Carbohydrate Polymers 90 (2012) 993–1002

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

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

ESIMS analysis of fucoidan preparations from *Costaria costata*, extracted from alga at different life-stages

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ARTICLE INFO

Article history:
Received 16 April 2012
Received in revised form 10 June 2012
Accepted 13 June 2012
Available online 20 June 2012

Keywords:
Fucoidan
Oligosaccharides
Structure
MS/MS
Brown algae
Acid hydrolysis

ABSTRACT

Four fucoidan fractions from brown alga *Costaria costata*, collected at different life-stages: vegetative, May (**5F2** and **5F3**) and generative, July (**7F1** and **7F2**) collections were characterized. It was found that seaweed synthesizes different set of fucoidans – one with high fucose content and substantial percentage of hexoses and uronic acid and lower sulfate content (**7F1**, **5F2** and **5F3**) and other – highly sulfated galactofucan (**7F2**). Structural features of fractions **7F2** and **5F3** were predominantly determined by mass spectrometric analysis of low-molecular-weight (LMW) oligosaccharide fragments, obtained by autohydrolysis of **7F2** and mild acid hydrolysis of **5F3** fucoidans. It was found that oligosaccharides from **7F2** fractions were mainly built up of sulfated at C-2 and/or at C-2/C-4 (1→3)-linked α-L-fucopyranose residues. D-Galactose residues, sulfated either at C-2 or C-6, were found as parts of mixed di- and trisaccharides at both termini and, probably, internal. Fucose residues in **5F3** fucoidan fragments were sulfated at C-2 and sometimes at C-4. Galactose residues were sulfated at C-4 and less frequently at C-2. Resistant to hydrolysis fraction was probably a core, built up with fucose, mannose and glucuronic acid. Presumably, oligosaccharide fragments were branches at C-4 of GlcA. They were sulfated at C-2 and sometimes at C-4 (1→3)- and/or (1→4)-linked fucooligosaccharides (sometimes terminated with (1→3)-linked galactose) and sulfated at C-4 or C-2 (1→4)- or, probably, (1→6)-linked galactooligosaccharides, probably, with own branches, formed by (1→2)-linked galactose residues. Unsulfated xylose residues were probably terminal in chains built up of fucose. It was confirmed, that monosaccharide content and structure of fucoidans of vegetative algae changed following its life stage. Generative alga in general produced highly sulfated galactofucan having lower MW along with less sulfated mannoglucuronofucan with higher MW, which was extensively synthesized by vegetative algae.

1. Introduction

At present time brown algae attract much attention since they are the rich source of structurally different polysaccharides – alginic acids, laminarans and fucoidans, exhibiting various biological activities. Fucoidans are sulfated heteropolysaccharides, mainly built up of α-L-fucopyranose (α-L-Fuc*p*) residues. Being non-toxic, they possess wide variety of biological activities, including immunomodulating (Khil'chenko et al., 2011), anticoagulant (Ushakova et al., 2008), antiviral (Ponce, Pujol, Damonte, Flores, & Stortz, 2003), antioxidant (Xue et al., 2001) and antitumor activities (Ermakova et al., 2011; Moon et al., 2009). It is clear that activity is related to structural features of the fucoidans: degree of sulfation (Teruya, Konishi, Uechi, Tamaki, & Tako, 2007), molecular weight (Nishino, Aizu, & Nagumo, 1991) and linkage pattern (Anastyuk et al., 2012). The establishment of the relationship between amount and structural characteristics of fucoidans and life stage of an algae and determination of physiological period, when selected algae produces polysaccharide with specific structural characteristics is aimed to the selection of optimal time for collection. Precise determination of mentioned-above parameters is required for drug development.

Selected specie of alga may synthesize structurally different fucoidans, depending significantly on its age (Honya, Mori, Anzai, Araki, & Nishizawa, 1999; Imbs et al., 2009; Skriptsova, Shevchenko, Zvyagintseva, & Imbs, 2009) and less significantly on the environment (Zvyagintseva et al., 2003). Furthermore, extraction conditions may affect the polysaccharide composition (Ponce et al., 2003). Thus, it is hard to simultaneously take into account such amount of variable parameters. Classic approach using NMR and methods of carbohydrate chemistry for the analysis of large amount of samples thus becomes time-consuming. Hence, new procedure for rapid analysis of material is required.

Mass spectrometry (MS) with matrix-assisted laser desorption/ionization (MALDIMS) and especially electrospray ionization (ESIMS) are important tools for the analysis of heterogeneous

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0144-8617/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2012.06.033

anionic carbohydrates (Zaia, 2004). Its speed, sensitivity and accuracy fit the above-mentioned requirements. Though its application for the fucoidan analysis was successful, (Anastyuk, Shevchenko, Nazarenko, Dmitrenok, & Zvyagintseva, 2009; Daniel et al., 2007; Shevchenko et al., 2007), it is yet impossible to directly analyze large highly charged molecules having molecular weight (MW) from tens to thousands kDa. Polysaccharide thus should be depolymerized to oligosaccharides, having MW 100 Da to 4 kDa. Since there are no widely available enzymes (Kusaykin et al., 2008), catalyzing specific transformation of fucoidans, acid hydrolysis is often performed. But, it also requires time-consuming experiments for optimal conditions selection, since sulfates are cleaved readily during acid hydrolysis (Pomin, Valente, Pereira, & Mourao, 2005). Recently, we used autohydrolysis[1] as an alternative strategy for fucoidan decomposition. It was found, that it is a reproducible method for preparation of multisulfated oligosaccharides, well reflecting the structure of the source polysaccharide. In this way fragments of fucoidans from brown algae *Saccharina cichorioides* (Anastyuk et al., 2010) and *Fucus evanescens* (Anastyuk et al., 2012) were successfully analyzed by tandem ESI and MALDIMS. Structural features of oligosaccharides matched with that observed previously for corresponding fucoidans using independent methods (Bilan et al., 2002; Zvyagintseva et al., 2003).

Herein we report mass spectrometric elucidation of the structural properties of oligosaccharides, obtained by mild acid hydrolysis and autohydrolysis from insufficiently studied fucoidan preparations from the brown seaweed *C. costata*, collected at different life stages.

2. Experimental

2.1. Materials

Brown alga *C. costata was* collected at May (vegetative) and July (generative) in 2008 and 2010 in Peter the Great Bay, Sea of Japan.

All MS experiments were performed using ultra pure water, produced with Direct-Q 3 equipment (Millipore, USA). Monosaccharides – L-rhamnose (L-Rha), D-ribose (D-Rib), D-mannose (D-Man), L-fucose (L-Fuc), D-galactose (D-Gal), D-xylose (D-Xyl) and D-glucose (D-Glc), used as standards for HPLC and GC were purchased from SIGMA (USA).

Arabinoosazone (phenylosazone of D-arabinose) was synthesized as described (Chen, Baker, & Novotny, 1997).

2.2. Instruments

MALDI-TOFMS spectra were recorded with an Ultra Flex III MALDI-TOF/TOF mass spectrometer (Bruker, Germany) with a nitrogen laser (337 nm), reflector and potential LIFT tandem modes of operation.

ESIMS spectra were recorded with an ESI Q-TOF mass spectrometer (Agilent 6510 LC Q-TOF, USA) with a dual electrospray-ionization source.

Molecular weights (MWs) of the polysaccharides were determined by HPLC in a Shimadzu LC-20A instrument with a RID-10A refractometric detector.

The alditol acetate derivatives (Sawardeker, Sloneker, & Jeanes, 1965) were analyzed by gas chromatography (GC) using a Hewlett-Packard 6850 (USA) chromatograph equipped with HP-5MS capillary column (30 m × 0.2 mm) using a temperature gradient of 150 → 230 °C at 3 °C min^{-1}. Infrared spectra were recorded with a Vektor-22 (Bruker, Germany) FT-IR spectrometer.

2.3. General methods

The content of carbohydrates was determined using the phenol–sulfuric acid test (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The content of sulfates in the polysaccharides was determined by a turbidimetric method of Dodgson and Price. The method is based on precipitation of inorganic sulfate by barium chloride–gelatin reagent in the presence of 4% trichloroacetic acid (Dodgson, 1961). Analysis of monosaccharide composition of polysaccharide and oligosaccharide fractions was performed by alditol acetate method (Sawardeker et al., 1965). The total acid 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 uronic acid, the hydrolysates were reduced with $NaBD_4$, co-evaporated with 1 N HCl 5 times to convert the uronic acid to urono-1,4-lactone, reduced with $NaBD_4$, and acetylated (Jones & Albersheim, 1972).

