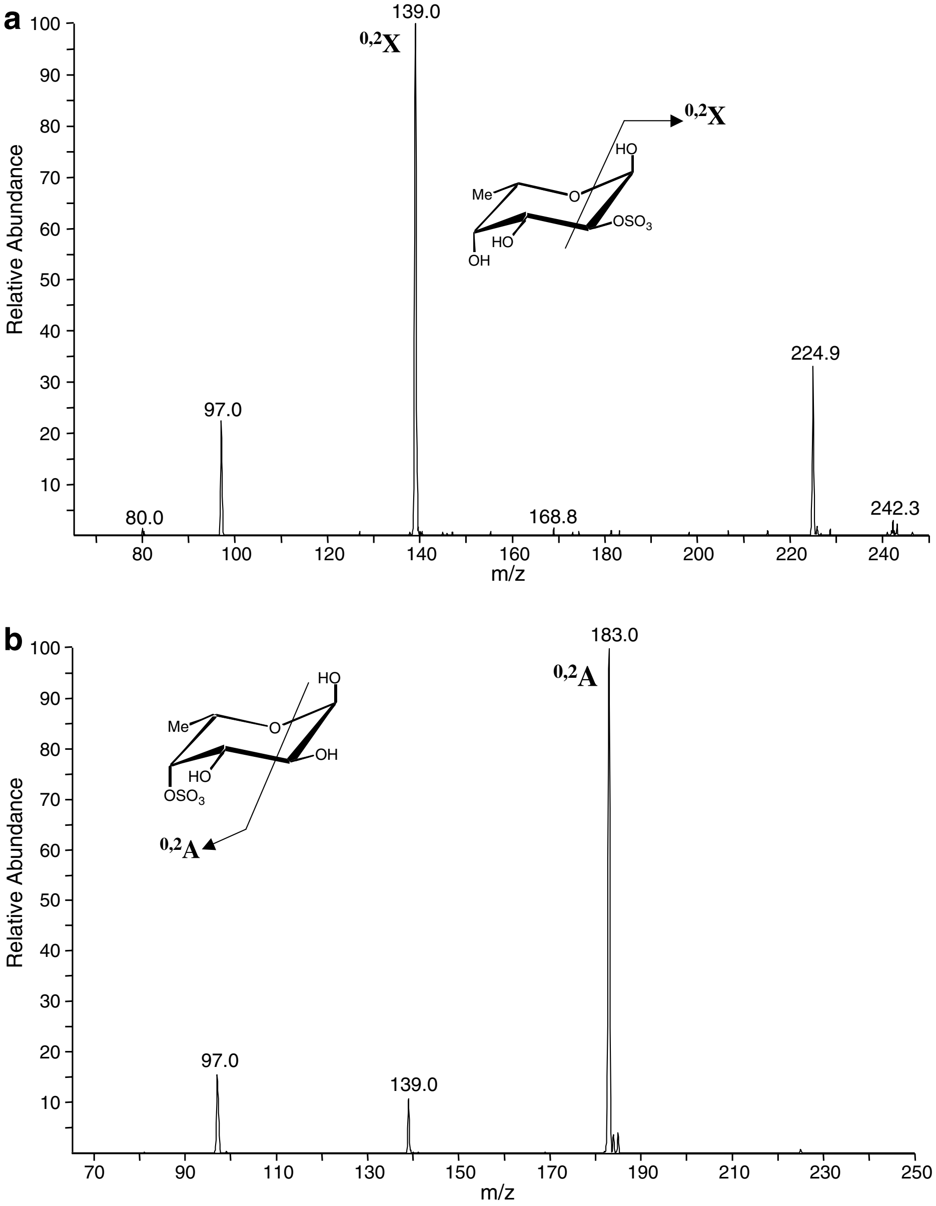

Figure 3. Negative ESIMS/MS spectra of the monosulfated fucose ion at *m*/*z* 243. (a) Monosulfated fucose issued from the algal fucoidan (fraction OF5); (b) 4-O-sulfated fucose issued from sea cucumber fucosylated chondroitin sulfate.

that the water elimination may occur through the formation of a cyclic ether. Similarly, dehydrated sulfated fucose kept only one hydroxyl group (*m*/*z* 226) because the fragmentation with dehydratation occurred via the formation of an epoxide as previously proposed.[11]

