Carbohydrate Research 344 (2009) 779–787

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Structural analysis of a fucoidan from the brown alga *Fucus evanescens* by MALDI-TOF and tandem ESI mass spectrometry

Stanislav D. Anastyuk *, Natalia M. Shevchenko, Evgeny L. Nazarenko, Pavel S. Dmitrenok, Tatyana N. Zvyagintseva

Pacific Institute of Bioorganic Chemistry, Russian Academy of Sciences, 100 Let Vladivostok Ave. 159, 690022, Vladivostok, Russian Federation

ARTICLE INFO

Article history:
Received 8 December 2008
Received in revised form 26 January 2009
Accepted 28 January 2009
Available online 31 January 2009

Keywords:
Fucoidan
Sulfated polysaccharide
Structure
ESIMS
MALDI-TOFMS
Brown alga

ABSTRACT

A fucoidan, a heterogeneous sulfated polysaccharide from the brown alga *Fucus evanescens*, was depolymerized under solvolytic conditions, and its ethanol-extracted low-molecular-weight fraction was analyzed by MALDI-TOFMS and ESIMS/MS. It was found that the mixture contained unsulfated oligosaccharides including some monosulfated components, which were shown to consist of mainly (1→3)-linked 2-O-sulfonated fucose residues (from 1 to 4). Minor components of the mixture were shown to contain 2-O- and 4-O-sulfonated xylose and galactose residues. Among them, mixed monosulfonated fucooligosaccharides were detected and characterized: Xyl-(1→4)-Fuc, Gal-(1→4)-Fuc, Gal-(1→4)-Gal-(1→4)-Fuc, Gal-(1→4)-Gal. Fucose, galactose, and xylose residues were shown to be mainly 2-O-sulfonated with traces of 4-O-sulfonation. Glucuronic acid was also found as a part of non-sulfated fucooligosaccharides: Fuc-(1→3)-GlcA, Fuc-(1→4)-Fuc-(1→3)-GlcA, Fuc-(1→3)-Fuc-(1→4)-Fuc-(1→3)-GlcA.

1. Introduction

Fucoidans belong to a family of sulfated homo- and heteropolysaccharides possessing diverse biological activities.[1,2] They include polysaccharides with a high content of glucuronic acid and a low content of fucose and sulfates, and polysaccharides built as sulfated homopolymers of fucose (fucans). Galactose, mannose, xylose, and rhamnose moieties have also been found in various fucoidans.[3–8] Although fucoidans have been known since 1913, only a few structures have been fully established. Brown algae synthesize highly branched polysaccharides with species-specific sugar compositions,[3,5,7,8] and for this reason, fucoidan structures are extremely diverse. It was recently shown that fucoidans have α-(1→3)-backbones or repeating disaccharide units of α-(1→3)- and α-(1→4)-linked fucose residues with O-2 branches. Depending on the structure of the main chain, fucoidans may be sulfonated at O-4, O-2, or at both positions of fucose units.[5,6,9–12] Some fucoidans are both sulfated and acetylated.[9,13] Generally, brown seaweeds belonging to the orders Chordariales and Laminariales (Phaeosporophyceae) produce polysaccharides built of (1→3)-linked α-L-Fuc*p* residues in the main chains.[5,11,13–15] Side chains consist of D-glucuronate (*Cladosiphon okamuranus*) or fucose residues (*Chorda filum*), and are attached to the O-2 position of the main chain's fucose residues in a regular manner. However, this regularity is masked by random sulfation and acetylation. An exception is the fucoidan from *Laminaria gurjanovae*,[16] which is, in fact, a galactofucan similar to the fucoidan from *Undaria pinnatifida*.[17] The main chains of the fucoidans from brown seaweeds of the order Fucales (Cyclosporophyceae) are built mainly of alternating (1→3)- and (1→4)-linked α-L-fucosyl residues. Fucoidan from *Fucus evanescens*, belonging to the same order, also was shown to contain a linear backbone of alternating 3- and 4-linked α-L-fucopyranose 2-sulfate residues. Additional sulfate occupies position 4 in some 3-linked fucose residues.[6,9,18]

Chemical methods of structural analysis, as well as NMR spectra of native algal fucoidans, usually give only partial information on their structures. In order to simplify the structure of the polysaccharide and obtain interpretable NMR spectra, some chemical modifications are applied to native fucoidan: acid hydrolysis, desulfation and deacetylation.[19,20] The yield of polysaccharide after these procedures often does not exceed 2–7% of the crude preparation.[9,21] Thus, a large amount of structural information on minor components found in known seaweed fucoidan samples (galactose, mannose, xylose and glucose) is being lost.

Along with NMR spectroscopy, MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) and ESI (electrospray-ionization) mass spectrometry are analytical methods of high sensitivity and selectivity. Their benefits include wide dynamic range, the ability to observe a diverse number of molecular species, and reproducible quantitative analysis. They are perfectly suitable for anionic carbohydrates analysis.[22] Recent MS studies on

* Corresponding author. Tel./fax: +7 4232 310705.
E-mail address: sanastyuk@piboc.dvo.ru (S.D. Anastyuk).

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doi:10.1016/j.carres.2009.01.023

fucoidans[16,23,24] have shown the capabilities of MS methods to effectively reveal additional structural and compositional information.

The present work is devoted to the structural features analysis (in addition to structures, established mostly with NMR by Bilan et al.[9] and Kusaykin et al.[18]) and determination of the exact location of minor structural elements of the fucoidan isolated from the Pacific brown alga *F. evanescens* (order Fucales, Cyclosporophyceae, Phaeophyta), by MALDI-TOFS and ESIMS/MS.

2. Results and discussion

The fucoidan from *F. evanescens*, which was shown[9] to be a linear polymer with alternating (1→3)- and (1→4)-linked fucose residues, sulfonated mainly at O-2 and partially acetylated, substantially differed from the fucoidan fraction under study. Our sample was shown to contain nearly 3.5 times more (1→3)-linked fucose residues over (1→4)-linked units.[18] Fucoidan was depolymerized under solvolytic conditions (see Section 4) and low-molecular-weight oligosaccharides suitable for MS analysis were obtained. It was previously shown by our group (for the fucoidan from the brown seaweed *L. gurjanovae*) that products of desulfation contained low-molecular-weight unsulfated fragments of fucoidan as the main product of reaction, along with desulfated polysaccharide. The mixture still contained some sulfated oligosaccharides, detected by MALDI-TOFMS in the negative-ionization mode.[16] Although it was known that during solvolysis some cleavages of glycosidic bonds might occur,[9,21] this is the first work, where low-molecular-weight unsulfated fragments of fucoidan (still containing a minor percent of sulfated oligosaccharides) were extracted and then analyzed by MALDI-TOF and tandem ESI mass spectrometry.