2.3.1. Extraction of polysaccharides

Fractions of water-soluble polysaccharides from *C. costata* **5FLM** (algae collected in May) and **7FLM** (algae collected in July) were extracted as described earlier (Imbs et al., 2009).

2.3.2. Anion exchange chromatography

Solution of polysaccharides **5FLM** in 0.1 M HCl (1.9 g in 50 mL) was applied onto a DEAE-cellulose column (Cl^- form, 3.5 cm × 22 cm) equilibrated with 0.05 M HCl. Laminaran was eluted with 0.05 M HCl, and the column was then successively eluted with 0.5, 1.0 and 2.0 M NaCl solutions, each time until the disappearance in eluate of positive reaction for carbohydrates with phenol and sulfuric acid. Sugar-containing fractions were purified by ultrafiltration with NMWL 1000 membrane (Millipore, USA) and lyophilized. Fucoidan preparations **5F1** of 350 mg, **5F2** of 320 mg and **5F3** of 250 mg were purified from **5FLM** polysaccharide mixture. Fucoidan preparations **7F1** of 110 mg, **7F2** of 221 mg were eluted with 1.5 M and 2.0 M NaCl solutions, respectively, from the mixture of polysaccharides **7FLM** as described earlier (Imbs, Shevchenko, Semenova, Sukhoverkhov, & Zvyagintseva, 2011).

2.3.3. Depolymerization of fucoidan by mild acid hydrolysis

Mild acid hydrolysis of fucoidan fraction **5F3** (10 mg) carried out using trifluoroacetic acid (1 N; 60 min; 60 °C, 5 mg/mL). The mixtures were neutralized with 5% NH_4OH solution in water and lyophilized. LMW fraction, **5F3–HL** (supernatant, yield 60%), was obtained by fractionation in H_2O/EtOH (1:10, w/w). The monosaccharide composition of **5F3–HL** (mol%): Fuc, 72.1; Gal, 20.2; Man, 0.6; Rha, 1.4; Xyl, 1.3; GlcA, 3.6. The monosaccharide composition of pellet (yield 30%) **5F3–HH** (mol%): Fuc, 56.6; Glc, 12.5; Man, 17.3; GlcA, 13.6.

2.3.4. Depolymerization of fucoidan by autohydrolysis

Autohydrolysis (Anastyuk et al., 2010) was used to obtain oligosaccharides suitable for MS analyses (10 mg): an aliquot of 2 mL of fucoidan **7F2** (5 mg/mL in water) was changed to the H^+-form using a minicolumn of cation exchange (Timberlite CG-120, 200–400 mesh, Serva, Germany) and left for 40 h at 37 °C. The mixture was then neutralized with 5% NH_4OH solution in water and lyophilized. LMW fraction **7F2–AHL** (yield 62%) was obtained by fractionation in H_2O/EtOH (5:1, w/w). The monosaccharide composition of supernatant **7F2–AHL** (mol%): Fuc, 74.0 and Gal, 19.0. The monosaccharide composition of pellet **7F2–AHH** (mol%): Fuc, 53.0 and Gal, 42.0.

[1] 'Autohydrolysis' is used here to denote acidic polysaccharide hydrolysis under very mild conditions using –SO_3H groups of the compound as the source of acid.

2.3.5. Molecular weight estimation

Fucoidan samples were dissolved in highly purified water and filtered through a membrane filter (0.45 μm pore size). Polysaccharides were separated over successively connected columns of Shodex Asahipak GS-520 HQ and GS-620 HQ (7.5 mm × 300 mm) at 50 °C with elution by H_2O (0.8 mL/min). Columns were calibrated using standard pullulans of MWs from 180 to 667 kDa (Polymer Laboratories, USA) and blue dextran (Amersham, Sweden).

2.4. Mass spectrometric analysis

2.4.1. MALDI-TOFMS

Spectra were acquired in the negative-ion mode: accelerating voltage, −25 kV; laser power, 30%; number of shots, 250; laser shot rate, 66 Hz. Sample preparation: a mixture containing 1 μL of sample (0.1 mg/mL) and 1 μL of arabinoosazone matrix solution in ethanol (10 mg/mL) was introduced onto the sample plate and air dried.

2.4.2. ESIMS

Spectra were acquired in both positive and negative-ion modes with pre-calibration using a standard "HP-mix". Capillary voltage was set to 4000 V, and the drying gas temperature was 325 °C. Fragmentor voltage was set to 160 V. The isolation window for MS/MS experiments was set to 1.3 mass units for singly charged ions and 4 mass units for multiply charged ions. Collision energy was optimized between 10 and 45 V to reach maximum intensity of fragments. 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 (KD Scientific, USA).

3. Results and discussion

C. costata is an annual plant with a short vegetative period. It grows most actively from January through April and reaches its maximum size in the first half of summer. Spore-bearing tissue begins to appear in June. Ripening of alga zoospores is completed on the shores of Primorye (Far East of Russia) in June–July, after which they are dispersed. By August the alga's thallus is destroyed. Earlier it was shown that highest yields of fucoidan (predominantly galactofucan) in the adult seaweeds were collected in June–July, when the thalli matured for sporulation. However, during other periods of life-stage, seaweed may synthesize fucoidans with different structure and thus with different pharmacological properties (Imbs et al., 2009).

3.1. Purification of water-soluble polysaccharide fractions

Water-soluble polysaccharide fractions, extracted from the brown algae *C. costata*, which were collected in May (fraction **5FLM**) and in July (fraction **7FLM**) were purified with anion-exchange chromatography. Subfractions **5F1**, **5F2** and **5F3** were obtained from fraction **5FLM** and subfractions **7F1** and **7F2** were obtained from **7FLM** fraction. Yields, MWs and monosaccharide content of obtained fractions are listed in Table 1. All fractions contained predominantly fucose, galactose, mannose and glucuronic acid, except for **7F1**, with highest mannose content. Common **5FLM** and **7FLM** preparations had MW range from 200 to 800 and from 20 to 300 kDa, respectively. It should be noted that polysaccharides with high glucuronic acid content were found to have alternative core, built up of glucuronic acid and different hexoses, along with fucose residues (Bilan et al., 2010; Duarte, Cardoso, Noseda, & Cerezo, 2001).

Fucoidans **7F2** and **5F3** that were eluted with 1.5 M and 2.0 M solutions, respectively, had more homogeneous monosaccharide composition. They contained fewer amounts of uronic acids and more sulfate groups. Since it was recently shown that sulfated fucans but not fucomannoglucuronans determine biological activities (Croci et al., 2011), these fractions were selected for further mass spectrometric analysis after brief characterization with aid of independent methods.

