Carbohydrate Polymers 87 (2012) 186–194

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Anticancer activity *in vitro* of a fucoidan from the brown alga *Fucus evanescens* and its low-molecular fragments, structurally characterized by tandem mass-spectrometry

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ARTICLE INFO

Article history:
Received 20 April 2011
Received in revised form 18 June 2011
Accepted 20 July 2011
Available online 28 July 2011

Keywords:
Fucoidan derivatives
Structure
MS/MS
Anticancer activity

ABSTRACT

Fucoidan was isolated and purified from the brown algae *Fucus evanescens* and subjected to autohydrolysis to obtain low-molecular-weight fragments. MALDI-TOFMS analysis has shown that monosulfated fucose and more heavily sulfated (up to 5) fucooligosaccharides with polymerization degree (DP) 2, 4 and 6, including galactose-containing sulfated oligosaccharides were the products of autohydrolysis. The structural features of these fragments were elucidated by negative-ion potential lift tandem MALDI-TOF mass-spectrometry using arabinoosazone as a matrix, which allowed reducing the in-source fragmentation. Native fucoidan has been shown to exert anticancer activities in both human malignant melanoma cell lines SK-MEL-28 and SK-MEL-5. Low-molecular-weight fragments exhibited almost no action to cell proliferation in both cell lines and colony formation on SK-MEL-5 cells, but its inhibition activity to the colony formation of SK-MEL-28 cell lines was as high as was demonstrated by native fucoidan (70%). Probably, the inhibiting activity for SK-MEL-28 depended on the presence of sulfates and (1 → 4)-linked α-L-Fuc*p* residues in the main chain of fucoidan/oligosaccharides.

1. Introduction

Cancer is known to be one of the diseases that most badly threaten to human's life. Unfortunately drugs, which are used for cancer therapy, are toxic and affect not only cancer cells but also normal cells and tissues. That is why the search and isolation of new effective nontoxic compounds from natural sources is actual problem. At last decade marine brown algae attract much attention because they represent a rich and easily regenerated source of polysaccharides with various structures and biological activities: alginic acids, laminarans and fucoidans. The sulfated polysaccharides (fucoidans), which mainly build up of α-L-fucopyranose (α-L-Fuc*p*) residues, have wide variety of biological activities, including anticoagulant, antivirus, immunomodulating and antitumor activities (Khotimchenko, 2010; Wijesekara, Pangestuti, & Kim, 2011). In particular, the antitumor activity has attracted considerable attention. Several investigations have been reported that fucoidans effectively inhibited proliferation and colonies formation of cancer cells *in vitro* (Ermakova et al., 2011; Jiang et al., 2010), as well as inhibitory activity in tumors growing *in vivo* (Ye, Wang, Zhou, Liu, & Zeng, 2008).

Previous studies indicated that the sulfate content (Haroun-Bouhedja, Ellouali, Sinquin, & Boisson-Vidal, 2000), molecular weight (Nishino, Aizu, & Nagumo, 1991; Yang et al., 2008), monosaccharide composition, and structure of main polymer's chain of fucoidans (Patankar, Oehninger, Barnett, Williams, & Clark, 1993) had great influence on their biological activities. Thus highly sulfated fucoidans, mainly contained fucose residues possessed higher antitumor activities than heterofucans with low degree of sulfation (Haroun-Bouhedja et al., 2000; Koyanagi, Tanigawa, Nakagawa, Soeda, & Shimeno, 2003). Because the structures of fucoidans are complex and heterogeneous and as many studies of biological activities were carried out using relatively crude polysaccharide preparations, it is presently not easy to determine the overall relationship between activity and structure of polysaccharides.

Matrix-assisted laser desorbtion/ionization time-of-flight mass-spectrometry (MALDI-TOFMS) and collisionally induced dissociation tandem electrospray ionization mass-spectrometry (CID ESI MS/MS) are valuable addition to classic methods of carbohydrate chemistry and nuclear magnetic resonance (NMR) method for structure elucidation. Although anionic polysaccharides, such as chondroitin sulfates, heparins and carrageenans cannot be directly studied by mass spectrometry without decomposition, the availability of specific enzymes (McCleary & Matheson, 1986), effectively depolymerizing such carbohydrates can explain the

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doi:10.1016/j.carbpol.2011.07.036

success in investigations (Harvey, 2011; Zaia, 2004). In case of fucoidans there are no such effective enzymes described yet (Kusaykin et al., 2008). That is because sulfated oligosaccharides, suitable for mass-spectral investigations were reported to obtain from native fucoidan either by mild acid hydrolysis (Daniel et al., 2007; Shevchenko et al., 2007), or under solvolytic desulfation conditions (Anastyuk, Shevchenko, Nazarenko, Dmitrenok, & Zvyagintseva, 2009). In our recent work (Anastyuk et al., 2010) we employed autohydrolysis as an alternative method for decomposition of a fucoidan from the brown alga *Laminaria cichorioides*. It was shown that the developed procedure was a reliable method for preparation of mutlisulfated fucooligosaccharides. The fast and precise characterization of such mixtures by methods of mass-spectrometry is of practical significance for drug development, since highly sulfated low-molecular weight fucoidans and fucooligosaccharides, as mentioned above, are also possessing valuable biological activities and could show better biocompatibility than high-molecular-weight fucoidans (Cole & Jayson, 2008; Yang et al., 2008).

In the present work we report the negative-ion mode MALDI-TOF/TOFMS analysis of low-molecular derivatives of a fucoidan from the brown alga *Fucus evanescens* and their anticancer activity *in vitro*.

2. Experimental

2.1. Materials

Brown alga *F. evanescens* was collected in July 2008. The samples were collected at Iturup Island, Sea of Okhotsk, Far East of Russia.

The Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), L-glutamine, gentamicin, Basal Medium Eagle (BME), and trypan blue were purchased from Biolot (Russia).

All experiments were performed using ultra-pure water, produced with Direct-Q 3 equipment (Millipore, USA).

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

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).

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.

Monosaccharide composition was determined on a Biotronik IC-5000 carbohydrate analyzer (Wissenschaftliche Geräte, Germany) using a Shim-pack ISA-07/S2504 (0.4 cm × 25 cm) column eluted with a potassium borate buffer at an elution rate of 0.6 mL/min. Detection was carried out by bicinchoninate method and integration, on a Shimadzu C-R2 AX system.