The low-molecular-weight mixture, extracted from products of desulfation with EtOH, **dSlmf**, was analyzed by MALDI-TOFMS in the positive-ion mode without any purification. A distribution of singly charged ions corresponding to fucose oligomers $[Fuc_n + Na]^+$ (n = 1–6) was detected (see insertion in Fig. 1). There were some less-intensive signals in the spectrum, which were found to be ions from mixed oligosaccharides when one of the fucose residues in the chain was substituted by another sugar residue, that is, $[Fuc_1Pent_1 + Na]^+$ at m/z 319.0 and $[Fuc_1Hex_1 + Na]^+$ at m/z 349.1 differed from $[Fuc_2 + Na]^+$ at m/z 333.1 by −14 and +16 Da, respectively. Thus, pentose- and hexose-containing oligosaccharides were detected in a positive MALDI-TOFMS of **dSlmf** as $[Fuc_nPent + Na]^+$, n = 1–6 and as $[Fuc_nHex + Na]^+$, n = 1–6. Oligosaccharides, containing uronic acid, were not detected. Then, a sample was separated using reversed-phase HPLC (see Section 4). An anionic fraction 1, which was not retained on the column (**AF1**), was exposed to ESIMS analysis in the negative-ion mode to investigate if some sulfated oligosaccharides remained in the mixture.

The high sensitivity of the ESI Q-TOF instrument allowed us to acquire interpretable MS/MS spectra of sulfated sugar compounds with even trace quantities, including those detected in this case, uronic acid-containing oligosaccharides (see Fig. 1). The fact that sulfated oligosaccharides were detected indicates that the desulfation reaction was not exhaustive. Due to the monosaccharide composition analysis, pentose was assigned to xylose (Xyl), hexose was assigned to galactose (Gal), hexuronic acid was assigned to glucuronic acid (GlcA), (see Section 4). Full information on the fragments found in **AF1** is depicted in Table 1. For the first time, to our knowledge, we have shown that the fucoidan (fraction **AF1**) from *F. evanescens* contained sulfated xylose (at m/z 229.006) and galactose (at m/z 259.018) residues. Free uronic acid was not detected, but was found in fragments $[Fuc_nGlcA–Na]^-$, n = 1–3 for the first time.

It is clear that each peak in the mass spectrum of **AF1** may contain a number of isomers, which may differ in sulfation pattern, type of linkages, etc. But by having characteristic ions of each isomer in a collision-induced dissociation (CID) MS/MS spectrum, we often may reveal the structural features of separate components. Recent studies proposing the formation of characteristic ions during negative CID MS/MS on sulfated fucose isomers[23] and sulfated fucooligomers[24] were used in the present work. It was shown that the formation of the $^{0,2}A_2$ ion (following Domon and Costello's nomenclature[25] see Fig. 2) required an available hydrogen at the C-3 hydroxyl group in order to assist breaking of the bonds be-

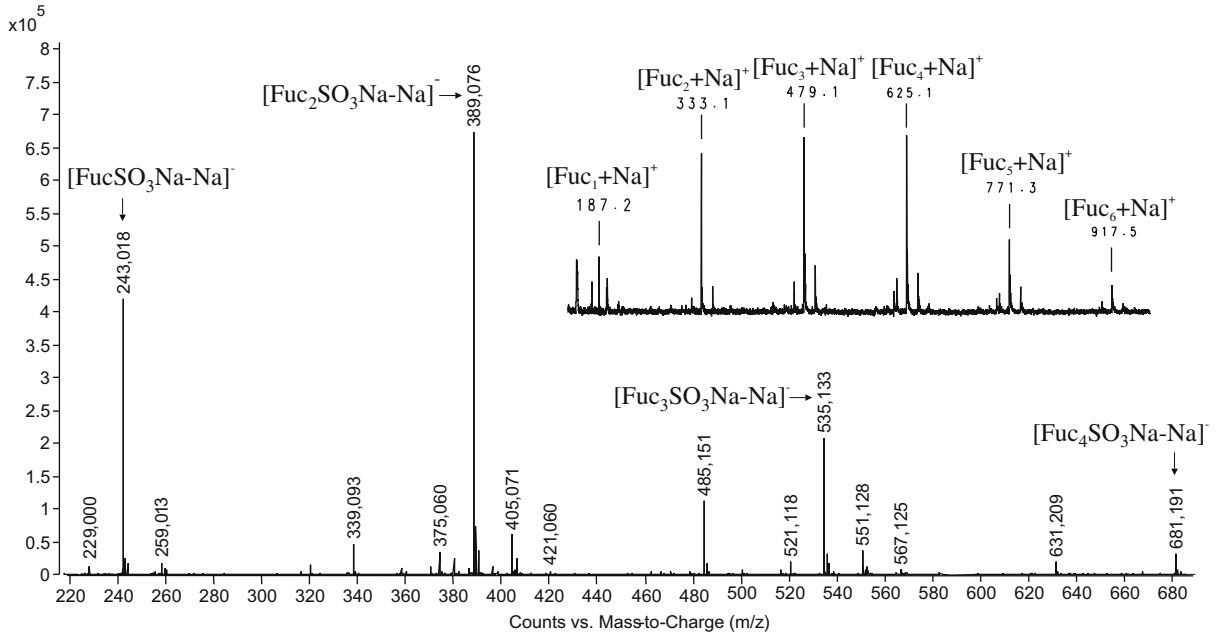

Figure 1. Negative-ion mode ESIMS spectrum of sulfated oligosaccharides of fraction AF1, obtained from the **dSlmf** fraction by HPLC on a C18-column (inset contains a positive-ion mode MALDI-TOFMS of the oligomeric fraction **dSlmf**, obtained under solvolytic conditions).

Table 1
ESIMS observed *m/z* values of the main and minor (*) oligosaccharides of fraction **AF1** from the fucoidan from brown alga *F. evanescens*

m/z	Composition
229.000*	$[Xyl_1SO_3Na–Na]^-$
243.018	$[Fuc_1SO_3Na–Na]^-$
259.013*	$[Gal_1SO_3Na–Na]^-$
339.093*	$[Fuc_1GlcUA_1]^-$
375.060*	$[Fuc_1Xyl_1SO_3Na–Na]^-$
389.076	$[Fuc_2SO_3Na–Na]^-$
405.071*	$[Fuc_1Gal_1SO_3Na–Na]^-$
421.060*	$[Gal_2SO_3Na–Na]^-$
485.151	$[Fuc_2GlcUA_1]^-$
521.118*	$[Fuc_2Xyl_1SO_3Na–Na]^-$
535.133	$[Fuc_3SO_3Na–Na]^-$
551.128*	$[Fuc_2Gal_1SO_3Na–Na]^-$
567.125*	$[Fuc_1Gal_2SO_3Na–Na]^-$
631.209*	$[Fuc_3GlcUA_1]^-$
681.191*	$[Fuc_4SO_3Na–Na]^-$

tween C-2 and C-3; hence, no $^{0,2}A_2$ ions appeared upon fragmentation of (1→3)-linked disaccharides.[26]

Figure 3 shows the CID MS/MS fragmentation of the ion at *m/z* 229.003, corresponding to $[XylSO_3Na–Na]^-$. The most abundant ion at *m/z* 96.963 suggested the loss of HSO_4^- (this ion was present on every MS/MS spectrum of sulfated compounds and will not be mentioned further), and less-intensive fragments at *m/z* 138.970 and 168.979 from cross-ring cleavage were seen. The first one was assigned to the $^{0,2}X$-type ion, indicating sulfation of the xylose at position 2, and the second ion was assigned to the $^{0,2}A$-type fragment, suggesting sulfation at position 4. The $^{0,3}X$-type ions were not detected. Thus, the mixture contained 2-O- and 4-O-sulfonated xylose. Again, to the best of our knowledge, this is the first detection of sulfated xylose residue in the fucoidan under study.