3.2. Preliminary analysis of fucoidan fractions

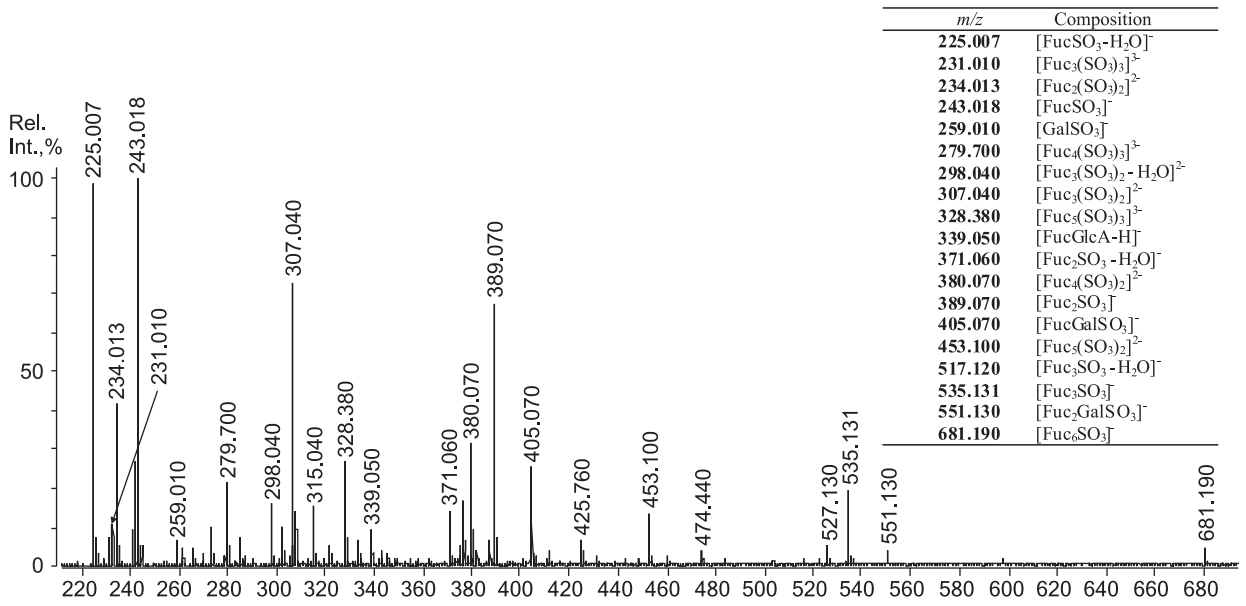
Fucoidan fraction **7F2** had high sulfate content and had monosaccharide composition, similar to fucoidans fractions from sphorofills of *U. pinnatifida* (Mori, Kamei, Nishide, & Nishizawa, 1982), thalli of *Laminaria gurjanovae* (Shevchenko et al., 2007), *Laminaria japonica* (Honya et al., 1999; Ozawa, Yamamoto, Yamagishi, Yamazaki, & Nishizawa, 2006; Xue et al., 2001) and *Ecklonia kurome* (Nishino & Nagumo, 1991). These fucoidans had high fucose and galactose content as well as sulfates and low content of uronic acids and other monosaccharides. Molar ration of Fuc:Gal in these fucoidans rated as 1.0:(0.2–1.1) and they were classified as galactofucans. Galactofucan fraction from *L. japonica* was shown to have a backbone of 3-linked α-L-Fuc*p* residues (characteristic to Laminariales order; Cumashi et al., 2007), which could be sulfated at C-2, C-4 or 2,4-disulfated. Sulfates also were found at C-3 or at C-4 of →6)-β-D-Gal*p*(1→ chains, that were attached at C-4 of a backbone as branching points (Wang, Zhang, Zhang, Zhang, & Niu, 2010).

Fucoidan fraction **5F3** had higher percentages of glucuronic acid and fewer sulfate groups, α-L-Fuc*p* was the major component but other sugars like galactose, mannose, glucuronic acid, glucose and xylose were also in substantial amounts, similarly to one of fucoidan sets from brown alga *Saccharina latissima*. It was recently found, that along with fucan fraction (sulfated at C-4 and/or at C-2,3-linked α-L-fucan), *S. latissima* had other fucoidans, found in total preparation: (I) a fucogalactan having a backbone of 6-linked β-D-Gal*p* residues branched mainly at C-4 and containing both terminal galactose and fucose residues; (II) a fucoglucuronomannan having a backbone of alternating units of →2)-α-D-Man(1→ and →4)-β-D-GlcA*p*(1→ with α-L-Fuc*p* residues as single branches at C-3 of α-D-Man*p*; (III) a fucoglucuronan having a backbone of 3-linked

Table 1
Properties of fucoidan fractions after anionic-exchange chromatography of **7FLM** (generative) and **5FLM** (vegetative) preparations of water-soluble polysaccharides from brown alga *C. costata*.

Fraction	Yield (%)[a]	Avg. MW (kDa)	Fuc:Gal	SO_4^{2-} (%)[b]	Monosaccharide composition, molar ratio (%)						
					Fuc	Gal	Man	Rha	Xyl	Glc	GlcA
5F2 (1.5 M)	16.4	380	1:0.3	15.0	30.5	8.5	15.3	7.8	16.0	6.6	14.7
5F3 (2.0 M)	12.8	800	1:0.5	15.4	39.7	21.4	12.0	0	11.6	5.6	7.5
7F1 (0.5 M)	9.1	620	1:0.3	6.7	22.6	6.4	36.9	4.2	4.6	9.7	15.5
7F2 (1.5 M)	18.4	160	1:0.3	23.8	70.2	19.8	7.0	0.0	0.0	0.0	3.0

[a] % of FLM fraction weight.
[b] % of fucoidan fraction weight.



m/z	Composition
225.007	$[FucSO_3\text{-}H_2O]^-$
231.010	$[Fuc_3(SO_3)_3]^{3-}$
234.013	$[Fuc_2(SO_3)_2]^{2-}$
243.018	$[FucSO_3]^-$
259.010	$[GalSO_3]^-$
279.700	$[Fuc_4(SO_3)_3]^{3-}$
298.040	$[Fuc_3(SO_3)_2\text{-}H_2O]^{2-}$
307.040	$[Fuc_3(SO_3)_2]^{2-}$
328.380	$[Fuc_5(SO_3)_3]^{3-}$
339.050	$[FucGlcA\text{-}H]^-$
371.060	$[Fuc_2SO_3\text{-}H_2O]^-$
380.070	$[Fuc_4(SO_3)_2]^{2-}$
389.070	$[Fuc_2SO_3]^-$
405.070	$[FucGalSO_3]^-$
453.100	$[Fuc_5(SO_3)_2]^{2-}$
517.120	$[Fuc_3SO_3\text{-}H_2O]^-$
535.131	$[Fuc_3SO_3]^-$
551.130	$[Fuc_2GalSO_3]^-$
681.190	$[Fuc_6SO_3]^-$

Fig. 1. Negative-ion ESIMS of the low-molecular-weight oligosaccharide fraction **7F2–AHL**, obtained from a fucoidan of *C. costata* by autohydrolysis.

β-D-GlcA residues with α-L-Fuc*p* residues as single branches at C-4 (Bilan et al., 2010). Thus, assuming all mentioned above, we can expect structural features of our samples **7F2** and **5F3** to be similar with galactofucans and fucoglucuronomannans, respectively.

Preliminary investigations of **7F2** and **5F3** fractions with NMR ^{13}C (see supplementary material) have shown the following. Spectra contained number of badly resolved peaks that was similar to the most of native fucoidan spectra. Anomeric carbons resonance region (δ 100–105 ppm) was typical for α-L-fuco- and β-D-galactopyranosides and high-field area (δ 17.0–17.44 ppm) for α-fucopyranosides. Doublet at δ 62.56 ppm indicated the presence of hexapyranosides with unsubstituted CH_2OH-groups. Signal at δ 21.66 and 174 ppm indicated the presence of *O*-acetyl groups in the polysaccharide. ^{13}C NMR spectra of **5F3** fucoidan gave similar information. In addition, intensive signal at δ 176.06 ppm indicated the presence of carbonyl groups, confirming that polysaccharide contained some amount of glucuronic acids.

IR spectrum of **7F2** fraction had characteristic absorption at 1254 cm^{-1} (1264 for **5F3**) (S=O-oscillation). Sample had the axial sulfate band at 823 cm^{-1} and a small shoulder of equatorial sulfate at 846 cm^{-1}. Analysis of contour by curve fitting of a band at 823 cm^{-1} of **5F3** sample has shown this band to contain two components with oscillations at 846 and 818 cm^{-1}. S_{846}/S_{818} was calculated as 1.23, hence, sulfates were predominantly at C-4/C-3 (about 80%) and C-2 (about 20%) (Zvyagintseva, Shevchenko, & Popovnich, 1999).

3.3. Mass spectrometric investigation of fucoidan fraction *7F2*

Galactofucan fraction **7F2**, extracted from generative algae had high sulfate content and simple monosaccharide composition. It was subjected to autohydrolysis to obtain oligosaccharides, suitable for mass spectrometric investigation. Autohydrolysis was recently employed as an alternative method for preparation of multisulfated fucooligosaccharides from fucoidans from brown algae (Anastyuk et al., 2012, 2010). In this way galactofucan **7F2** was decomposed. High-MW fraction **7F2–AHH** (pellet) and LMW fraction **7F2–AHL** (supernatant) were obtained by addition of aq. ethanol (Section 2.3.4) to the reaction mixture.