The alditol acetate derivatives were analyzed by gas liquid chromatography (GLC) using a Hewlett-Packard 6850 (USA) chromatograph equipped with HP-5MS capillary column (30 m x 0.2 mm) using a temperature gradient of 150 → 230 °C at 3 °C min^{-1}.

2.3. General methods

The content of carbohydrates was determined using the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The content of sulfates in the polysaccharides was determined by a turbidimetric method (Dodgson, 1961). Analysis of monosaccharide composition of polysaccharide and oligosaccharide fractions was performed by the alditol acetate method (Sawardeker, Sloneker, & Jeanes, 1965). 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 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

Thalli of algae were extracted twice with EtOH for 3 h at 40 °C (alga/EtOH 1:0.8, w/w) to remove low-molecular-weight compounds. Then algae were dried in air and in vacuum, ground to particle size <1 mm, and extracted for 3 h twice with HCl (pH 2.0–2.3) at 60 °C (ratio 1:20). The extracts were combined, concentrated to one fifth the volume, neutralized with 3% aq $NaHCO_3$ until the pH was 5.7–6.1, dialyzed against 10 volumes of distilled water, and lyophilized to obtain **FeFL** preparation.

2.3.2. Anion exchange chromatography

A solution of polysaccharides **FeFL** in 0.05 M HCl (1.0 g in 50 mL) was applied onto a DEAE-cellulose column (Cl^- form, 3.2 cm × 22 cm) equilibrated with 0.01 M HCl. Laminaran was eluted with 0.05 M HCl (FeL of 43 mg), and the column was then successively eluted with 0.5, 1.5 and 2.0 M NaCl solutions, each time until the disappearance of carbohydrate in the eluate as determined by phenol–sulfuric acid test. The fractions were purified by ultrafiltration and lyophilized to get the polysaccharide preparations **FeF0** (53 mg), **FeF1** (537 mg) and **FeF2** (42 mg). The yield of the **FeF1** fraction from the **FeFL** fraction was 53.7%. The monosaccharide composition of the **FeF1** fraction (mol%): L-Fuc, 87.1, D-Xyl, 1.8, D-Man, 4.4, D-Glc, 1.3, D-Gal, 1.6, D-GlcA, 2.0. The sulfate content of the **FeF1** fraction was 28% from the fraction weight. The average molecular weight of fucoidan from *F. evanescens*, estimated by our group earlier, was about 60 kDa (Kusaykin et al., 2006).

2.3.3. Depolymerization of fucoidan by autohydrolysis

Autohydrolysis procedure (Anastyuk et al., 2010) was used to obtain oligosaccharides suitable for MS analyses: an aliquot of 12 mL of fucoidan **FeF1** (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. The low-molecular-weight fraction, **FeF1-AHL** (40.7 mg), was obtained from **FeF1** (54 mg) by fractionation in H_2O/EtOH (5:1, w/w). The yield of **FeF1-AHL** fraction (supernatant) from the **FeF1** fraction was 75.4%. The monosaccharide composition of the **FeF1-AHL** fraction (mol%): L-Fuc, 88.2, D-Gal, 4.9. The yield **FeF1-AHH** of high-molecular fraction (pellet) was 23%. The monosaccharide composition of the **FeF1-AHH** fraction (mol%): L-Fuc, 22.7, D-Xyl, 15.2, D-Man, 38.4%, D-Gal 4.5, D-GlcA, 7.4.

2.3.4. Derivatization of oligosaccharides

An aliquot of the oligosaccharide mixture (0.5 mL), obtained in autohydrolysis conditions without neutralization was evaporated on the rotary evaporator 3 times with addition of 0.5 mL of ethanol at 40 °C and finally neutralized with 5% NH_4OH solution in water and lyophilized.

2.3.5. Mass spectrometric analysis

Instrument settings for the positive-ion mode: accelerating voltage, 25 kV; laser power, 30%; number of shots, 100; laser shot rate, 66 Hz. Sample preparation for a positive-ion mode: a mixture containing 1 μL of sample (0.1 mg/mL) and 1 μL of 0.5 M DHB matrix solution in MeOH was introduced onto the sample plate

and air dried, followed by recrystallization with MeOH. Instrument settings for the negative-ion mode: accelerating voltage, −25 kV; laser power, 17%; number of shots, 100; 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. Anticancer activity in vitro

2.4.1. Cell culture

The SK-MEL-5 (ATCC # HTB-70), SK-MEL-28 (ATCC # HTB-72) human malignant melanoma cell lines were grown in monolayer in DMEM supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, and 1% penicillin–streptomycin at humidified atmosphere containing 5% CO_2.

2.4.2. Cell viability assay

To estimate cell cytotoxicity of fucoidans, SK-MEL-5 and SK-MEL-28 cells were seeded in density of 4.0×10^3 cells/200 μL of complete DMEM/10%FBS medium in 96-well plates. After 24 h the medium was removed and cells were treated with serial fucoidans dilutions of 2-fold dilution steps covering range from 100 to 800 μg/mL. After incubation for 24 h the cells (both in suspension and attached) were harvested by trypsinization and centrifuged. The cell pellet was resuspended in 200 μL of phosphate buffer and stained with trypan blue. The number of viable and dead cells was counted using a hemacytometer. Cell viability is the percentage of viable cells among the total (viable and dead) number of cells.

For determination of cell proliferation, SK-MEL-5 and SK-MEL-28 cells (3.0×10^3) were seeded in 96-well plates and after 24 h and cells were treated with fucoidans (100–400 μg/mL) for 48 h. After incubation the cells were treated with trypan blue. The number of viable and dead cells was counted as described above.

2.4.3. Soft agar clonogenic assay

Soft agar assay was performed on human malignant melanoma SK-MEL-5, SK-MEL-28 cell lines. In brief, cells (2.4×10^4/mL) were grown in 1 mL of 0.3% Basal Medium Eagle (BME) agar containing 10% FBS. The culture was maintained at 37 °C in 5% CO_2 incubator for 2 weeks and the cell colonies were scored using a microscope and the ImageJ computer software program as described by Colburn, Wendel, and Abruzzo (1981).

2.5. Data analysis

All figures shown in this manuscript were representative of at least two independent experiments with similar results. Statistical differences were evaluated using the Student's *t*-test and considered significant at $p \leq 0.05$.