The MS spectrum of the preceding fraction (OF3) exhibited two main peaks at *m*/*z* 469 and 234 corresponding to a singly charged anion $[M-H]^-$ and to a doubly charged ion $[M-2H]^{2-}$ of the disulfated difucose, respectively (Fig. 5, inset). Fragmentation of the singly charged anion at *m*/*z* 469 was relatively uninformative with a single fragment at *m*/*z* 389 corresponding to the monosulfated disaccharide, just indicating the desulfation of the precursor ion. The fragmentation of the doubly charged anion at *m*/*z* 234 (Fig. 5) led to fragment ions arising from the loss of sulfate (hydrogenosulfate anion at *m*/*z* 97 and dehydrated monosulfated disaccharide at *m*/*z* 371) and from cross-ring and glycosidic bond cleavages. The cleavage of the glycosidic bond led to monosulfated fucose (*m*/*z* 243 and dehydrated form at *m*/*z* 225) as well as to disulfated fucose observed as the doubly charged anion at *m*/*z* 161. The relative intensity of the peak at *m*/*z* 161 varied depending on the preparation. Nevertheless this result evidences that the disulfated disaccharides contained in this fraction presented two different patterns of sulfation: one with a single sulfate group on each fucose residue (structure **I**), and another one with two sulfate

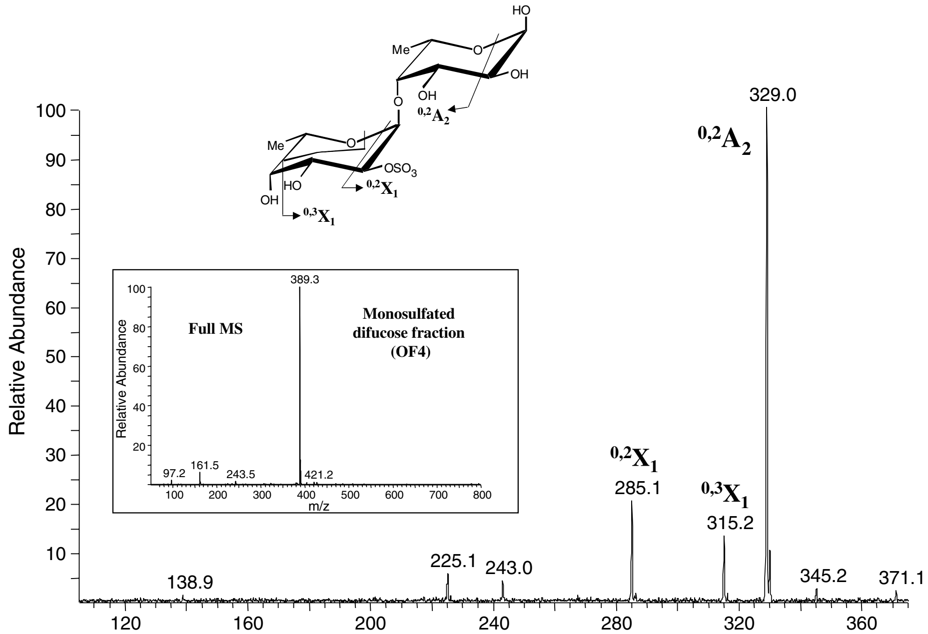

Figure 4. Negative ESIMS/MS spectrum of the monosulfated difucose ion at *m*/*z* 389 obtained from size exclusion chromatography (Biogel P4 fraction OF4, inset: full MS spectrum).