MALDI-TOFMS (not shown) and ESIMS (Fig. 1) analyses revealed that oligosaccharide mixture **7F2–AHL** contained set of fucooligosaccharides with DP 2–6 and up to 3 sulfate groups per molecule, including monosulfated fucose $[FucSO_3]^-$ ion at *m/z* 243.020 which had highest intensity. Doubly sulfated fucose ion $[Fuc(SO_3)_2Na]^-$ was detected by MALDIMS only. Hexose-containing (D-Gal, due to monosaccharide composition analysis) oligosaccharides were also detected by both ESIMS (see table in Fig. 1) and MALDIMS as satellite $[M-Na+16]^-$ signals, where M represented sodium salt of sulfated fucooligosaccharides (*m/z* 405.07, 507.00, 653.06 and 799.12). The fact of partially acetylation was observed by the presence of $[AcFuc_5(SO_3)_2]^{2-}$ ion at *m/z* 474.100 by ESIMS. MALDI-TOFMS revealed, probably, fragment ions $[M+42-Na]^-$ from mentioned-above ion: $[AcFuc_2SO_3]^-$ at *m/z* 431.09, $[AcFuc_3SO_3]^-$ at *m/z* 577.14, $[AcFuc_3(SO_3)_2Na]^-$ at *m/z* 679.09. Intact ion $[AcFuc_5(SO_3)_2Na]^-$ at *m/z* 971.2 also had low intensity. Thus, we could presume that at least one residue from five in the chain is acetylated. A signal of unknown ion $[M-Na+18]^-$ was detected at *m/z* 407.13 by MALDIMS only. Fragment ions from the loss of water molecules (in-source fragmentation) were observed by both MALDI as $[M-Na-18]^-$ signals (*m/z* 225.00, 371.01, 473.00, 517.12, 559.13, etc.) and ESIMS (see table in Fig. 1).

Similar composition of fucooligosaccharide mixture (Anastyuk et al., 2010) after autohydrolysis had fucoidan from *S. cichorioides* (except for fragments with galactose), structurally characterized as a linear (1→3)-linked α-L-fucan in which α-L-Fuc*p* residues were 2,4-disulfated (Zvyagintseva et al., 2003). As already mentioned, this type of linkage was shown as the prevalent structure of fucan and galactofucan backbone for the order Laminariales. Negative-ion ESIMS/MS of monosulfated fucose $[FucSO_3]^-$ at *m/z* 243.018 was similar to that observed for *S. cichorioides*, where signals from C-4 (*m/z* 182.995) and C-2 (*m/z* 138.968) sulfation of α-L-Fuc*p* residues were equal (Anastyuk et al., 2010). No evidence of sulfation at C-3 was detected, since corresponding fragment ion at *m/z* 168.9 was not observed (Tissot, Salpin, Martinez, Gaigeot, & Daniel, 2006). ESIMS/MS analysis of the most abundant mono- and multisulfated oligosaccharides (Table 2) revealed almost 100% match with those obtained for *S. cichorioides*. All the spectra featured lack of characteristic to the (1→4)-type of linkage $^{0,2}A$-type ion series, which were intensive in the case of ESIMS/MS of oligosaccharides from *A. nodosum* (Daniel et al., 2007) and *F. evanescens* (Anastyuk et al., 2012), rich of (1→4)-linked α-L-Fuc*p* residues. Note that all of oligosaccharides with determined structures (both for *C. costata*

Table 2
Proposed structural features of some oligosaccharides, obtained from **7F2** (generative) and **5F3** (vegetative) fucoidan fractions of brown alga *C. costata* by autohydrolysis and mild acid hydrolysis, respectively.

Ion (*m*/*z*)	Structural features of mono- and oligosaccharides	
	7F2, autohydrolysis	**5F3**, acid hydrolysis
231.010	Fuc2S-(1,3)-Fuc2S-(1,3)-Fuc2S	–
234.13	Fuc2S-(1,3)-Fuc2S Fuc4S-(1,3)-Fuc2S Fuc(2,4)S-(1,3)-Fuc	–
243.018 259.010	Fuc2S, Fuc4S Gal2S, Gal4S	Fuc2S, Fuc4S Gal2S, Gal4S
307.040	Fuc2S-(1,3)-Fuc2S-(1,3)-Fuc Fuc4S-(1,3)-Fuc2S-(1,3)-Fuc	–
339.050	–	Fuc-(1,4)-GlcA
355.089	–	Gal-(1,4)-GlcA Gal-(1,3)-GlcA
389.070	Fuc2S-(1,3)-Fuc	Fuc2S-(1,4)-Fuc Fuc4S-(1,4)-Fuc Fuc2S-(1,3)-Fuc
405.070	Gal2S-(1,3)-Fuc Gal6S-(1,3)-Fuc Fuc2S-(1,3)-Gal	Gal2S-(1,3)-Fuc Gal4S-(1,3)-Fuc Fuc2S-(1,3)-Gal Fuc-(1,3)-Gal4S Gal-(1,4)-Fuc2S
421.065	–	Gal2S-(1,4)-Gal Gal2S-(1,2)-Gal Gal-(1,3)-Gal4S Gal2S-(1,6)-Gal
474.440	$[AcFuc_5(SO_3)_2]^{2-}$, structure unknown	–
551.130	Gal6S-(1,3)-Fuc-(1,3)-Fuc Gal2S-(1,3)-Fuc-(1,3)-Fuc Gal-(1,3)-Fuc2S-(1,3)-Fuc Fuc-(1,3)-Gal2S-(1,3)-Fuc Fuc-(1,3)-Fuc-(1,3)-Gal2S	–

and *S. cichorioides*) were always sulfated at non-reducing end and less frequently at the reducing end. This observation is in accord with the data on previous studies of mild acid hydrolysis of 3-linked sulfated fucans from invertebrates with different sulfation pattern (Pomin et al., 2005). It was found that hydrolysis is performed between residues with different sulfation pattern and sulfate at C-2 is cleaved first. Probably, desulfation at C-2 followed by hydrolysis of glycosidic linkage involves the same mechanism in both mild acid hydrolysis and autohydrolysis. Hence, α-L-Fuc*p* residues in a galactofucan from generative brown alga *C. costata* were predominantly linked with (1→3)-type of linkage and sulfate groups occupied mostly C-2 or C-2/C-4 positions. All defined structures of fucooligosaccharides are collected in Table 2.

Further investigations were concentrated on the minor components of oligosaccharide mixture – D-Gal*p*- and D-GlcA-containing oligosaccharides. ESIMS/MS of monosulfated galactose ion $[GalSO_3]^-$ at *m*/*z* 259.010 have shown fragment ions, characteristic to the sulfation at C-2 at *m*/*z* 138.980 ($^{0,2}X$) and C-4 at *m*/*z* 198.990 ($^{0,2}A$) of equal intensity. Satellite signal from the ion at *m*/*z* 180.980 ($^{0,2}X$-H_2O), observed upon fragmentation of galactose sulfated at C-4 (observed at in $ESIMS^3$ experiment, though) had relatively high intensity. Thus, Gal residues could be probably sulfated at C-2 or at C-4 (Minamisawa & Hirabayashi, 2005). Tandem ESIMS of $[FucGlcA-H]^-$ at *m*/*z* 339.050 was not interpreted since it overlapped with other minor sulfated compound. The only ion from GlcA-containing fragment was Y_1 at *m*/*z* 193.043.