3. Results and discussion

3.1. Depolymerization of fucoidan in mild conditions

The application of autohydrolysis[1] in our recent work (Anastyuk et al., 2010) for depolymerization of the fucoidan from the brown alga *L. cichorioides* was successful, since we have obtained a low-molecular-weight mixture of multisulfated fucooligosaccharides which were shown by tandem ESIMS and MALDI-TOFMS to well reflect the structure of the polysaccharide, established with NMR and methylation analysis (Zvyagintseva et al., 2003).

Using even milder conditions of autohydrolysis, the sample of highly purified fucoidan from *F. evanescens* **FeF1** (5 mg/mL in water), which was also extracted in mild conditions than in earlier report (Anastyuk et al., 2009), was depolymerized to obtain the low-molecular-weight mixture, suitable for mass spectrometric analysis.

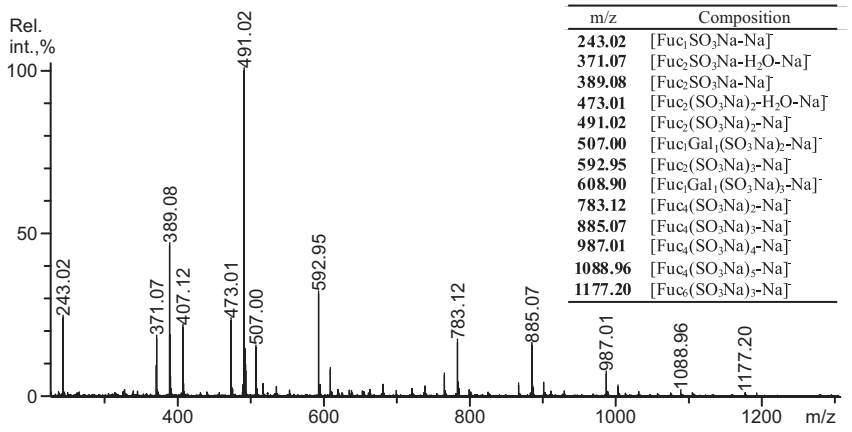
3.2. MALDI-TOFMS analysis of the oligosaccharide mixture

The composition of fraction **FeF1-AHL** (yield 75.4%), obtained by water–ethanol fractionation of the autohydrolysis reaction mixture was analyzed by negative-ion mode MALDI-TOFMS. The analysis revealed that the **FeF1-AHL** fraction contained a set of fucooligosaccharides with DP 2–6 and up to 5 sulfate groups per molecule, including monosulfated fucose residues at *m*/*z* 243.0 (Fig. 1). The main component, due to mass-spectral investigation of the mixture, was disulfated fucobiose at *m*/*z* 491.0 instead of monosulfated fucose, which was the main component of autohydrolysate mixture, obtained from the fucoidan of *L. cichorioides* (Anastyuk et al., 2010). The signals of the ions of sulfated fucotrioses $[Fuc_3(SO_3Na)–Na]^-$ at *m*/*z* 535.1, $[Fuc_3(SO_3Na)_2–Na]^-$ at *m*/*z* 637.1 and $[Fuc_3(SO_3Na)_3–Na]^-$ at *m*/*z* 739.1 had very low intensity as well as the signals from the sulfated fucopentaoses, indicating that oligosaccharides with even DP hydrolyzed faster. This fact was not observed in the MALDI-TOFMS spectrum of fucooligosaccharide mixture from *L. cichorioides*, having backbone structure of a 3-linked, 2,4-disulfated α-L-Fuc*p* residues (Anastyuk et al., 2010; Zvyagintseva et al., 2003). Probably, the (1 → 3)-type of linkage was less-stable in the autohydrolysis conditions. The multisulfated oligosaccharides, composed of fucose and hexose (D-Gal, due to the monosaccharide composition analysis) were also detected: $[FucGal(SO_3Na)_2–Na]^-$ at *m*/*z* 507.0 and $[FucGal(SO_3Na)_3–Na]^-$ at *m*/*z* 608.9 (Fig. 1). It must be noted that sulfated oligosaccharides, simultaneously containing galactose and fucose residues, were detected by ESIMS in our previous report, but they were singly-sulfated (Anastyuk et al., 2009). In contrast, mixed oligosaccharides, containing along with fucose glucuronic acid, xylose and galactose, which were detected in the previous work, were not observed. However, it was found, that high-molecular fraction **FeF1-AHH** contained minor constituents of **FeF1** fucoidan – xylose, mannose and glucuronic acid. Thus, they were not hydrolyzed under mild autohydrolysis conditions. The mass-spectrum also contained $[M–H_2O]^-$ signals, which, probably were the fragment ions $[M(SO_3Na)_n–NaHSO_4–Na]^-$ from the sulfate loss, $n \geq 2$, where *n* – number of sulfate groups per molecule and M represents sodium salt of oligosaccharides.

3.3. Tandem MALDI-TOFMS analysis of the oligosaccharides

To elucidate the structural features of the most abundant oligosaccharides, revealed in fraction **FeF1-AHL**, MALDI-TOF/TOFMS technique was applied. For interpretation of MS/MS spectra, the following data of the former studies used: the formation of characteristic ^{0,2}A-/^{0,2}X-ions during negative-ion CID MS/MS required available proton at the C-3 hydroxyl group (following nomenclature, introduced by Domon & Costello, 1988). This fact was confirmed by the recent studies of the fucooligosaccharides from *L. cichorioides*, predominantly linked with (1 → 3)-type of linkage. The intensities of the ^{0,2}A-/^{0,2}X ions were very low during CID MS/MS and MALDI TOF/TOFMS (Anastyuk et al., 2010; Zvyagintseva et al., 2003). On the contrary, CID MS/MS spectra of fucooligosaccharides from *Ascophyllum nodosum*, which contained both (1 → 3)- and predominantly (1 → 4)-linked α-L-Fuc*p* residues, demonstrated intensive ^{0,2}X- and ^{0,2}A-type ions (Daniel et al., 2007). The assignment of the fragment ions, having the same *m*/*z* but arising from the different termini was based on the results

[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.