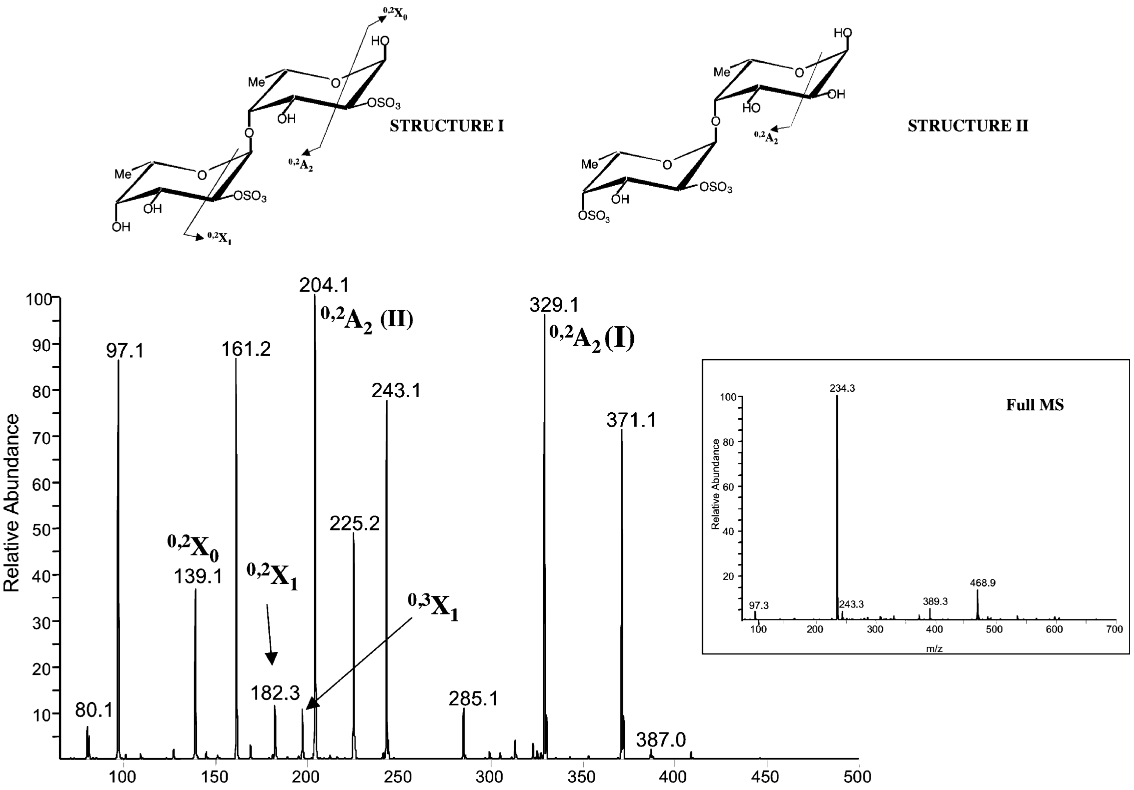

Figure 5. Negative ESIMS/MS spectrum of the disulfated difucose ion at *m*/*z* 234 ($[M-2H]^{2-}$) obtained from size exclusion chromatography (Biogel P4 fraction OF3, inset: full MS spectrum).

groups carried by one of the fucose residues (structure **II**). Disaccharides with structure **I** have been previously encountered in disaccharide fractions that we have previously characterized by NMR.[16] These fractions contained four different disaccharides either (1→3)- or (1→4)-linked, and showing various patterns of sulfation. Here, the doubly charged fragments $^{0,2}X_1$ (*m*/*z* 182, $z = 2$) and $^{0,3}X_1$ (*m*/*z* 197, $z = 2$) indicated the presence of disulfated disaccharides in which the nonreducing fucose was sulfated in position 2. The $^{0,3}X_1$ ion could also arise from a nonreducing residue sulfated in position 3. These disaccharides were sulfated on the reducing residue either at position 3 as indicated by the ion at *m*/*z* 169 ($^{0,3}X_0$), or at position 2 as indicated by the cross-ring cleavage products at *m*/*z* 139 ($^{0,2}X_0$) and 329 ($^{0,2}A_2$). The presence of the fragment $^{0,2}A_2$ indicated a (1→4) linkage (see above). When gathered, these data lead to several structural combinations for disaccharides with structure **I** in agreement with our previous NMR analysis.[16] Among them, the following combination [α-L-Fuc*p*-2-OSO_3^--(1→4)-L-Fuc*p*-2-OSO_3^-] corresponding to the most prevalent sulfation pattern in algal fucoidan has been depicted in Figure 5 (structure **I**). Again, the presence of (1→3)-linked disaccharides cannot be ruled out since both (1→4) and (1→3) glycosidic bonds have been shown to be present in *A. nodosum* fucoidan. However, the (1→3)-linked disaccharides cannot be determined due to the lack of cross-ring fragmentation of their reducing moiety and of characteristic fragments.[10,16]