The MS/MS spectrum of the xylose-containing fragment $[Fuc_1Xyl_1SO_3Na–Na]^-$ at *m/z* 375.059 exhibited a complicated fragmentation pattern (see Fig. 4). Fragment ions at *m/z* 210.993 and 225.006 were assigned to Z-type ions arising from glycosidic bond cleavage of dehydrated xylose and fucose sulfates, respectively. It is worth noting, that the residue of xylose sulfate on the reducing end exhibited much less Y_1-type fragmentation (Y'_1 at *m/z* 228.996) than a fucose sulfate residue at *m/z* 243.014. Abundant $^{0,2}X_0$ ions at *m/z* 138.972 suggested that reducing sugars were sulfonated at the O-2 position; however, the presence of $^{0,2}A_1$-type ions of low intensity at *m/z* 168.976 and 182.994 indicated sulfonation at the O-4 position of some nonreducing xylose and fucose residues, respectively. The $^{0,2}X_1$ fragment ions found at *m/z* 271.008 and 285.032 might also arise from disaccharides where sulfate occupied the C-2 position of a nonreducing sugar residue. The $^{0,3}X_0$ fragment ion at *m/z* 301.012 from cross-ring cleavage of structure with reducing fucose and nonreducing sulfated xylose residue showed that this structural fragment might be (1→3)-linked. Other strong evidence of (1→3)-linkage was absent. The relatively high abundance of $^{0,2}X_0$ and $^{0,2}A_2$ fragments (second one from both reducing xylose and fucose residue, when the nonreducing sugar is sulfated) indicated that (1→4)-types of linkages of the both structure variants were present. The CID MS/MS spectrum of the longer xylose-containing fragment $[Fuc_2Xyl_1SO_3Na–Na]^-$ at *m/z* 521.126 (data not shown) yielded ambiguous data. It was not even possible to distinguish the position of xylose because of the same molecular weight of the key fragments.

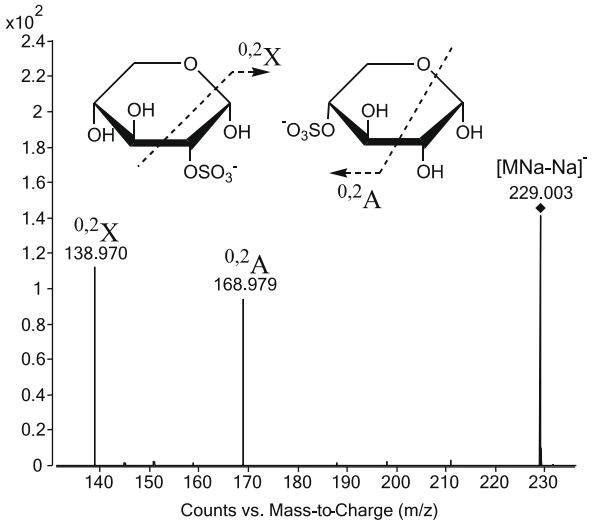

Figure 3. Negative-ion mode CID ESIMS/MS spectrum of the ion $[XylSO_3Na–Na]^-$ at *m/z* 229.003.

Figure 5 shows the CID MS/MS spectrum of $[Fuc_1GlcA–Na]^-$ at *m/z* 339.093. The most abundant $^{0,2}X_1$ fragment at *m/z* 235.046 arose from cross-ring cleavage of the nonreducing end fucose residue. Fragment ions at *m/z* 175.025 and 193.036 were assigned to Z_1 and Y_1 ions (GlcA and GlcA–H_2O), respectively, arising from cleavage of the glycosidic bonds. The ion at *m/z* 261.061 was assumed to be a $^{2,5}A_2$-type ion, arising from cross-ring cleavage of the reducing end GlcA. Such a unique cleavage has been reported to be from reducing uronic acids, only.[27] Thus, assuming all the above-mentioned and low-intensity $^{0,2}A_2$-type fragments, found at *m/z* 279.067, (arising from reducing GlcA), we could presume that the mixture contained (1→3)-linked fucose and GlcA residues as prevalent components. The fragmentation pattern of the next uronic acid-containing trisaccharide $[Fuc_2GlcA–Na]^-$ ion at *m/z* 485.158 and $[Fuc_3GlcA–Na]^-$ ion at *m/z* 631.204 was similar, so the discussion would proceed with the last tetrasaccharide, whose CID MS/MS spectrum is shown in Figure 6. The major fragmentation of the selected ion yielded Y- and Z-type ions as the main fragments, corresponding to successive cleavages of glycosidic bonds of the

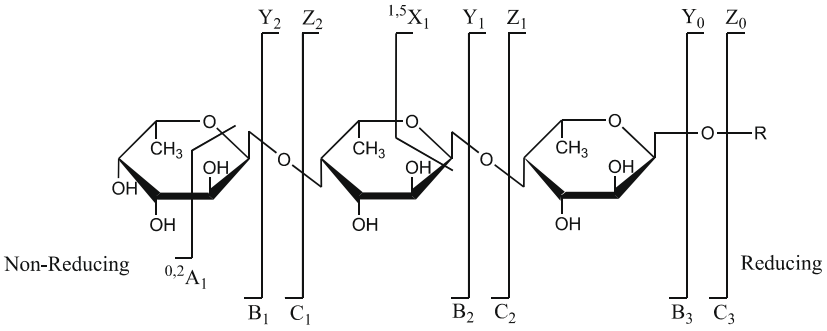

Figure 2. Nomenclature according to Domon and Costello.[25]

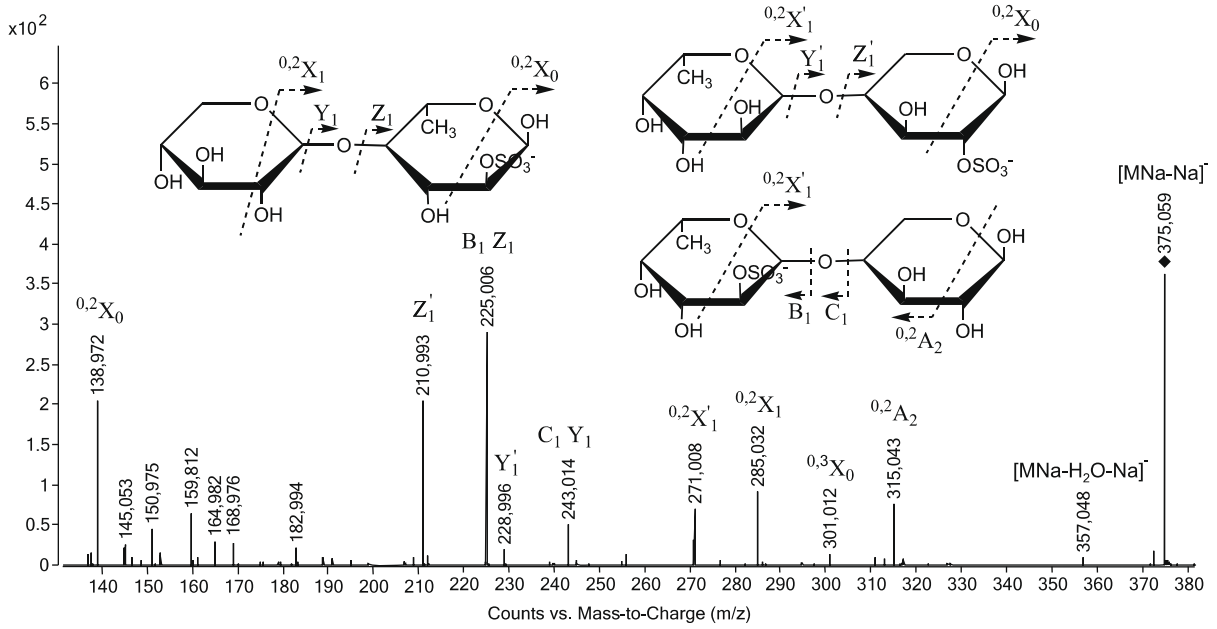

Figure 4. Negative-ion mode CID ESIMS/MS spectrum of $[Xyl_1Fuc_1SO_3Na–Na]^-$ at *m*/*z* 375.059.