m/z	Composition
243.02	[Fuc$_1$SO$_3$Na-Na]$^-$
371.07	[Fuc$_2$SO$_3$Na-H$_2$O-Na]$^-$
389.08	[Fuc$_2$SO$_3$Na-Na]$^-$
473.01	[Fuc$_2$(SO$_3$Na)$_2$-H$_2$O-Na]$^-$
491.02	[Fuc$_2$(SO$_3$Na)$_2$-Na]$^-$
507.00	[Fuc$_1$Gal$_1$(SO$_3$Na)$_2$-Na]$^-$
592.95	[Fuc$_2$(SO$_3$Na)$_3$-Na]$^-$
608.90	[Fuc$_1$Gal$_1$(SO$_3$Na)$_3$-Na]$^-$
783.12	[Fuc$_4$(SO$_3$Na)$_2$-Na]$^-$
885.07	[Fuc$_4$(SO$_3$Na)$_3$-Na]$^-$
987.01	[Fuc$_4$(SO$_3$Na)$_4$-Na]$^-$
1088.96	[Fuc$_4$(SO$_3$Na)$_5$-Na]$^-$
1177.20	[Fuc$_6$(SO$_3$Na)$_3$-Na]$^-$

Fig. 1. Negative-ion MALDI-TOFMS of the low-molecular-weight oligosaccharide fraction. **FeF1-AHL**, obtained from a fucoidan of *F. evanescens* by autohydrolysis.

of mass-spectral elucidation of the structural features of the modified fucooligosaccharides (Anastyuk et al., 2010; Zvyagintseva et al., 2003).

A tandem MALDI-TOFMS spectrum of the monosulfated fucose ion at *m/z* 243 (not shown) had almost no difference with similar spectrum, obtained in our previous work by ESIMS/MS (Anastyuk et al., 2009). Its fragment ions were ^{0,2}X at *m/z* 138.9 (100%), ^{0,2}A at *m/z* 182.9 (80%), $[M–H_2O]^-$ at *m/z* 225.0 (30%), and HSO_4^- at *m/z* 96.9. The first two fragment ions suggested sulfation of the α-L-Fuc*p* residue at C-2 and C-4, respectively. It must be noted, that $[M–H_2O]^-$ signal had very low intensity at ESIMS/MS spectrum (Anastyuk et al., 2009). A tandem MALDI-TOFMS spectrum of the disulfated fucobiose at *m/z* 344.9 featured the presence of $[M–NaHSO_4]^-$ at *m/z* 225.0 (100%). The rest of the fragment ions of the selected ion had intensities below 3% of the ion at *m/z* 225.0. There were no ^{0,3}X fragment ions detected at *m/z* 168.9 that would have suggested sulfation at C-3 of the α-L-Fuc*p* residues (Tissot, Salpin, Martinez, Gaigeot, & Daniel, 2006).

A tandem MALDI-TOFMS spectrum (not shown) of the monosulfated fucobiose at *m/z* 389.0 was similar to that observed by ESIMS/MS in our previous work (Anastyuk et al., 2009). However, a signal of the ^{0,3}X-type ion from the cross-ring cleavage at *m/z* 315.0 was absent at the MALDI-TOFMS/MS spectrum. The MS/MS spectrum of disulfated fucobiose at *m/z* 491.0 (Fig. 2) also did not contained mentioned above ion. The intensity of the fragment ions from the cleavage of monosulfated fucose residues from the reducing end (Y_1 at *m/z* 243.0) and non-reducing end (B_1 at *m/z* 225.0) were almost equal (Fig. 2, at the right). It must be noted, that the intensity of the fragment ion $^{0,2}A_2$ at *m/z* 329.1 was highest, as observed at ESIMS/MS spectrum of doubly-charged ion of disulfated fucobiose from the fucoidan of *A. nodosum* (Daniel et al., 2007), where (1 → 4)-type of glycosidic linkage between α-L-Fuc*p* residues predominated. The fragment ion $^{0,2}X_0$ of low intensity at *m/z* 138.9 from the cross-ring cleavage of the α-L-Fuc*p* residue at the reducing end, together with fragment ion Y_1, indicated sulfation of the fucose residue at C-2. Again, such fragment ion could be observed if the fucose residues of selected ion were (1 → 4)-linked (Daniel et al., 2007). Fragment ions $^{0,2}X_1$ at *m/z* 285.0 and $^{0,2}X'_1$ at *m/z* 386.9 indicated sulfation at C-2 of the fucose residue at the non-reducing end, suggesting the following structural variant of the selected ion: α-L-Fuc*p*-2-OSO_3^--(1 → 4)-α-L-Fuc*p*-2-OSO_3^- (Fig. 2, at the right). The fragment ions B'_1 at *m/z* 326.9 and $^{0,2}A_2$ at *m/z* 431.1 suggested another structural variant (Fig. 2, at the left). Thus, the α-L-Fuc*p* residues in the most abundant component of the oligosaccharide mixture was predominantly linked with (1 → 4)-type of linkage, proving that (1 → 3)-type of glycosidic linkage hydrolyzed faster in the selected conditions.

The interpretation of the MALDI-TOFMS/MS of triply-sulfated fucobiose at *m/z* 593.0 (Fig. 3) was similar. It was revealed, that selected ion had two structural variants. Sulfate groups occupied position C-2 and C-4 of the α-L-Fuc*p* residue at the reducing end that was confirmed by observation of the Y'_1 fragment ion at *m/z* 344.9. The presence of this ion revealed structural variant having (1 → 3)-type of linkage between α-L-Fuc*p* residues (Fig. 3, at

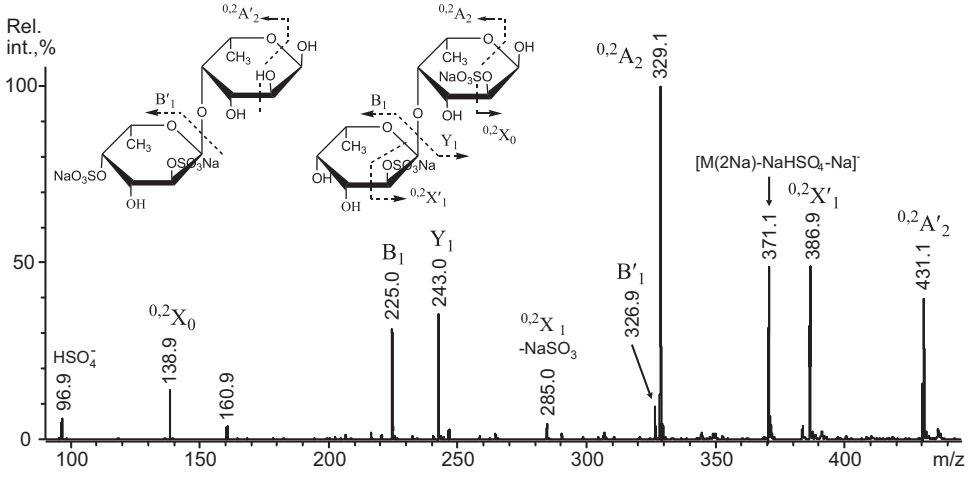


Fig. 2. Negative-ion tandem MALDI-TOFMS of the $[Fuc_2(SO_3Na)_2–Na]^-$ ion at *m/z* 491.0. M represents sodium salt of oligosaccharides.