Disaccharides in which one fucose residue is disulfated (structure **II**) yielded two major fragment ions at *m*/*z* 161 and at *m*/*z* 204 resulting from glycosidic bond cleavage and from cross-ring cleavage, respectively. The ion at *m*/*z* 161 corresponded to the disulfated fucose anion and the ions at *m*/*z* 204 was assigned to the disulfated fragment $^{0,2}A_2$ (doubly charged). The $^{0,2}A_2$ ion indicated that both sulfate groups were on the nonreducing fucose linked by a (1→4) glycosidic bond. In our previously reported NMR study of disaccharide fractions, we have showed the presence of disulfated disaccharides presenting 2,3-di-O-sulfated and 2,4-di-O-sulfated nonreducing fucose.[16] Based on our previous NMR determination of the major structural features of *A. nodosum* fucoidan,[3] which showed that (1→4) linkage might occur with 2,4-O-disulfated fucose, we propose the structure [α-L-Fuc*p*-2,4-OSO_3^--(1→4)-L-Fuc*p*] as one of the disaccharides **II** (Fig. 5).

The MS spectrum of the higher MW fraction (OF2) exhibited, in addition to the peaks assigned to the disulfated trifucose (deprotonated molecule $[M-H]^-$at *m*/*z* 615 and the doubly charged ion $[M-2H]^{2-}$ at *m*/*z* 307.5), two ions at *m*/*z* 549 and 274 corresponding to trisulfated difucose (respectively, the $[M-H]^-$ deprotonated molecule and the $[M-2H]^{2-}$ doubly charged ion) (Fig. 6). These latter ions provide additional evidence for disulfated fucosyl units in fucoidan. Figure 7 shows the mass spectrum of a less resolved size exclusion–fraction (OF1) containing mainly sulfated tetra- and pentasaccharides. Some of these oligosaccharides are heavily sulfated like the pentasulfated pentafucose detected at *m*/*z* 573 and *m*/*z* 382 ($[M-2H]^{2-}$ and $[M-3H]^{3-}$, respectively) and the pentasulfated tetrafucose detected at *m*/*z* 500 and *m*/*z* 333 ($[M-2H]^{2-}$ and $[M-3H]^{3-}$, respectively). This demonstrates that highly sulfated oligosaccharides from fucoidan can be analyzed by ESIMS.

Finally, our previous report concerning the ESIMS differentiation of sulfated fucose isomers[12] indicated that MS^n mass spectrometry would be helpful to analyze the sulfation pattern of oligosaccharides. This was illustrated by MS^3 experiments performed on the

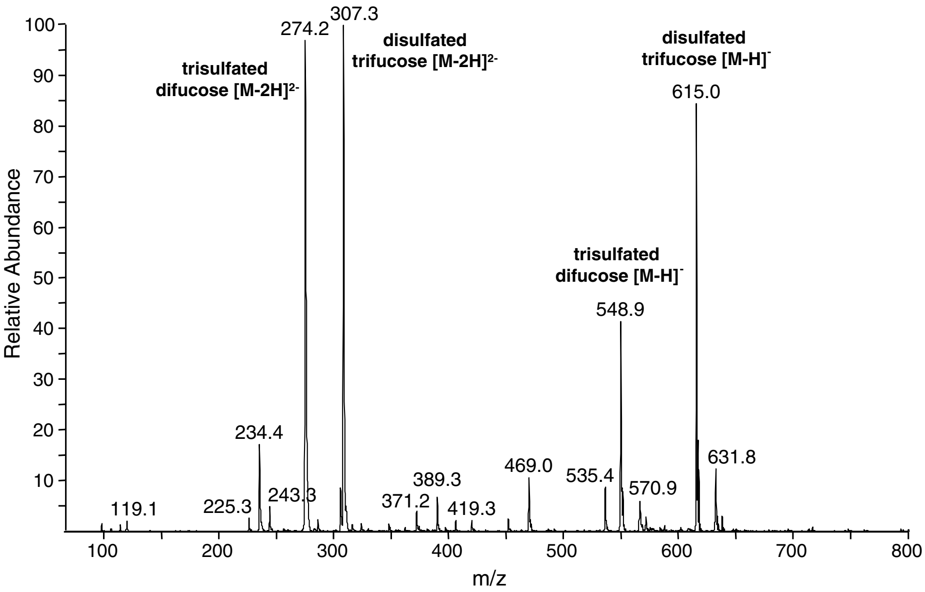

Figure 6. Negative ESIMS spectrum of the sulfated di/trifucose-containing fraction (Biogel P4 fraction OF2).