ESIMS/MS of $[FucGalSO_3]^-$ at *m*/*z* 405.070 (Fig. 2) was simple and contained only one intensive signal B_1 at *m*/*z* 241.000 from glycosydic bonds breaking and cleavage of dehydrated

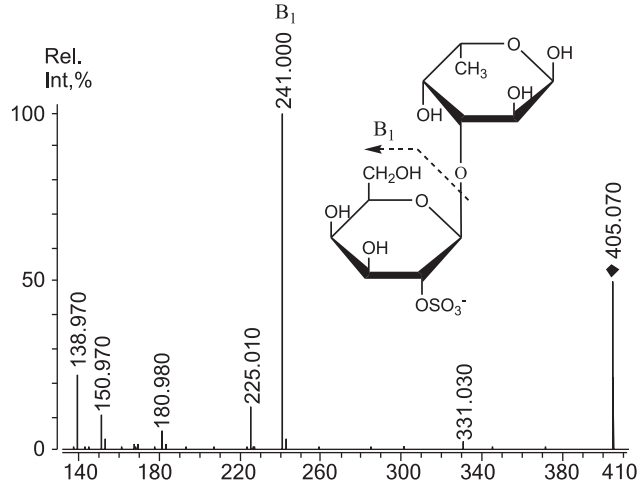


Fig. 2. Negative-ion ESIMS/MS of the ion $[FucHexSO_3]^-$ at *m*/*z* 405.070. M represents sodium salt of oligosaccharides (fraction **7F2–AHL**).

sulfated galactose residue. This glycosidic cleavage observation is consistent with the previously proposed (Saad & Leary, 2004) sulfate-mediated hydrogen transfer for B_1-type ion formation. This mechanism justifies the observation that if oligosaccharide has the sulfate group spatially closer to the glycosidic linkage, it undergoes easier B_1-type fragmentation. Similar fragmentation pattern was observed upon CID ESIMS/MS and MS^n fragmentation of D-Gal*p*-2-OSO_3^--(1→3)-3,6-AnGal*p*OH (Goncalves, Ducatti, Grindley, Duarte, & Noseda, 2010), where all four monosulfation possibilities of that disaccharide were examined. Since no evidence of sulfation of the reducing end was observed (no Y-type ion at *m*/*z* 259), and no $^{0,2}A_2$-type ion detected, fragment ion at *m*/*z* 138.970 was either a product of secondary cleavage (Anastyuk et al., 2010), or, along with minor signals at *m*/*z* 150.970 and 180.970 suggested sulfation at C-6 of non-reducing galactose residue as reported (Goncalves et al., 2010). Small signal at *m*/*z* 225.010 similarly indicated that fucose residue sulfated at C-2 could also occupy the non-reducing end. Hence, at least three variants of selected ion can co-exist in the mixture (Table 2, *m*/*z* 405).

ESIMS/MS of monosulfated trisaccharide $[Fuc_2Gal_1SO_3]^-$ of low intensity at *m*/*z* 551.130 was more complex (Fig. 3). Signals from fragment ions of B-type were most intensive, they indicated structural variants with sulfated at C-2 of galactose (*m*/*z* 241.000) and fucose (*m*/*z* 225.010) residues, which were located at non-reducing end (Fig. 3, structure in the center and left, respectively). B'_2-type ion at *m*/*z* 371.0 is not intensive, hence, internal fucose residue in trisaccharide variant Fuc-Fuc-Gal is likely unsulfated (Saad & Leary, 2004). Structural variant with sulfated D-Gal*p* residue at the reducing end was also possible, due to the presence of Y-type series ions with low intensities (Fig. 3, structure at the right). Again, mass spectrum contained minor signals at *m*/*z* 150.970 and 138.970, which, along with $^{0,2}A_1$-type ion at *m*/*z* 198.990 suggested, probably, sulfation at C-6 (Goncalves et al., 2010) of galactose residue at non-reducing end. Sulfation at C-4 could suggest ion at *m*/*z* 152.9, but none was detected. The presence of Y_2 could suggest fragment ion at *m*/*z* 138.970 to be $^{0,2}X_0$ type ion, but it is not confirmed by corresponding $^{0,2}A$-type ion. Thus, no evidence of (1→4)-type of linkage was found. Structural variant with internal galactose residue could not be excluded, since B_2-type ion at *m*/*z* 387.060 could not unambiguously suggest which internal residue (Gal or Fuc) was sulfated at C-2. It is important to place some discussion here on $^{0,2}X_1$-type (*m*/*z* 447) of cleavage for non-reducing sugar. In work of Daniel et al. (2007) it is clearly seen that intensity of $^{0,2}X_1$ at *m*/*z* 285 of monosulfated fucobiose is higher than

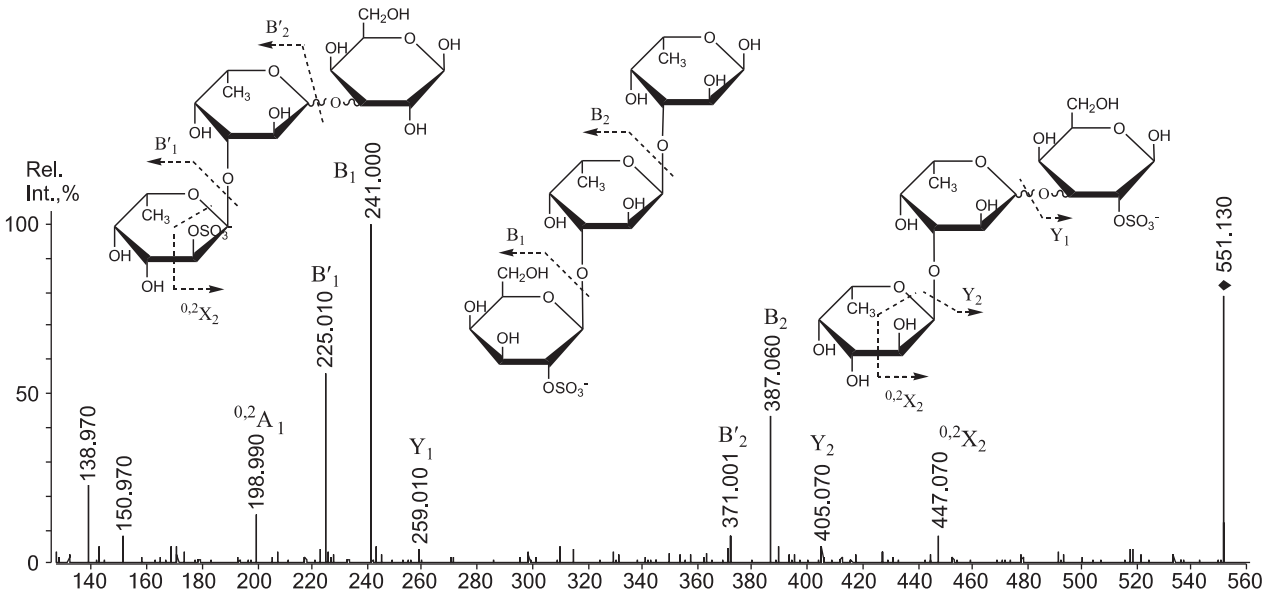


Fig. 3. Negative-ion ESIMS/MS of the ion $[Fuc_2HexSO_3]^-$ at *m*/*z* 551.130. M represents sodium salt of oligosaccharides (fraction **7F2–AHL**).

intensity for the same fragment for doubly sulfated fucobiose. The same effect was observed by our group when more heavily sulfated fucooligosaccharides were analyzed (Anastyuk et al., 2010). Thus, it can be proposed that sulfate group at C-2 of non-reducing end, probably, prevents the formation of $^{0,2}X_1$ rather than promotes. Furthermore, fragment ions of similar type from non-reducing termini of unsulfated, GlcA-containing disaccharides in present work (see below) and (Anastyuk et al., 2009) indicates that these fragments could occur without sulfate group at all. All other variants of selected ion are collected in Table 2 at *m*/*z* 405.

Despite relatively high content of galactose in the monosaccharide composition of **7F2–AHL** fraction (19%), fragments with higher DP, built up of fucose and galactose or galactose only were not observed even in acidic hydrolysis products, performed in the same conditions as it was done for **5F3** fraction (data not shown). Fragment $[FucGalSO_3]^-$ at *m*/*z* 405 was also observed instead and its intensity was higher in comparison to the same fragment, obtained by autohydrolysis. Its ESIMS/MS was the same as for autohydrolysis (data not shown). Thus, it is probably no prolonged blocks (or branches), built up of galactose, which were readily observed by MALDI-TOFMS in the mixture after mild acid hydrolysis of galactofucan from *S. gurjanovae* (Shevchenko et al., 2007).