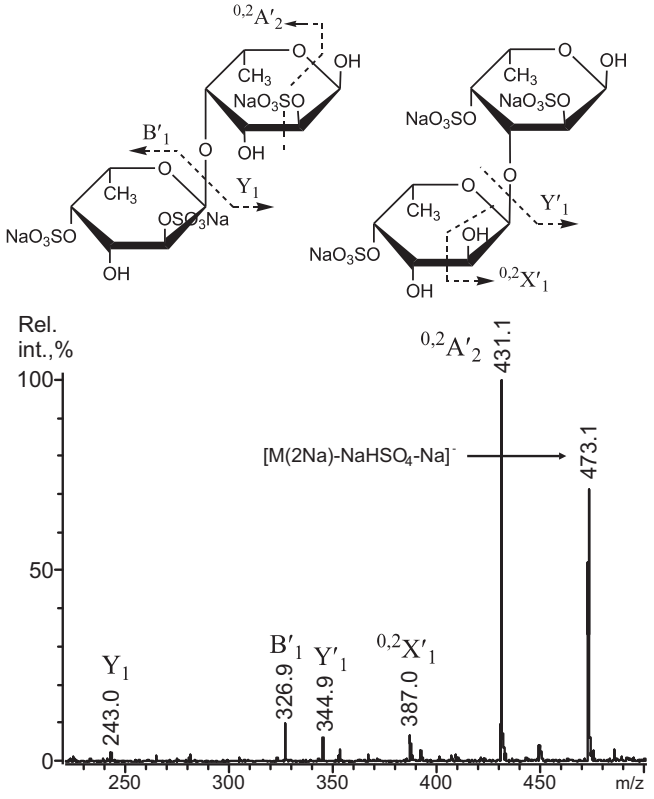


Fig. 3. Negative-ion tandem MALDI-TOFMS of the $[Fuc_2(SO_3Na)_3–Na]^-$ ion at *m*/*z* 593.0. M represents sodium salt of oligosaccharides.

the right), since no mass-spectral (see above) or literature (Bilan et al., 2002) data on the 3-O-sulfation of the α-L-Fuc*p* residues were found. The signal of $^{0,2}A'_2$ fragment ion at *m*/*z* 431.1 was in this case most intensive, suggesting another structural variant of α-L-Fuc*p*-2,4-di-OSO_3^--(1 → 4)-α-L-Fuc*p*-2-OSO_3^- (Fig. 3, at the left).

A tandem MALDI-TOF/TOFMS of the $[FucGal(SO_3Na)_2–Na]^-$ ion at *m*/*z* 507.0 (Fig. 4.) exhibited rather complex fragmentation pattern. The most intensive was B_1 fragment ion at *m*/*z* 241.0 from the cleavage of the dehydrated monosulfated hexose (D-Gal, due to monosaccharide composition analysis) residue from the non-reducing end. In contrast, B_1-type ion at *m*/*z* 225.0 from the cleavage of the dehydrated monosulfated α-L-Fuc*p* residue had low intensity. However, the intensities of the fragment ions of Y-type at *m*/*z* 243.0 and 259.0 from the cleavage of the monosulfated α-L-Fuc*p* and hexose residues, respectively, were almost equal. The fragment ion $^{0,2}X'_1$ at *m*/*z* 403.0 had relatively high intensity. Probably, the abundance of such fragment ion depend on the 2-O-sulfation of the non-reducing residue of the molecule since such fragments were intensive while observed upon fragmentation of the ESIMS/MS of the oligosaccharides with GlcA at the reducing end, such as Fuc-(1 → 4)-Fuc-(1 → 3)-GlcA (Anastyuk et al., 2009). Also, this presumption can be confirmed with the low intensity of the $^{0,2}X_1$ ions at *m*/*z* 285.0, which were low or absent along all MALDI-TOF/TOF spectra in the present work in except of MS/MS of the monosulfated fucobiose at *m*/*z* 389.0 (data not shown). The presence of the fragment ion Y'_1 c *m*/*z* 360.9 and the lack of the corresponding *A*-type ion, suggested (1 → 3)-type of linkage (Fig. 4, at the right) between the fucose and hexose residues, while fragment ion of $^{0,2}A'_2$ type at *m*/*z* 447.0 had relatively high intensity (Fig. 4, at the middle). The low intensity of the $^{0,2}X_0$ at *m*/*z* 138.9 comparatively to the intensities of the fragment ions of Y-type indirectly suggested the existence of the third structural variant (Fig. 4, at the left). The fragment ions B'_1 at *m*/*z* 342.9 and $^{0,2}A_1$ at *m*/*z* 198.9 suggested 2,4-di-O-sulfation of the hexose residue on the non-reducing end. Thus, assuming all the mentioned above and the highest intensity of the B_1 fragment ion, it was concluded, that structural variant with hexose at the non-reducing end predominated. Probably, sulfated D-Gal residues could be attached randomly to the backbone as the single branching points, being (1 → 3)- or (1 → 4)-linked to the α-L-Fuc*p* residues of the main chain.