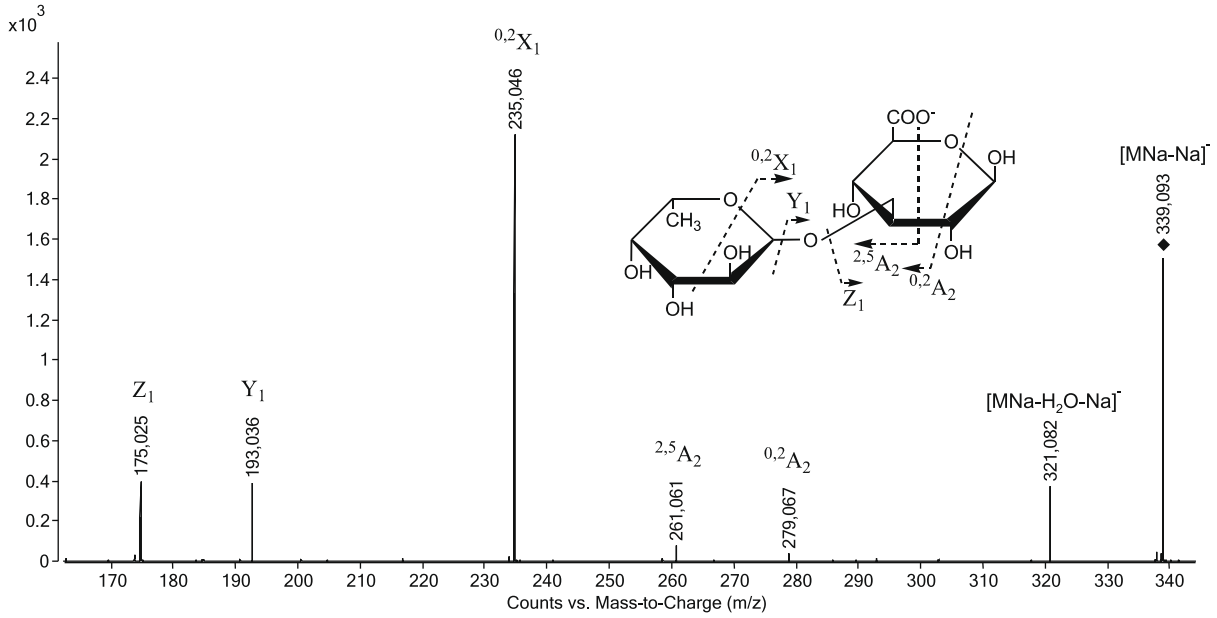

Figure 5. Negative-ion mode CID ESIMS/MS spectrum of $[Fuc_1GlcA–Na]^-$ at *m*/*z* 339.093].

oligosaccharide chain: Z_3 and Y_3 ions found at *m*/*z* 467.140 and 485.147 suggest cleavage of fucose and its dehydrated form from the nonreducing end; Z_2 and Y_2 ions, found at *m*/*z* 321.083 and 339.097 suggest cleavage of fucobiose and its dehydrated form from the nonreducing terminus and so on. Thus, while the spectrum contained characteristic $^{2,5}A_4$ and $^{0,2}A_4$ ions of glucuronic acid (see discussion of the $[Fuc_1GlcA–Na]^-$ fragment) found at *m*/*z* 553.174 and 571.189 and contained no ^{0,3}A- or ^{0,3}X-type ions, that suggested branching, we had concluded that the selected ion had a linear structure with a reducing end of glucuronic acid. The lack of interference from the sulfates and suitable position of the glucuronic acid allowed us to analyze the intensities of ^{0,2}X-fragments of the fucotriose chain. The $^{0,2}X_3$ fragment ion at *m*/*z* 527.159, arising from a nonreducing-end terminal fucose residue, had the highest abundance, because the fucose residue had an available hydrogen of the hydroxyl at C-3 (see above), that would assist in breaking bonds between C-2 and C-3. The $^{0,2}X_2$ fragment ion at *m*/*z* 381.102 of the second fucose residue from the nonreducing end had a dramatically lower intensity, having the prevalence of the (1→3)-type linkage over the (1→4)-type. In the same manner, the $^{0,2}X_1$ fragment ion at *m*/*z* 235.045 of the third fucose residue from the nonreducing terminus had an abundance of about three-times higher than previous fragment, having a prevalence of the (1→4)-type linkage over a (1→3)-linkage. The low-abundant $^{0,2}A_4$ fragment ion at *m*/*z* 571.204 suggested that the Fuc residue, following the GlcA residue, was mainly (1→3)-linked. Summarizing all the facts mentioned above, we have concluded that the prevalent structure of the selected ion was Fuc-(1→3)-Fuc-(1→4)-Fuc-(1→3)-GlcA. As mentioned above, the similar mass spectrum of the $[Fuc_2GlcA–Na]^-$ (data not shown) ion at *m*/*z* 485.151 also contained Fuc-(1→4)-