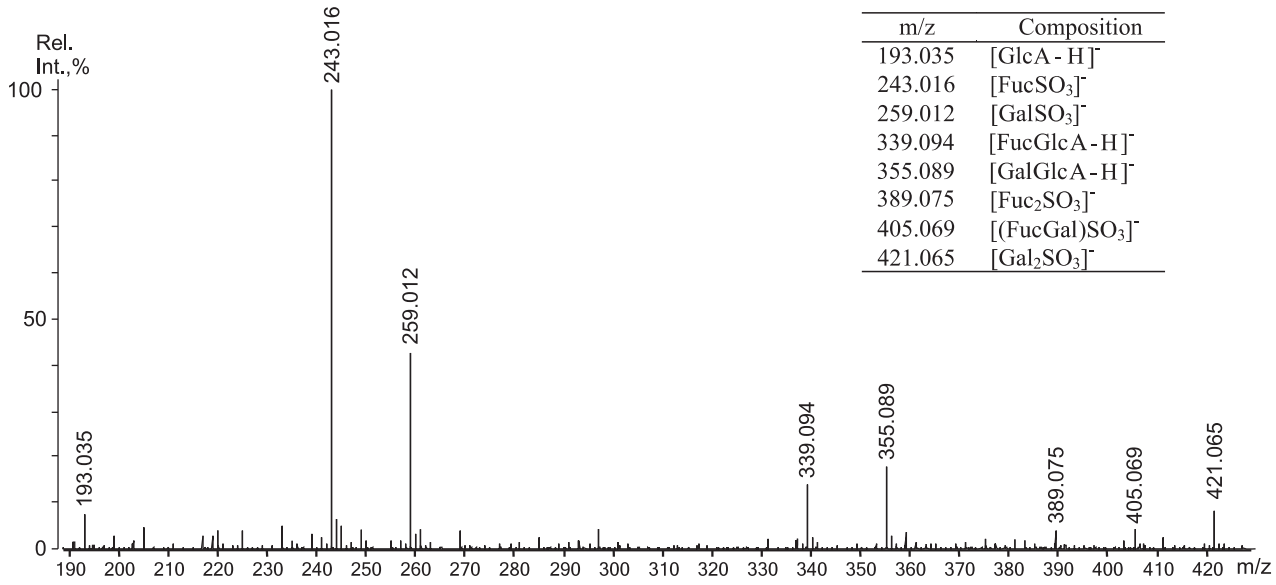
3.4. Mass spectrometric investigation of fucoidan fraction *5F3*

Heterogeneous fucoidan **5F3**, which was extensively produced by vegetative alga, was subjected to autohydrolysis, but reaction was ineffective due to low content of sulfates (Table 1). Thus, mild acid hydrolysis was employed instead. Sample was hydrolyzed for 10–70 min and aliquots were taken in 10 min steps. Fraction of 60 min of hydrolysis, obtained by addition of aq. ethanol, was found to contain highest percentage of oligosaccharides by ESIMS analysis, including GlcA-containing oligosaccharides. The reaction mixture was separated by ethanol precipitation and two fractions – **5F3–HL** (supernatant) and **5F3–HH** (pellet) were obtained, see Section 2.3.3. Note that monosaccharide composition of high-MW fraction **5F3–HH**, which was resistant to hydrolysis under selected conditions, along with highest content of fucose contained large amounts of mannose, glucose and glucuronic acid. Presumably, the core of **5F3** fucoidan was built up of mannose and/or glucuronic acid likely to the fucoidan fractions from *S. latissima* (Bilan et al., 2010), probably, with incorporations of glucose, likely to the complex fucoidans from *Sargassum stenophyllum* (Duarte et al., 2001).

Negative-ion mode ESIMS of **5F3–HL** has shown (Fig. 4) that main component of the mixture was $[FucSO_3]^-$ at *m*/*z* 243.016. Mass spectrum also contained the following less-intensive ions: $[Gal_1SO_3]^-$ at *m*/*z* 259.012, [FucGlcA−H]$^-$ c *m*/*z* 339.094, $[Gal_1GlcA-H]^-$ at *m*/*z* 355.089, $[Fuc_2SO_3]^-$ at *m*/*z* 389.075, $[FucGalSO_3]^-$ at *m*/*z* 405.069, $[Gal_2SO_3]^-$ at *m*/*z* 421.065 and a minor [GlcA−H]$^-$ *m*/*z* 193.035. Note that there were no signals of ions, corresponding to xylose-containing oligosaccharides, but, one was detected as $[XylFuc+Na]^+$ ion at *m*/*z* 319.101 in a positive-ion mode ESIMS. Along with sodium-salt forms of mentioned-above ions, detected in negative-ion mode, unsulfated galactose at *m*/*z* 203.054 (highest), fucose at *m*/*z* 187.059, fucobiose at *m*/*z* 333.116 and galactobiose at *m*/*z* 365.104 were also found in a positive-ion ESIMS (see supplementary materials for full spectrum). Probably, unsulfated xylose residues were terminal in chains built up of fucose (Duarte et al., 2001).

Negative-ion ESIMS/MS of monosulfated fucose $[FucSO_3]^-$ at *m*/*z* 243.016 was similar to that observed for fucoidan fraction **7F2** (see above) but with a small signal at *m*/*z* 168.9 indicating sulfation at C-3 of α-L-Fuc*p* residues (Tissot et al., 2006). This observation is in accord with IR data.

ESIMS/MS of sulfated fucobiose ion $[Fuc_2SO_3]^-$ at *m*/*z* 389.074 (Fig. 5) almost matched with that observed for the same monosulfated fucobiose from *A. nodosum* (Daniel et al., 2007), also obtained by mild acid hydrolysis. Our spectrum in addition contained only two intensive ions at *m*/*z* 138.972 (probably, $^{0,2}X_0$) and 182.996 ($^{0,2}A_1$). The latter signal suggested sulfation at C-4 of fucose residue on the non-reducing end. The former signal (*m*/*z* 138.972), along with $^{0,2}A_2$ *m*/*z* 329.059, should indicate (1→4)-type of linkage between fucose residues. However, despite of high intensity of the ion at *m*/*z* 138.972, corresponding Y-ion was not detected. Thus α-L-Fuc*p* residue at the reducing end was likely unsulfated and "$^{0,2}X_0$" signal was probably the result of unspecific cleavage (involving sulfate at C-4) or secondary cleavage from non-reducing α-L-Fuc*p* residue, which was found to be sulfated at C-2, since spectrum contained internsive B_1 ion at *m*/*z* 225.017 (Saad & Leary, 2004). Thus, the following prevalent variants of monosulfated fucobiose were detected: α-L-Fuc*p*-4-OSO_3^--(1→4)-α-L-Fuc*p*, α-L-Fuc*p*-2-OSO_3^--(1→4)-α-L-Fuc*p*. It must be noted that $^{0,3}X_1$ fragment ion at *m*/*z* 315.039 was observed upon fragmentation of the same



m/z	Composition
193.035	$[GlcA-H]^-$
243.016	$[FucSO_3]^-$
259.012	$[GalSO_3]^-$
339.094	$[FucGlcA-H]^-$
355.089	$[GalGlcA-H]^-$
389.075	$[Fuc_2SO_3]^-$
405.069	$[(FucGal)SO_3]^-$
421.065	$[Gal_2SO_3]^-$

Fig. 4. Negative-ion ESIMS of the LMW oligosaccharide mixture (**5F3-HL**), obtained from a fucoidan fraction **5F3** by mild acid hydrolysis.

ion obtained from **7F3** fraction, containing α-(1→3)-bound L-Fuc*p* residues (see supplementary data). Hence, this type of linkage could also occur. Again, it should be noted that disaccharides from *A. nodosum*, obtained by mild acid hydrolysis, were also sulfated predominantly at C-2 of non-reducing residues (Daniel et al., 2001, 2007).

ESIMS/MS spectra of GlcA-containing oligosaccharides were interesting (Fig. 6A and B). Both of them had intensive Y_1 ion at *m*/*z* 193.035 and low signal at *m*/*z* 175.027 from glycosidic bonds cleavage. All that signals, together with characteristic $^{2,5}A_2$, indicated that GlcA residue was predominantly located at the reducing end (Zhang et al., 2006). Present spectra featured $^{0,2}X_1$ signals at *m*/*z* 235.05 of low intensity, though that signal was highest in tandem spectrum of Fuc-GlcA disaccharide, found in oligosaccharides, derived from a fucoidan from *Fucus evanescens*, where signals at *m*/*z* 193.035 and 175.027 were equal in intensity. Possibly, that fragment had prevalent structure GlcA-(1→2)-Fuc, instead of Fuc-(1→3)-GlcA, as reported (Anastyuk et al., 2009). However, in this case we have direct confirmation of (1→4)-type of linkage between fucose residue and GlcA, since both $^{0,3}A_2$ and $^{0,2}A_2$ ion series were observed (Fig. 6A). Possibly, galactose-terminated disaccharide could be linked either by (1→4)- and (1→3)-type of linkages, because signals of A_2 ions were lower (Fig. 6B). Presumably, both fragment suggested branching points at C-4 of GlcA in the core instead of mannose, as it was shown for the core built up of mannose and glucuronic acid (Bilan et al., 2010), where only fucose residues were found as branches at C-4 of GlcA residues.