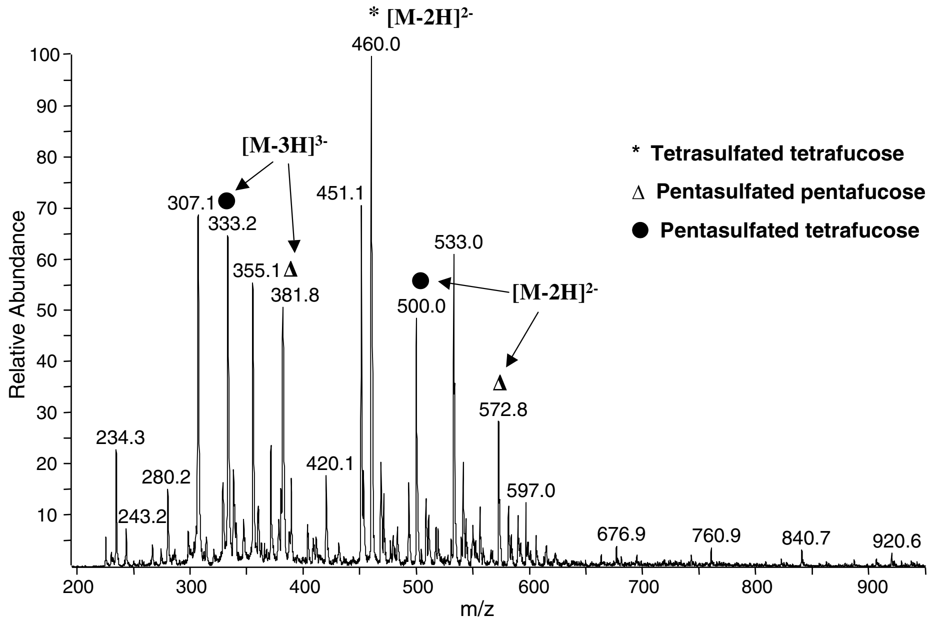

Figure 7. Negative ESIMS spectrum of the sulfated tetra/pentafucose-containing fraction (Biogel P4 fraction OF1).

disulfated disaccharide at *m*/*z* 234. The MS^2 daughter ion at *m*/*z* 243 corresponding to the monosulfated fucose was fragmented (Fig. 8). The resulting spectrum showed the above-mentioned fragment ions due to desulfation (*m*/*z* 97), dehydration (*m*/*z* 225), and cross-ring cleavage (*m*/*z* 139 and 183). The large abundance of the fragment at *m*/*z* 139 indicated that the disaccharide was mainly sulfated at position 2. Analysis of longer oligosaccharides using MS^n is currently in progress. The coupling of this strategy based on ion trap tandem MS with isotope labeling using ^{18}O and ^{2}H may allow the formation of diagnostic fragment ions useful for the elucidation of the sulfation pattern and of the glycosidic linkage. This study, based on the specificity of the fragmentation patterns according to the position of the sulfate group(s), provides the first ESIMS data on fucoidan oligosaccharides. The rules of fragmentation previously reported for heparin and chondroitin sulfated disaccharides[9,10] were applied here; however, for some of the observed fragments ions, a unique origin could still be not assert with certainty. Therefore, we are currently focusing our