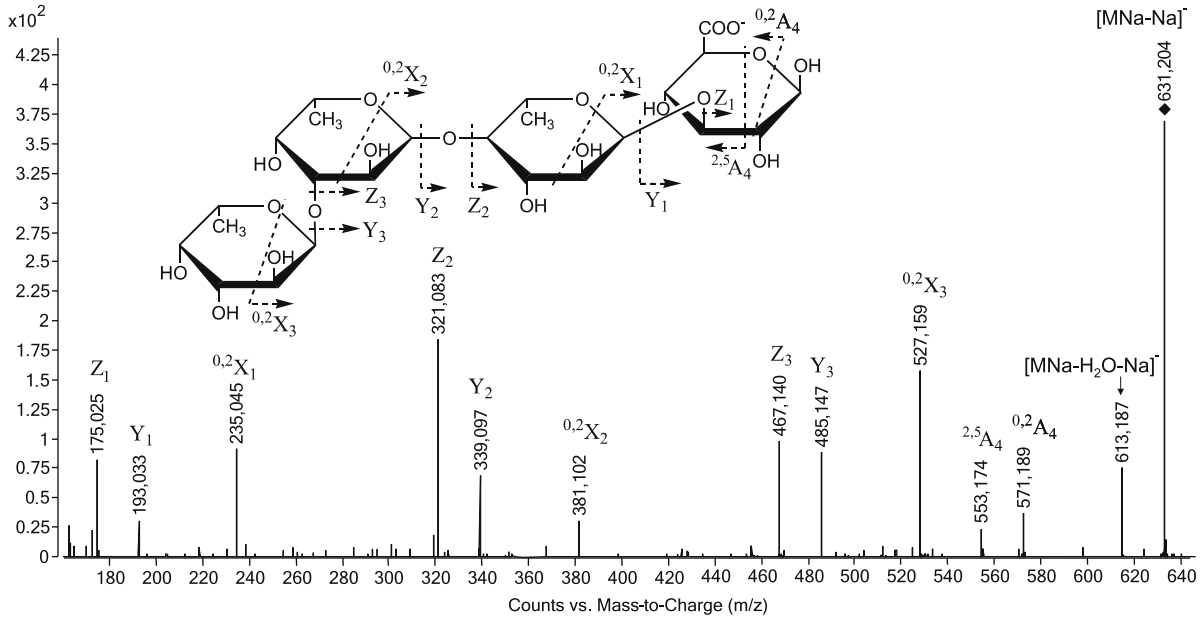

Figure 6. Negative-ion mode CID ESIMS/MS spectrum of $[Fuc_3GlcA–Na]^-$ ion at *m*/*z* 631.204.

Fuc-(1→3)-GlcA as a primary structural variant. Previously, the fucoidan was shown to contain a linear backbone of alternating 3- and 4-linked α-L-fucopyranose.[9] Hence, most probably, GlcA-containing fragments, found in the low-molecular-weight products of solvolysis, represented GlcA-ended backbone fragments.

After obtaining CID fragments of the abundant $[FucSO_3Na–Na]^-$ ion at *m*/*z* 243.016 (data not shown), we acquired essentially the same results as Daniel et al., 2007 with fucooligosaccharides from *Ascophillum nodosum*. The difference was that our sample contained both 2-O- and 4-O-sulfonated fucose in the mixture, since the intensities of the ^{0,2}A-ion at *m*/*z* 182.996 and ^{0,2}X-ion at *m*/*z* 138.971 were almost equal.

The fragmentation pattern of the most abundant $[Fuc_2SO_3Na–Na]^-$ ion at *m*/*z* 389.082 was in many ways similar to the $[Fuc_3SO_3Na–Na]^-$ ion at *m*/*z* 535.131 shown in Figure 7. CID MS/MS fragmentation exhibited the abundant ion at *m*/*z* 225.007, which was assigned as the fragment from glycosidic bond cleavage (dehydrated form of a sulfated fucose residue) as well as the ion at *m*/*z* 243.017 (sulfated fucose residue). Less-intensive fragments from glycosidic bond cleavages were found at *m*/*z* 371.065 and 389.074, assigned to a dehydrated fucobiose sulfate and fucobiose sulfate, respectively. The $^{0,2}X_0$-type ion at *m*/*z* 138.971 and $^{0,2}X_1$ at *m*/*z* 285.029 from cross-ring cleavage indicated that sulfonation might occur at the O-2-position of nonreducing fucose and at the same position of the second fucose from the reducing end, respectively. The $^{0,2}X_2$ ion at *m*/*z* 431.085 could arise both from the sulfonated O-2-position of the nonreducing-end fucose residue and from the O-2 positions of the other fucose residues, since no $^{0,3}A_2$

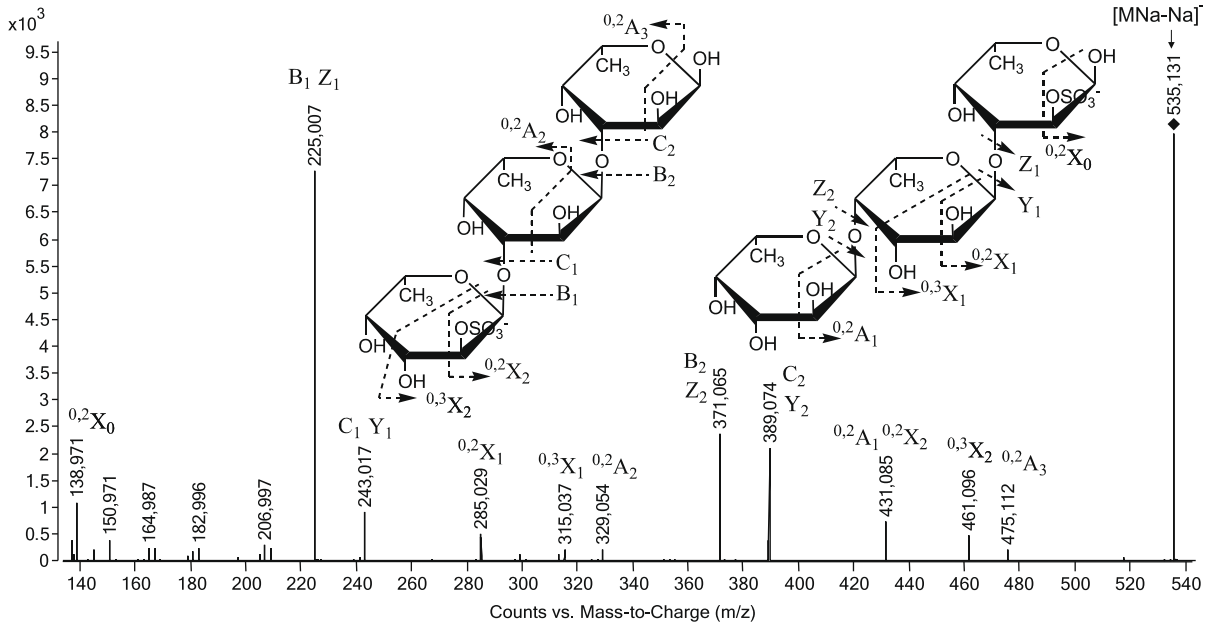

Figure 7. Negative-ion mode CID MS/MS spectrum of $[Fuc_3SO_3Na–Na]^-$ at *m*/*z* 535.131.

and $^{0,3}A_3$ fragments, indicating sulfonation at O-4, were found. However, a weak signal found at *m/z* 182.996 suggested that the nonreducing-end fucose residue of the parent ion might be 4-O-sulfonated. Thus, with an increase in the degree of polymerization it was harder to determine the sulfation pattern of the ions observed. The low-abundant $^{0,2}A_2$-ion at *m/z* 329.054 (compared to $^{0,2}X_2$-ion at *m/z* 431.085 from cross-ring cleavage of nonreducing-end fucose with a free hydroxyl group at C-3) illustrated that the parent ion with a possible structure, depicted in Figure 7 (structure to the left), had a prevalence of the (1→3)-type linkage over the (1→4)-type between two nonreducing fucose residues. However, the MS/MS spectrum contained a $^{0,3}X_1$-type ion, found at *m/z* 315.037 along with $^{0,2}X_1$ at *m/z* 285.029, confirming a (1→4)-type linkage between the nonreducing fucose residues (see Fig. 7, structure to the right). The low intensity of $^{0,2}A_3$ ion at *m/z* 475.112 and the above-mentioned $^{0,2}X_0$ also suggested the prevalence of a (1→3)-type linkage over a (1→4)-type. All the data gathered on the selected ion was in good agreement with the structural features of the fucoidan sample from *F. evanescens* reported by Kusaykin et al.[18] The longer sulfated fucooligosaccharide fragments were not analyzed due to the high complexity of the mass spectrum and the abundant noise signals.