ESIMS/MS of the ion $[FucGalSO_3]^-$ at *m*/*z* 405.069 (Fig. 7) was more complex, than observed for **7F2** sample. It also had intensive signals from sulfated at C-2 galactose (B'_1 ion at *m*/*z* 241.000) and lower signal from sulfated fucose residue (B_1 ion at *m*/*z* 225.008) both from non-reducing end. Signals of $^{0,2}A_1$-type, regarding to sulfation at C-4/C-6 of both residues at non-reducing end were absent, fragment ion $^{3,5}A$ at *m*/*z* 152.985 was detected instead It was impossible to unambiguously assign which if it arises from reducing or non-reducing sulfated at C-4 (Goncalves et al., 2010) galactose residue. Further interpretation of ESIMS/MS was similar to that observed earlier (Anastyuk et al., 2009). It must be noted again that $^{0,2}X_0$ at *m*/*z* 138.973 had high intensity, but Y-ion had low intensity instead, probably, due to mentioned-above reasons.

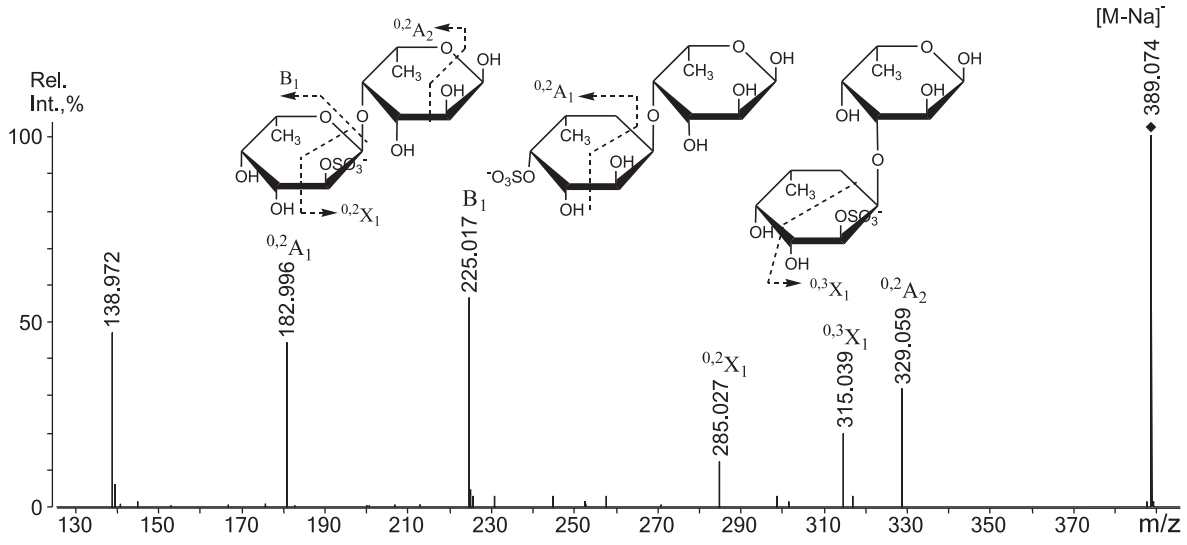


Fig. 5. Negative-ion ESIMS/MS of the ion $[Fuc_2SO_3]^-$ at *m*/*z* 389.074 (fucoidan fraction **5F3–HL**).

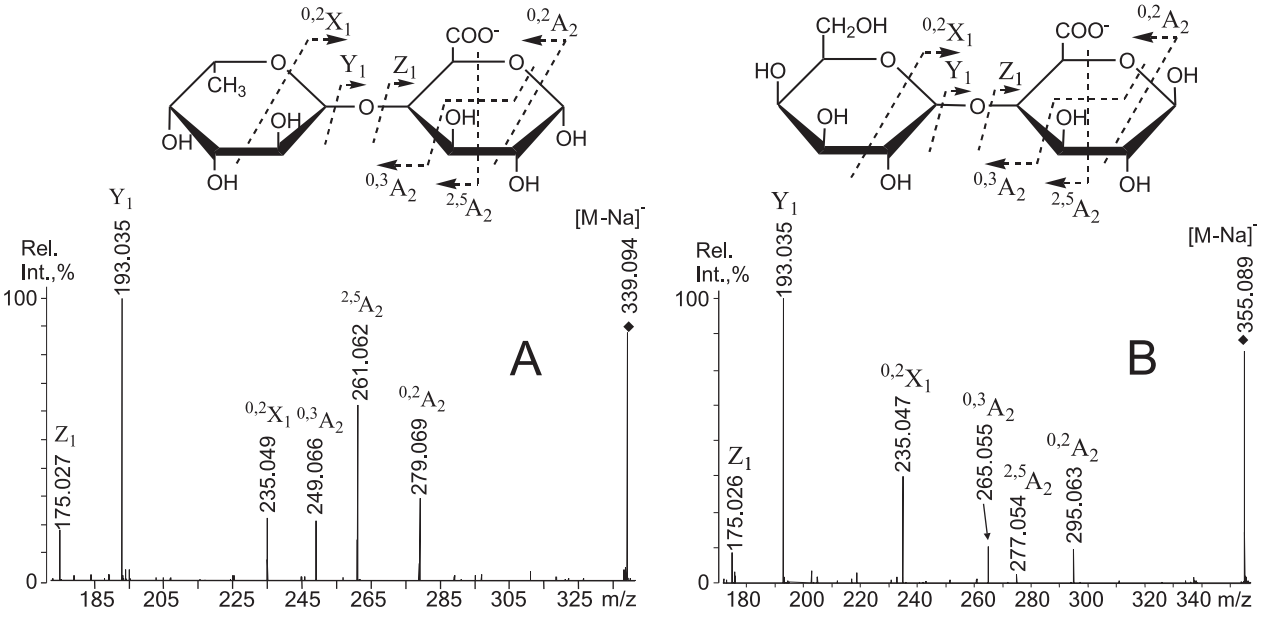


Fig. 6. Negative-ion ESIMS/MS of (A) ion $[FucGlcA–H]^-$ at *m/z* 339.094 and (B) ion $[GalGlcA–H]^-$ at *m/z* 355.089 (fucoidan fraction **5F3–HL**).

Thus, due to low intensity of the selected ion in **5F3–HL** mixture, it was presumed that either galactose residues, sulfated at C-2 could terminate short chains, built up of fucose residues and, probably, fucose residues were single branches at C-3 of mannose residues in the mannose-glucuronic acid core, since small amount (below 1%) of mannose residues was detected in the monosaccharide composition. But, fucose residues, probably, could also terminate small chains, built up of galactose as described (Bilan et al., 2010).

ESIMS/MS of the ion $[Gal_2SO_3]^-$ at *m/z* 421.065 (Fig. 8) demonstrated intensive ion signals from cross-ring cleavages. The most intensive was $^{0,2}X_1$ fragment ion at *m/z* 301.018. Intensity increase could be explained by the presence of the structural variant of disaccharide, in which galactose residues, similarly to observe in *S. latissima* (Bilan et al., 2010) were (1→2)-linked and the non-reducing one was sulfated. In that case $^{0,2}X_1$ cleavage could occur according to the mechanism, proposed in (Tissot et al., 2006). Y_1 ion from a sulfated galactose on the reducing end was found at *m/z* 259.013, having low intensity, though. B_1 ion at *m/z* 241.001 and intensive $^{0,2}A_2$ fragment ion at *m/z* 361.040 suggested structural variant Gal-(1→4)-Gal, in which non-reducing hexose could be sulfated at C-2 or C-4 that was supported by $^{0,2}A_2$ and $^{3,5}A$ ion signals (Fig. 8, at the left). Signal of $^{3,5}A$ series ion at *m/z* 152.987 could indicate sulfation at C-4 of both hexose residues, but its exact origin remained unknown. The rest diagnostic ions suggested second variant of the disaccharide structure (Fig. 8, at the left). However, low intensity of $^{0,2}X_0$ and the presence of $^{0,3}X_1$ ion signal could indirectly suggest (1→3)-type of linkage, since the origin of $^{0,3}X$ series is also still unknown and this signal was observed in tandem MS of (1→3)-linked sulfated fucooligosaccharides (Anastyuk et al., 2010). It must be noted that -(1→6)-type of linkage could not be excluded, but, characteristic signals of that variant are not yet described. Chains of 6-linked of galactose residues were found as branching points at C-3 of Man residues in fucoidan from *S. latissima*. We suppose that our sample featured (1→4)-linked galactose residues (with Gal branches at C-2), attached at C-4 of GlcA residues of the core, since, due to preliminary analysis, C-6 of hexoses was found unsubstituted, though there is high content of different hexoses in this sample.