A tandem MALDI-TOFMS at *m*/*z* 783.2 (Fig. 5) of the disulfated fucotetraose featured the following. The signals of the fragment ions $^{0,2}X_2$ at *m*/*z* 431.1 and $^{0,2}X'_2$ at *m*/*z* 533.1 were detected, as for the ion $^{0,2}A_2$ at *m*/*z* 329.0, indicating (1 → 4)-type of linkage between α-L-Fuc*p* residues at the non-reducing end. The signals of the fragment ions $^{0,2}X_0$ at *m*/*z* 138.9 and Y_1 c *m*/*z* 243.0 were

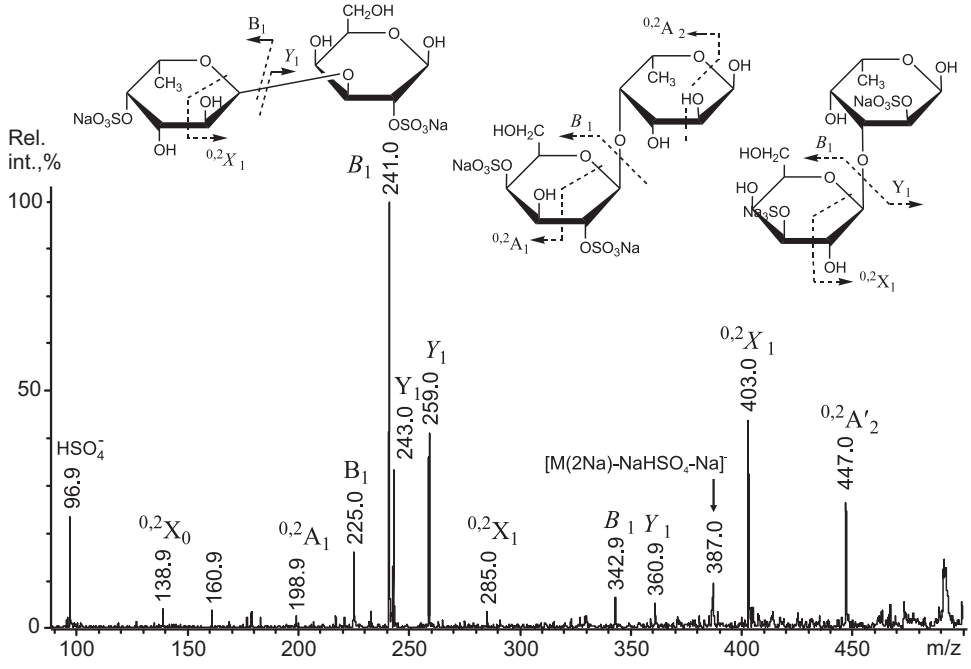


Fig. 4. Negative-ion tandem MALDI-TOFMS of the $[FucGal(SO_3Na)_2–Na]^-$ at *m*/*z* 507.0. M represents sodium salt of oligosaccharides.

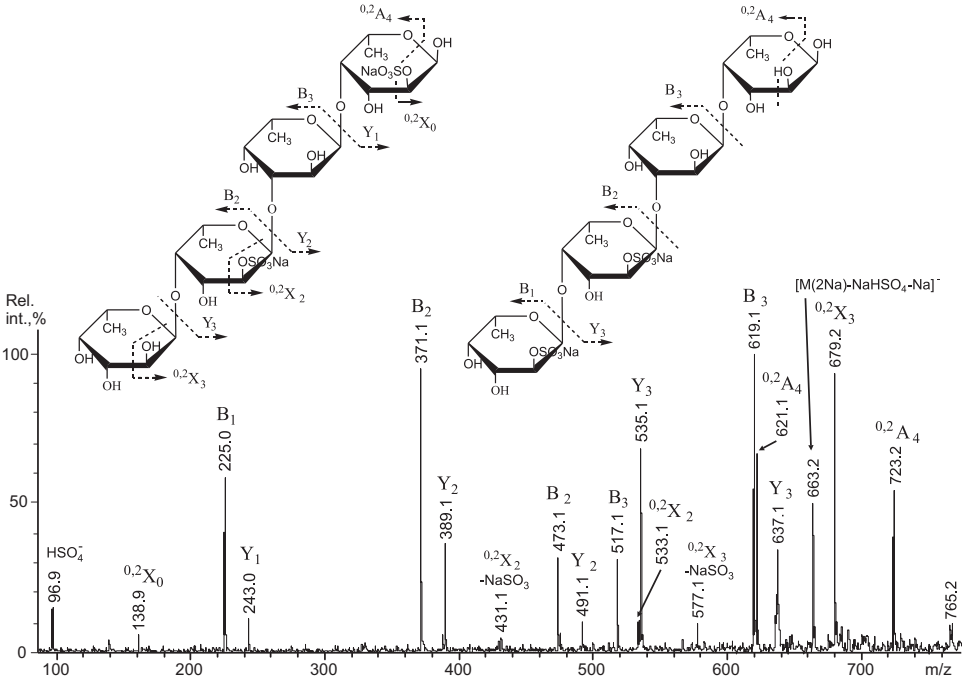


Fig. 5. Negative-ion tandem MALDI-TOFMS of the $[Fuc_4(SO_3Na)_2–Na]^-$ ion at *m*/*z* 783.12. M represents sodium salt of oligosaccharides.

low, probably indicating low degree of the sulfation of the reducing α-L-Fuc*p* residue. The differentiation between the reducing and non-reducing ends was suggested by MALDI-TOF/TOFMS of the corresponding ethylglycoside $[Et\text{-}Fuc_4(SO_3Na)_2–Na]^-$ ion at *m*/*z* 811.10 (data not shown).

The more heavily sulfated fucotetraoses were also examined (Fig. 6). Their tandem mass-spectra featured intensive ions from glycosidic bonds cleavage near the doubly-sulfated residues, probably, due to the high forces of charge repulsion. The $^{0,2}A_4$ ions from the cross-ring cleavage of the α-L-Fuc*p* residue at the reducing end were intensive in all spectra. It must be noted, that the intensity of $^{0,2}X$-type fragment ions signals decreased with the increasing of sulfation.

The intensity of the pentasulfated fucotetraose ion was low, but, nevertheless, its tandem MALDI-TOFMS at *m*/*z* 1086.90 was recorded (Fig. 7). The spectrum contained only 4 intensive signals, while others were low. The most intensive signals were Y_2 at *m*/*z* 592.9 and B_2 at *m*/*z* 473.0 from glycosidic bonds splitting and cleavage of triply-sulfated fucobiose from the reducing- and disulfated fucobiose from the non-reducing ends respectively, probably caused by the high forces of charge repulsion. The abundance of the signal of the ion, indicating the loss of $NaHSO_4$ at *m*/*z* 968.9 was as high as B_2-ion. Other signals, corresponding to desulfation, had low intensity (Fig. 7). The only fragment ion $^{0,2}A_4$ at *m*/*z* 926.9 from the cross-ring cleavage, having high intensity, indicated (1 → 4)-type of linkage between α-L-Fuc*p* residues at the reducing end. The other signal from the cross-ring cleavage and evidence of (1 → 4)-type of linkage was $^{0,2}X_2$ at *m*/*z* 634.9, however it was found desulfated ($^{0,2}X_2$-$NaSO_3$). Assuming all mentioned above, the following structure of the selected ion was suggested: α-L-Fuc*p*-2-OSO_3Na-(1 → 4)-α-L-Fuc*p*-2-OSO_3Na-(1 → 3)-α-L-Fuc*p*-2,4-di-OSO_3Na-(1 → 4)-α-L-Fuc*p*-2-OSO_3Na.