The fragmentation pattern of the $[GalSO_3Na–Na]^-$ ion at *m/z* 259.018 (data not shown) was similar to that reported earlier.[28] Again, the mass spectrum contained both an ^{0,2}A-ion at *m/z* 198.992 and an ^{0,2}X-ion at *m/z* 138.973 in high abundance, suggesting 2-O- and 4-O-, and, probably 6-O-sulfonation. The ^{0,3}A fragment ions at 168.979 arising from cross-ring cleavages of the pyranose ring could also be from 3-O-, 4-O-, and 6-O-sulfonation. It is worthwhile to point out that galactose produced more intense ^{0,3}A fragments than did fucose.

The CID MS/MS fragmentation of the very low-abundant $[Gal_2SO_3Na–Na]^-$ ion at *m/z* 421.060 (see Fig. 8) yielded an abundant ion at *m/z* 241.000 and a less intensive ion at *m/z* 259.012, arising from glycosidic bond cleavage. They were assigned to a dehydrated galactose sulfate and galactose sulfate residues, respectively. The appearance of an $^{0,2}X_0$-type ion at *m/z* 138.970 suggested sulfation at O-2 of the reducing-end galactose residue, while the $^{0,2}X_1$-type ion at *m/z* 301.022 and the $^{0,3}X_1$-type ion at *m/z* 331.032 could also suggest sulfonation at the O-2 of the nonreducing end galactose moiety. The last ion might also arise from a (1→3)-linked disaccharide, sulfonated at the O-2-position of the reducing galactose residue. The lack of characteristic ions, indicating sulfonation at O-4 of both reducing and nonreducing-end galactose residues among the intensive $^{0,2}A_2$-ions at *m/z* 361.040 allowed us to presume that structure Gal-2-SO_3^--(1→4)-Gal had a prevalence over the other structural variants of the $[Gal_2SO_3Na–Na]^-$ ion.

Looking to the disaccharides observed previously, we can predict that the selected $[Fuc_1Gal_1SO_3Na–Na]^-$ ion at *m/z* 405.070 might also consist of several isomers, both with reducing fucose and galactose residues, sulfonated at O-2- and O-4- of nonreducing residues and at the O-2-position of the reducing-end residues (see Fig. 9). Indeed, fragmentation exhibited an abundant ion at *m/z* 225.007 and 241.003 from glycosidic bond cleavage, corresponding to dehydrated fucose sulfate and galactose sulfate residues, respectively. The $^{0,2}X_0$ fragment ions at *m/z* 138.971 indicated sulfonation at O-2-position of the reducing moiety, while ^{0,2}X-type fragment ions found at *m/z* 285.029 and 301.021 revealed that nonreducing galactose and fucose residues might be also 2-O-sulfonated. The $^{0,3}A_1$- and $^{0,2}A_1$-type fragment ions found at *m/z* 168.979 and 198.992, respectively, possibly suggest (see above), 4-O-sulfonation of the nonreducing galactose residue. No ions, indicating 4-O-sulfonation of nonreducing fucose residues, were found. Besides the $^{0,2}A_2$-ion at *m/z* 345.047 and the above-mentioned $^{0,2}X_0$-type ion of a relatively high abundance that suggested a (1→4)-type linkage, we could not exclude a (1→3)-type linkage of the parent ion. The evidence for a (1→3)-linkage might be the $^{0,3}A_2$-type ion at *m/z* 315.042 of the structure shown in Figure 9 (bottom-left) that has the same *m/z* as the $^{0,3}X_1$-fragment of the other possible structure shown in the same figure.

CID MS/MS fragmentation of the $[Fuc_1Gal_2SO_3Na–Na]^-$ ion at *m/z* 567.124 shown in Figure 10 exhibited the most complex fragmentation pattern. The abundant ions at *m/z* 225.006 and 241.002 were assigned to Z-type ions from glycosidic bond cleavage fragments and represented dehydrated forms of sulfated fucose and galactose residues, respectively. Fragment ions at *m/z* 403.055 and 387.056 were assigned to cleavage of FucGal sulfate and its dehydrated form, respectively. A signal corresponding to cleavage of a sulfated galactose residue was found at *m/z* 259.009, and the corresponding fucose-sulfate signal was not detected. The low-abundant $^{0,2}X_1$-type fragment ions at *m/z* 285.030 and 301.026 (see Fig. 10, upper and lower structures) suggested that the

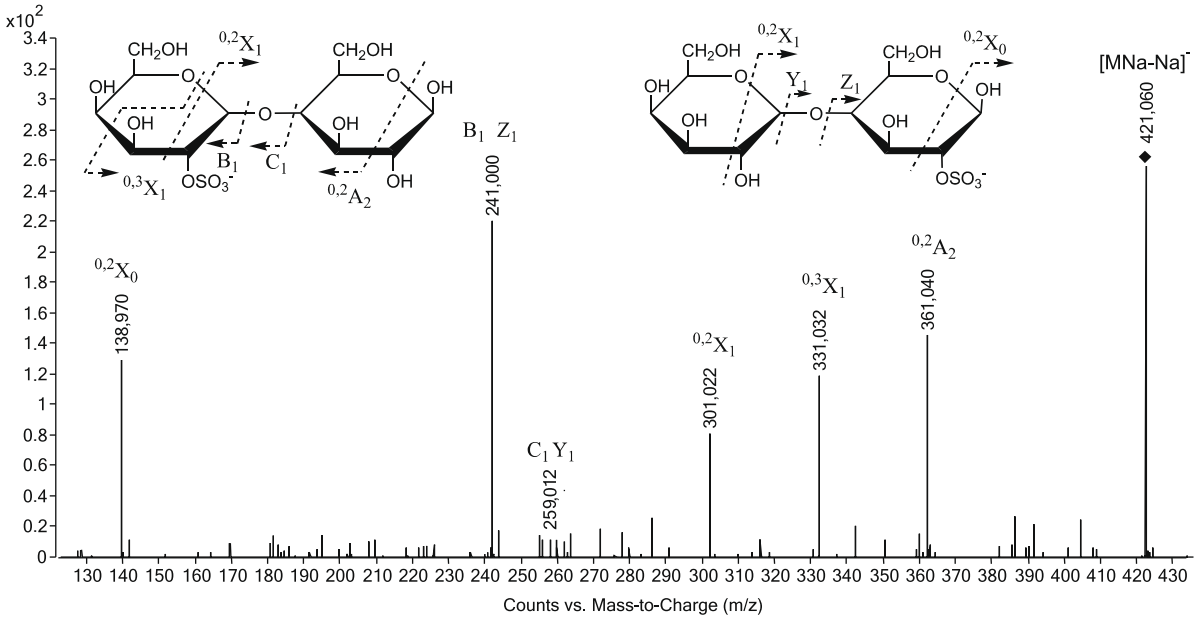

Figure 8. Negative-ion mode CID MS/MS spectrum of $[Gal_2SO_3Na–Na]^-$ ion at *m/z* 421.060.