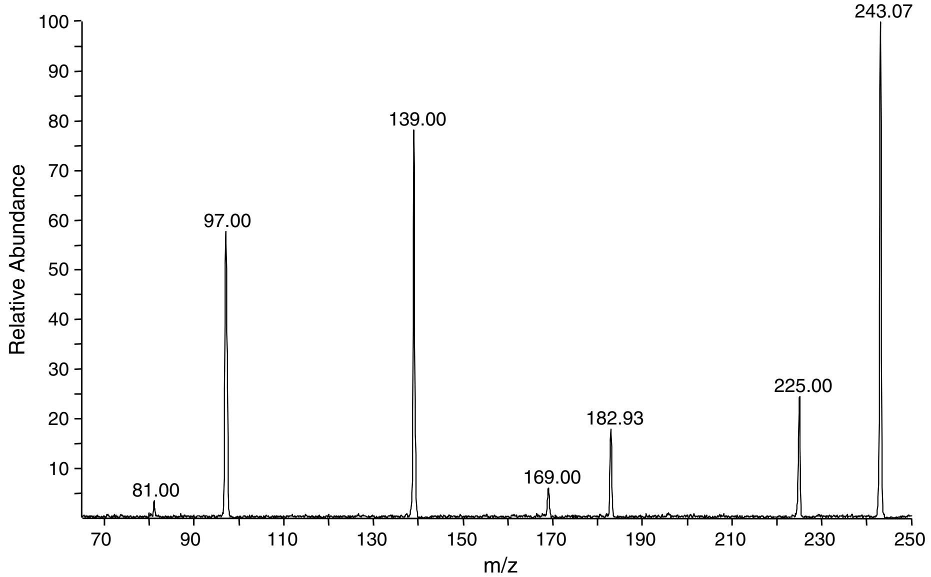

Figure 8. Negative ESIMS/MS of the monosulfated fucose fragment ion at *m*/*z* 243 resulting from the MS^2 fragmentation of the disulfated difucose precursor ion at *m*/*z* 234 (observed in fraction OF3, Fig. 5).

efforts towards the preparation of pure and structurally well-defined oligosaccharides in order to extend our results toward a rational interpretation of the MS fragmentation of fucoidan oligosaccharides.

3. Experimental

3.1. Oligofucoidan fractions

Fucoidan (M_r 25,000, fucose 47 g/100 g, sulfate 30 g/100 g) used in this study was extracted from the brown algae *A. nodosum* and purified as previously reported.[19,20] Low molecular weight fraction (LMWF) of sulfated oligofucoidan was obtained by acid hydrolysis of fucoidan in mild conditions in 0.75 mM H_2SO_4. Typically 2 g of fucoidan was suspended in 100 mL of 0.75 mM H_2SO_4 and incubated for hydrolysis for 60 min at 60 °C. The resulting hydrolysate was neutralized with $Ba(OH)_2$ and then filtered to remove the precipitate. The resulting solution was filtered through a Na-form cation-exchange column. A small aliquot (LMWF-1) was kept for ESIMS analysis and the remaining solution was ultra-filtered through a membrane (molecular weight cut-off 1000). The oligosaccharides contained in the filtrate (LMWF-2) were then purified by gel filtration on a Biogel P4 column (1.5 × 100 cm) equilibrated with 0.5 M ammonium hydrogenocarbonate buffer, pH 8.3 (flow rate 7 mL/h). Elution performed using the same buffer was followed by refractive index detection (RI-71, E. Merck). Oligosaccharides containing fractions (OF1–OF5) were freeze-dried with two intermediate lyophilization steps. 4-O-Sulfated fucose was obtained by acid hydrolysis of fucosylated chondroitin sulfate from sea cucumber as previously described.[14,15] Other chemicals and reagents were obtained from commercial sources at the highest level of purity available. All buffers and solutions were prepared with ultrapure water produced by a laboratory water purification system (Purite, UK).

3.2. Mass spectrometry analysis

ESIMS experiments were performed on a Finnigan LCQ ion trap mass spectrometer (ThermoQuest, Finnigan MAT, San Jose, CA) equipped with electrospray interface. The analyses were carried out in negative mode. The spray voltage was 3.5 kV and the capillary temperature was set to 200 °C. The isolation window for MS/MS and MS^3 experiments was 3 mass units wide, and the relative collision energy was optimized for each compound in the range between 5% and 25%. Dried samples were diluted in 1:1 MeOH–water and introduced into the mass spectrometer at a flow rate of 2 μL/min using a Havard Instruments infusion pump.

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