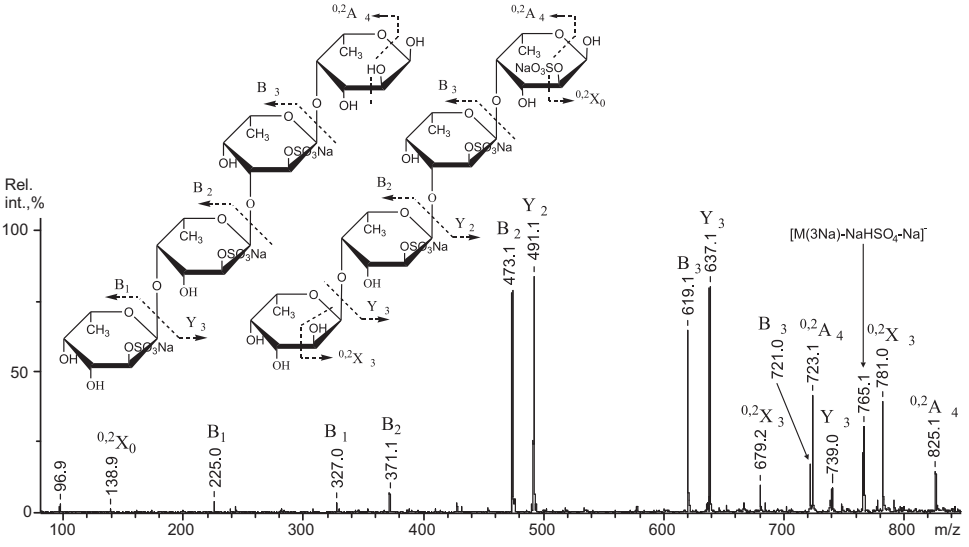


Fig. 6. Negative-ion tandem MALDI-TOFMS of the $[Fuc_4(SO_3Na)_3–Na]^-$ ion at *m*/*z* 885.07. M represents sodium salt of oligosaccharides.

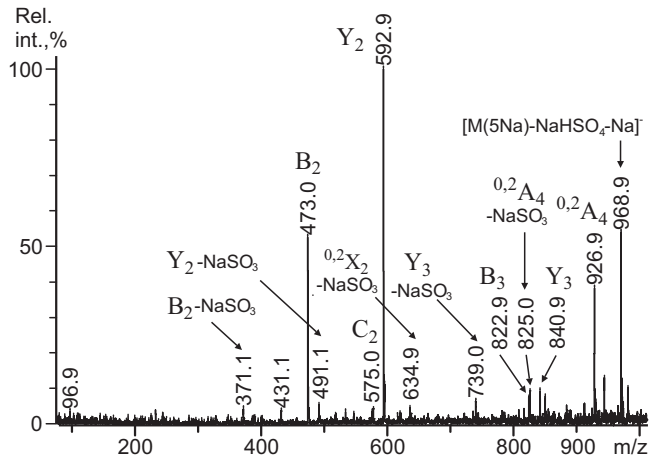


Fig. 7. Negative-ion tandem MALDI-TOFMS of the $[Fuc_4(SO_3Na)_5–Na]^-$ ion at *m/z* 1086.90. M represents sodium salt of oligosaccharides.

3.4. Anticancer activity of native fucoidan versus oligosaccharide mixture

We examined the effect of native fucoidan preparation from *F. evanescens* **FeF1** and oligosaccharide mixture **FeF1-AHL** (obtained by autohydrolysis of **FeF1** and characterized by mass-spectrometry) on the cytotoxicity of human SK-MEL-5 and SK-MEL-28 melanoma cells. Both native fucoidan and oligosaccharides did not show significant cytotoxicity after treatment for 24 h at concentration from 100 to 800 μg/mL (data not shown). Earlier we reported that fucoidan from *L. gurjanovae* also did not show cytotoxic effect against normal epidermal cells JB6Cl 41 (Lee et al., 2008). Next we determined the cell proliferation after treatment with preparations under study at concentrations from 100 to 400 μg/mL. The cells were treated with different doses of preparations for 48 h, and proliferation was determined as described under Section 2.4.2.

A dose-dependent decreasing in the rate of proliferation was found for native fucoidan for both cell lines. In contrast, **FeF1-AHL** showed slight inhibition of cell proliferation of SK-MEL-28 cells and did not show activity against SK-MEL-5 cells (Fig. 8A and B). Earlier observations showed that decrease (from over 1000 kDa) the molecular weight of fucoidans as well as oversulfation potentized the anticancer activity (Teruya, Konishi, Uechi, Tamaki, & Tako, 2007; Yang et al., 2008). It was also reported that low-molecular-weight fucoidans (18.6 kDa) from *A. nodosum* at the high concentration of 1.0 mg/mL showed almost 100% inhibition activity on CCL39 cell growth (Haroun-Bouhedja et al., 2000). Our sample of native fucoidan exhibited high activity being rather low-molecular weight (60 kDa) and highly sulfated (28%) polymer. On the contrary, low-molecular weight fragments probably were either not enough sulfated or had too low DP to exert high activity. It should be noted that antiproliferative effects of sulfated polysaccharides also could depend on cell lines (Jiang et al., 2010). Melanoma SK-MEL-28 cell lines were more sensitive for **FeF1** and **FeF1-AHL**, whereas melanoma SK-MEL-5 cell lines were rather resistant towards **FeF1-AHL.**

Effect of fucoidan **FeF1** and oligosaccharide mixture **FeF1-AHL** on colony formation of melanoma cell lines was carried out with soft agar assay. It is a well-developed model for studying a potential of antitumor agents. Results indicated that **FeF1** fucoidan from *F. evanescens* significantly inhibited colony formation of SK-MEL-5 and SK-MEL-28 cells (Fig. 8C and D). The inhibition was 63% and 70%, respectively, at 400 μg/mL. On the other hand, **FeF1-AHL** oligosaccharides inhibited colony formation of SK-MEL-28 and SK-MEL-5 cells for 65% and 38%, respectively, at the same dose. Recent studies on the inhibitory effect on the colony formation of SK-MEL-28 cell cultures by treating with different fucoidans (Ermakova et al., 2011) also showed noticeable results. The galactofucan fraction (probably, having branched structure from *Eclonia cava* with 22% of sulfates shown only 8% activity.