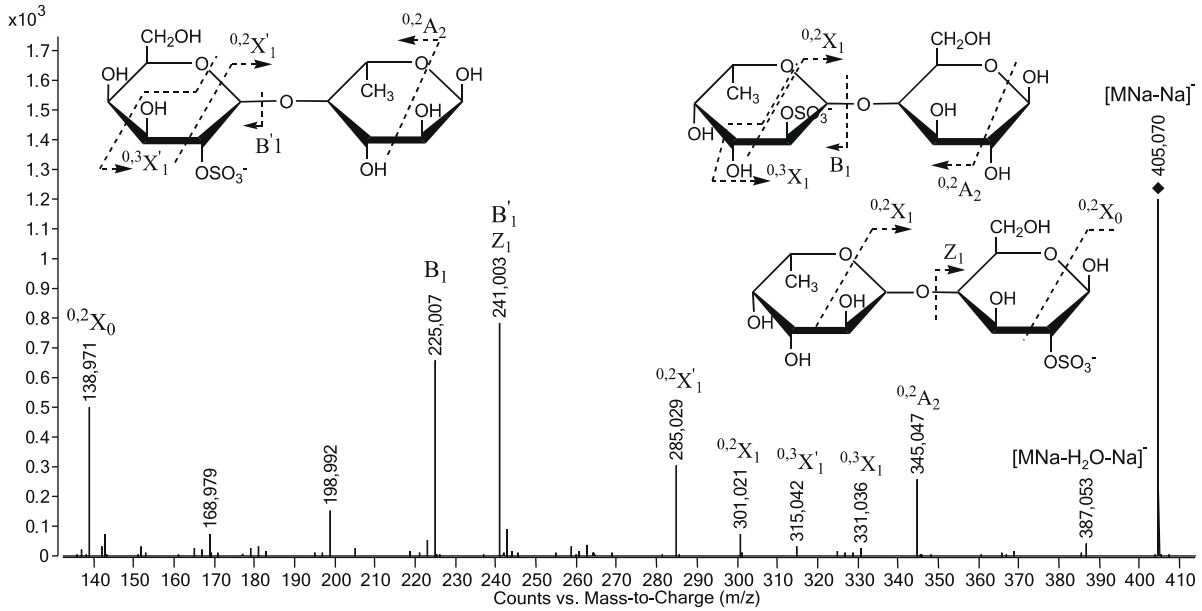

Figure 9. Negative-ion mode CID MS/MS spectrum of $[Fuc_1Gal_1SO_3Na–Na]^-$ ion at *m*/*z* 405.070.

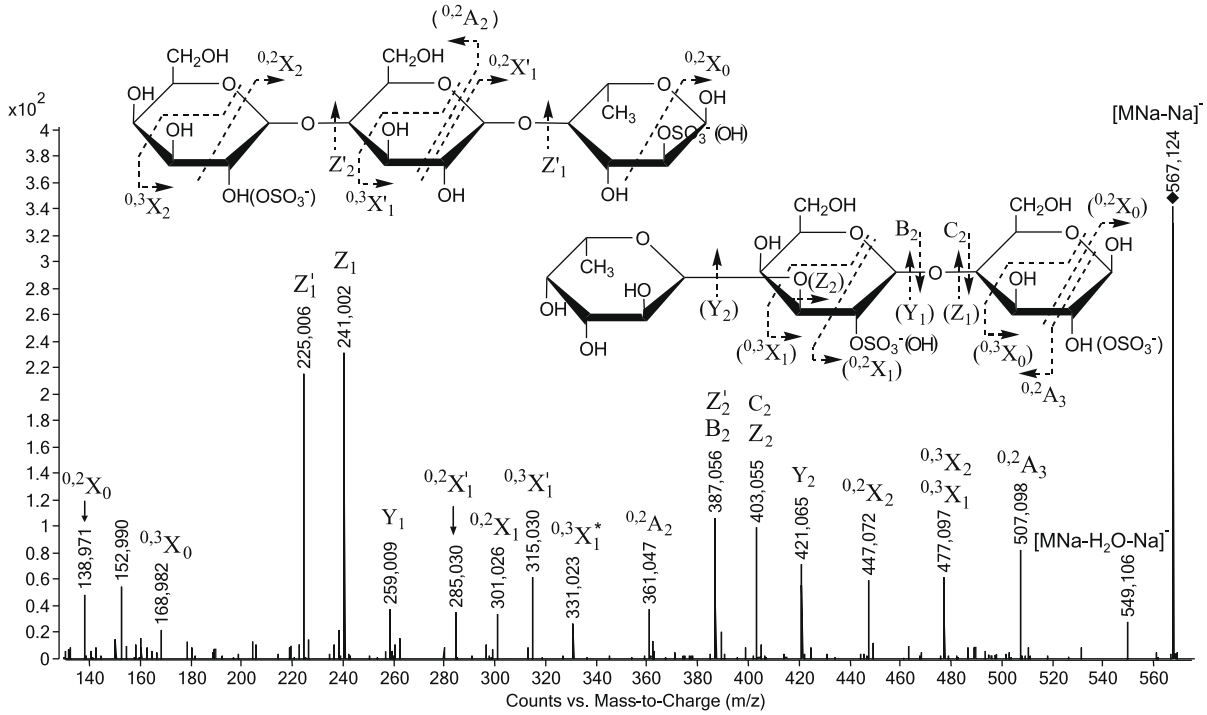

Figure 10. Negative-ion mode CID MS/MS spectrum of $[Fuc_1Gal_2SO_3Na–Na]^-$ ion at *m*/*z* 567.124. Additional structural variants with different sulfate positions and corresponding fragments are enclosed in brackets.

structural variants with reducing fucose and galactose existed simultaneously in the mixture, probably as the 2-O-sulfonated at the reducing-end sugar residue or at the same position of the prior sugar residue. The $^{0,2}X_0$ fragment at *m*/*z* 138.971 gave additional evidence of the 2-O-sulfonation of the reducing-end sugar residue, while the $^{0,3}X_0$ fragment at *m*/*z* 168.982 gave direct information of a reducing-end galactose sulfonation at O-2, since the fucose produced much fewer $^{0,3}X_0$-type ions. The nonreducing fucose residue (see Fig. 10, bottom structure) seemed to contain no sulfate groups, since no $^{0,2}A_2$ and or $^{0,2}X_2$-type ions were found. Galactose, as mentioned above, was shown to produce more cross-ring cleavages of the $^{0,3}X$ ($^{0,3}A$)-type. Thus, direct information on the type of linkage was collected: the ions of $^{0,3}X_1$-type were found at *m*/*z* 331.023 and 477.097. The first one, marked with asterisk, suggested a (1→4)-type linkage between the nonreducing fucose and galactose residues, while the last one, which is more abundant, suggested a (1→3)-type linkage. It is worth mentioning that the ion at *m*/*z* 477.097 could also arise from an $^{0,3}X_2$-type ion (cross-ring cleavage of the nonreducing-end galactose residue, see Fig. 10, upper structure). The $^{0,3}X_1$-type ion at *m*/*z* 315.030, along with the $^{0,2}A_2$-type ion at *m*/*z* 361.047, suggested that the structure, mentioned above, had a (1→4)-type linkage between the nonreducing galactose

residues. The $^{0,2}A_2$-type ion, along with the $^{0,2}X_2$-type ion at m/z 447.072, also suggested 2-O-sulfonation of the nonreducing-end galactose residue. The Y_2-type ion at m/z 421.065, which arose from glycosidic bond cleavage, represented a sulfated Gal_2-fragment. The $^{0,2}A_3$-type ion at m/z 507.098, along with the $^{0,3}X_0$-type ion at m/z 168.982, suggested that the second structural variant, shown in Figure 10 at the bottom, also had a (1→4)-type linkage between the reducing-end galactose residues. Assuming all the facts mentioned above, we have concluded that the selected ion had two prevalent structures: Gal-(1→4)-Gal-(1→3)-Fuc and Fuc-(1→3)-Gal-(1→4)-Gal, with the different sulfonation pattern (shown by the structures at the top and bottom of Fig. 10, respectively). The type of linkage between the fucose and galactose residues, most probably, might be both (1→3)- and (1→4)-type linkages.