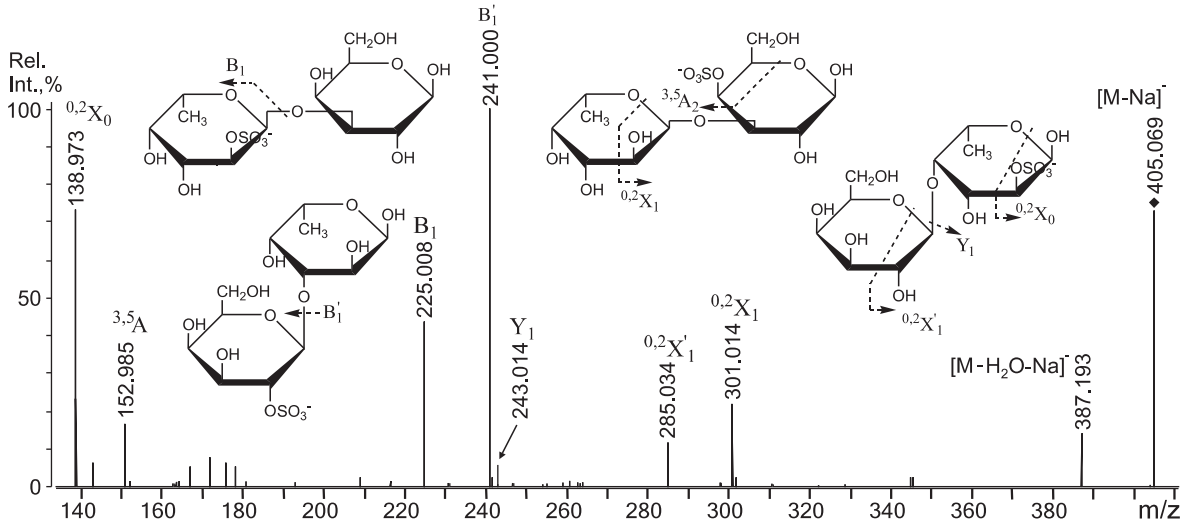


Fig. 7. Negative-ion ESIMS/MS of the ion $[FucGalSO_3]^-$ at *m/z* 405.069 (fucoidan fraction **5F3–HL**).

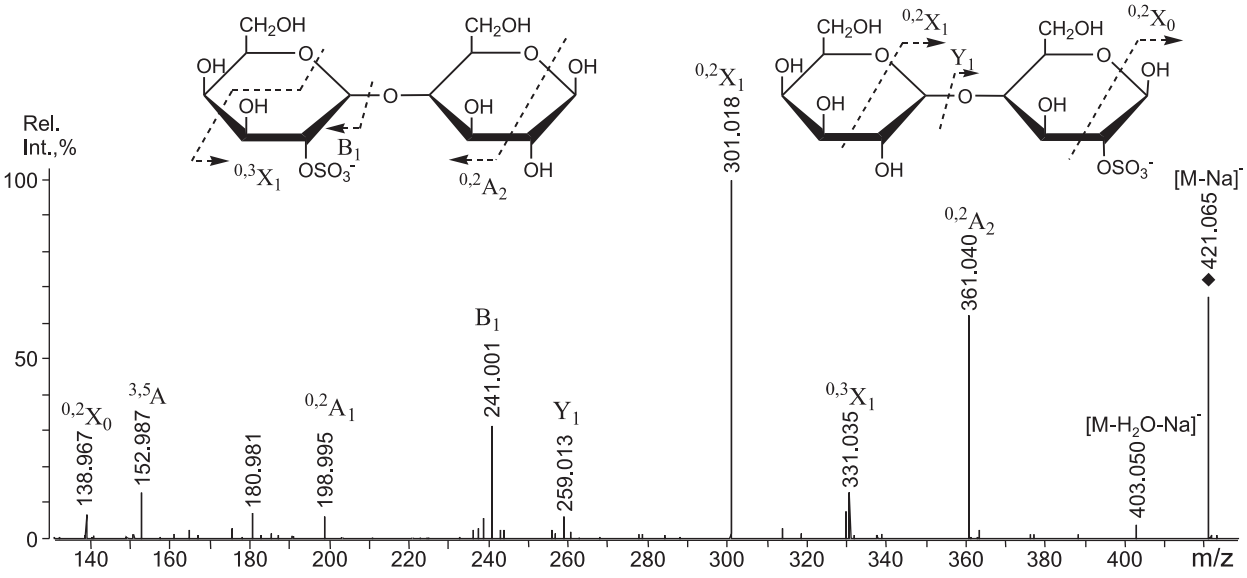


Fig. 8. Negative-ion ESIMS/MS of the ion $[Gal_2SO_3]^-$ at *m/z* 421.065 (fucoidan fraction **5F3–HL**).

4. Conclusion

Thus, four purified fucoidan fractions, extracted from vegetative brown alga *C. costata* in May (**5F2** and **5F3**) and generative in July (**7F1** and **7F2**) were examined. Structural features of polysaccharides in fractions **7F2** and **5F3** were predominantly determined by mass spectrometric analysis of LMW oligosaccharide fragments, obtained by autohydrolysis of **7F2** and mild acid hydrolysis of **5F3** (Table 2). It was shown that fucoidan fraction **7F2** from generative alga (sulfate content 23.8%) was mainly built of C-2 and sometimes C-4 sulfated and partially acetylated (1→3)-linked α-L-fucose residues, similar fucoidan fractions were found to be characteristic to the Laminariales order (Anastyuk et al., 2010; Bilan et al., 2010; Cumashi et al., 2007; Zvyagintseva et al., 2003). Galactose residues were sulfated mostly at C-2 and C-6 and were found in (1→3)-linked mixed with fucose di- and trisaccharides, located at both termini and even be internal in mixed trisaccharides. Fragments, suggesting that Gal residues were single branches from fucan chain were not detected. Fucoidan fraction **5F3** from vegetative alga had heterogeneous monosaccharide composition and lower sulfate content (15.4%), similarly to *S. latissima* (Bilan et al., 2010). α-L-Fucose was the major component, but other sugars – galactose, mannose, xylose, glucose and glucuronic acid also were in substantial amounts. Fucose and galactose residues were predominantly sulfated at C-2 or sometimes at C-4. Resistant to acidic hydrolysis fraction (with high fucose, glucose, mannose and glucuronic acid content), probably, contained a core, built up of glucuronic acid and mannose residues and/or 3-linked GlcA residues, similarly to fucoidan from *S. latissima* (Bilan et al., 2010). Short monosulfated (1→3)- and/or (1→4)-linked fucooligosaccharides (sometimes terminated with (1→3)-linked Gal residues) were found in hydrolysate, and, probably, were branch points at C-4 of GlcA in the core, since Fuc-(1→4)-GlcA fragments were found. Presumably, fucoidan also had additional branch points at C-4 of GlcA in the core, formed by (1→4)- (or (1→6)-) linked galactooligosaccharides, which were hydrolyzed under selected conditions. Possibly, galactan chains had own branches, formed by (1→2)-linked galactose residues. Unsulfated xylose residues were probably terminal in chains built up of fucose (Duarte et al., 2001), since fragment, built up of xylose and fucose was found in the positive-ion mode to be free of sulfates. The absolute configurations of all sugar residues were not established due to restrictions of MS-method, but, some information was obtained by NMR. Exact location of glucose residues in the core remained unknown.

Acknowledgements

The work was supported by RFBR (Projects No. 12-04-0669), FEB RAS grants and the program 'Molecular and Cellular Biology' for Basic Research of the Presidium of Russian Academy of Sciences.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2012.06.033.

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