3. Conclusions

The powerful capabilities of mass spectrometry (MS) allowed us to acquire unique data on the unsulfated oligomeric fraction, which is the major product of the solvolysis reaction (which usually goes to waste). MS data on some sulfated fucooligosaccharides, also detected in the mixture, were found to be in agreement with the results of former studies.[9,18] Precise information on the status of the xylose, galactose, and glucuronic acid was found as a result of our MS investigations: xylose (up to 1 residue) was found as $[Fuc_nXyl_1 + Na]^+$, $n = 2–6$ by MALDI-TOFMS analysis of minor low-molecular-mass solvolysis products. Galactose was found by the same method as $[Fuc_nGal_1 + Na]^+$, $n = 2–6$ (signals may overlap with K^+ adducts of Fuc_nGal_1). This information was extended by the ESIMS/MS data: xylose, found in the negative-ion registration mode, was shown to be mainly 2-O- and 4-O-sulfonated; also it was found in mixed oligosaccharides: $[Fuc_1Xyl_1SO_3]^-$, $[Fuc_2Xyl_1SO_3]^-$ (We were unable to unambigously establish the linkage type and sequence of the last ion.), having (1→4)- and probably (1→3)-type linkages between xylose and fucose. Fucose and xylose in these oligosaccharides were shown to be mainly 2-O- and sometimes 4-O-sulfonated. Galactose was found to be a 2-O- and probably a 4-O-sulfonated monosaccharide. Galactose-containing oligosaccharides were also found and characterized: $[Gal_2SO_3]^-$, $[Fuc_1Gal_1SO_3]^-$, $[Fuc_2Gal_2SO_3]^-$. Fucose and galactose were also shown to be 2-O- and sometimes probably 4-O-sulfonated. The (1→4)- and (1→3)-type linkage between Fuc and Gal was shown to be possible. Galactoses in $[Fuc_1Gal_2SO_3]^-$ and $[Gal_2SO_3]^-$ ions were shown to be (1→4)-linked. Structural features of the $[Fuc_2Gal_1SO_3]^-$ ion were not established precisely. Fucose oligomers $[Fuc_nSO_3]^-$, $n = 1–4$, were shown to be mainly 2-O-sulfonated and had a prevalence of (1→3)-type linkages over the (1→4)-type. Most possibly the structures of glucuronic acid-containing oligosaccharides found were Fuc-(1→3)-Fuc-(1→4)-Fuc-(1→3)-GlcA, Fuc-(1→4)-Fuc-(1→3)-GlcA, Fuc-(1→3)-GlcA.

4. Experimental

4.1. General methods

Analysis of monosaccharide composition was performed by the alditol acetate method.[29] The hydrolyses were carried out with 2 M trifluoroacetic acid at 105 °C for 4 h, followed by reduction in H_2O with $NaBD_4$ overnight at room temperature. To identify the uronic acid, the hydrolysates were reduced with $NaBD_4$, co-evaporated with 1 N HCl five times to convert the uronic acid to urono-1,4-lactone, reduced with $NaBD_4$, and acetylated.[30] The alditol acetate derivatives were analyzed by gas–liquid chromatography (GLC) using a Hewlett–Packard 6850 chromatograph equipped with HP-5MS capillary column (30 m × 0.4 mm) and using a temperature gradient of 150→230 °C at 3 °C min^{-1}. Monosaccharide samples were used as standards. HPLC was performed on an Agilent 1100 instrument equipped with a refractive index detector on a Silasorb C_{18} column (24 × 250 mm); flow rate 4.8 mL/min, 20 °C, isocratic mode, in water. The main fraction (**AF1**) was eluted with the column void volume. All experiments were performed using ultra pure water, produced with Direct-Q 3 equipment, manufactured by Millipore (USA).

4.2. Depolymerization of fucoidan by solvolysis

Fucoidan from the brown alga *F. evanescens* was extracted and purified as reported earlier.[18] Purified fucoidan was deacetylated by treatment with aq ammonia as described in the former studies.[13] A single-step solvolytic desulfation procedure[20] was used to obtain oligosaccharides, suitable for MS analyses: deacetylated fucoidan (100 mg) was changed to the pyridinium form[16,18,20] and dissolved in 18 mL of DMSO (abs) and 2 mL of pyridine by stirring, then heating at 100 °C for 10 h. The solution was diluted with water and lyophilized. The low-molecular-weight fraction, **dSlmf**, was extracted from the dry residue (~60 mg) with EtOH. The molecular mass distribution of **dSlmf**, measured by MALDI-TOFMS in the positive-ion mode, was in range of 187–917 Da (insertion at Fig. 2). Fraction **AF1** (~4 mg) was obtained from **dSlmf** using HPLC (see above). The molecular mass distribution of **AF1**, measured by ESIMS in negative-ion mode, was in the range of 229–681 Da (Fig. 2). The sugar composition of **AF1**, estimated by GLC of polyol acetates (molar %) was Fuc—72.8%, Xyl—5.2%, Gal—4.6%, GlcA—17.4%.

4.3. Mass spectrometric analysis

MALDI-TOFMS spectra were recorded with a BIFLEX-III MALDI-TOF mass spectrometer with delayed extraction and reflector modes (Bruker, Germany), equipped with nitrogen laser (337 nm), with accelerating voltage of 19 kV. DHB (2,5-dihydroxybenzoic acid) was used as the MALDI matrix in the positive-ion mode at a concentration of 10 mg/mL of 1:1 acetonitrile–water. Sample preparation: 1 μL of the matrix solution was applied to a stainless steel plate and air dried, then 1 μL of a sample solution in water (~1 mg/mL) was applied as a second layer. The mixture was air dried and then introduced into the mass spectrometer. Matrix recrystallization with methanol was applied when needed.

ESIMS spectra were recorded with an ESI Q-TOF mass spectrometer (Agilent 6510 LC Q-TOF) with a dual electrospray-ionization source. All spectra were acquired in the negative-ion mode, with pre-calibration with a standard "HP-mix" for negative-ion mode. The capillary voltage was set to 4000 V, and the drying gas temperature was 325 °C. The fragmentor voltage was set to −243 V. The isolation window for MS/MS experiments was set to 1.5 mass units. The collision energy was optimized between 10 and 30 V by fragmentation abundance. The dried sample was dissolved in 1:1 acetonitrile–water (concentration of the sample was approx. 0.01 mg/mL) and introduced into the mass spectrometer at flow rate of 5 μL/min using a syringe pump, manufactured by KD Scientific (USA).

Acknowledgments

The work was supported by RFBR (Projects No. 09-04-00761), FEB RAS grants and the program 'Molecular and Cellular Biology' for Basic Research of the Presidium of Russian Academy of Science.

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