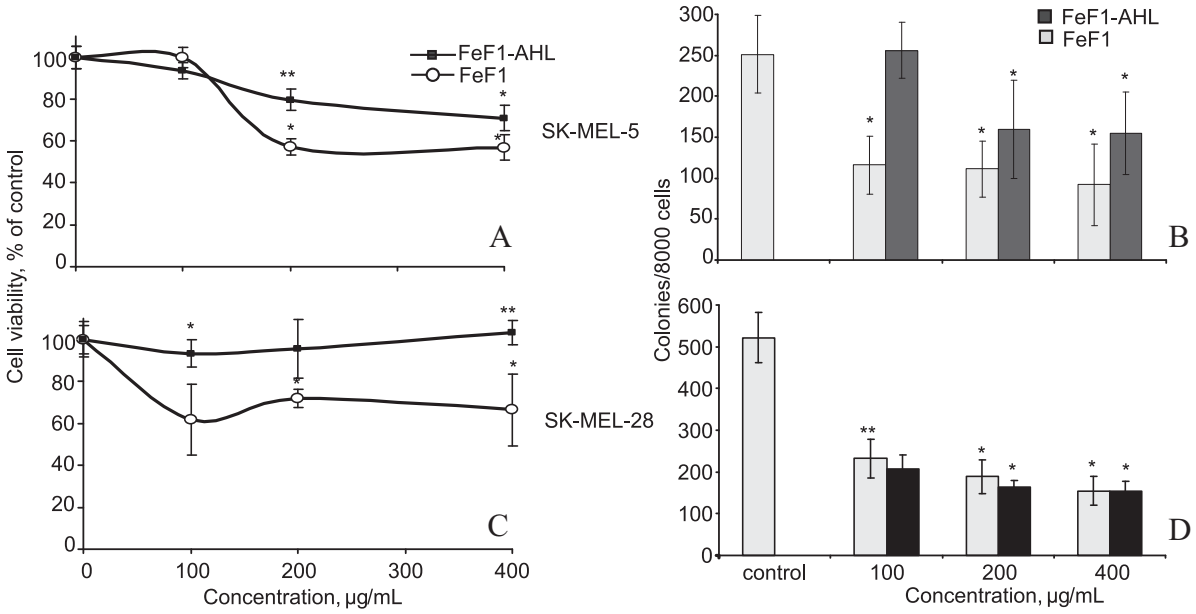


Fig. 8. Effect of native fucoidan (**FeF1**) and oligosaccharides (**FeF1-AHL**) from the brown algae *F. evanescens* on cell viability SK-MEL-5 (A) and SK-MEL-28 (B). Cells were treated with different concentrations of **FeF1** and **FeF1-AHL** (100, 200, and 400 μg/mL) for 48 h. Cell viability was determined by trypan blue staining and expressed as a percentage of viable cells in the total number of cells counted. (C and D) The inhibitory effects of **FeF1** and **FeF1-AHL** on colony formation in human skin melanoma cell lines SK-MEL-5 (C) and SK-MEL-28 (D) comparing untreated control cells. Cells (2.4×10^4/mL) treated with/without different concentrations of **FeF1** and **FeF1-AHL** (100, 200, and 400 μg/mL) were exposed in 1 mL of 0.3% BME's agar containing 10% FBS.

Other fucoidan fraction from *Eclonia cava* (high-molecular-weight galactofucan with 19% of sulfates), fucoidan fraction from *Sargassum hornery* (linear chain of (1 → 3)-linked α-L-Fuc*p* residues, with sulfate groups at positions 2; 14.9% of sulfates) and galactofucan from *Costaria costata* (19% of sulfates) had relatively low activity, below 30%. The unsulfated fucoidan fraction ShF2 from *S. hornery*, having linear chain of alternating (1 → 3)- and (1 → 4)-linked α-L-Fuc*p* residues as well as ShF3 fucoidan fraction (linear chain of (1 → 4)-linked α-L-Fuc*p* residues with sulfate groups at positions 2, 16.9% of sulfates) from the same algae exhibited higher activity (Ermakova et al., 2011). The fact, that sulfated low-molecular-weight fragments from the fucoidan of *F. evanescens* possessed almost the same activity against colony formation of SK-MEL-28 cell lines as native polymer could be explained by the structure of backbone, having alternating (1 → 3)- and (1 → 4)-linked α-L-Fuc*p* residues as the main chain. Furthermore, autohydrolysis enriched the content of (1 → 4)-linked α-L-Fuc*p* residues in the main chain due to MALDI-TOFMS/MS analysis of the products, since main component was doubly-sulfated (1 → 4)-linked fucobiose. Hence, probably, the inhibition activity of fucoidans for SK-MEL-28 cell lines depended on the presence of sulfates and (1 → 4)-linked α-L-Fuc*p* residues in the main chain of fucoidan/oligosaccharides.

4. Conclusion

The autohydrolysis at the safe (5 mg/mL in water) concentration was shown to effectively depolymerize the fucoidan of the brown alga *F. evanescens* to produce oligosaccharides with DP up to 6 and from 1 to 5 sulfate groups per molecule. The autohydrolysis was found to be sulfate-retaining depolymerizing reaction as the ratio of sulfate groups per monosaccharide remained almost the same as for native fucoidan (Bilan et al., 2002). The composition, sulfation pattern and sequence of the oligosaccharides were suggested by tandem MALDI-TOFMS. It was confirmed, that their structural properties well-reflected the structure of the backbone, elucidated by independent methods (Bilan et al., 2002). Moreover, D-Gal residues, natively occurring in fucoidan's monosaccharide composition were shown to be multiply sulfated and found as a part of the main chain as the following fragments were characterized: Gal-2-OSO_3Na-(1 → 3)-Gal-2-OSO_3Na, Gal-2,4-di-OSO_3Na-(1 → 4)-Fuc and Fuc-2-OSO_3Na-(1 → 4)-Gal-2-OSO_3Na. Absolute configuration of the glycosidic bonds was not established. The prevalence of oligosaccharides with even DP (except for monosulfated L-Fuc at *m*/*z* 243.02) in the reaction mixture after autohydrolysis could be explained by the tendency of the (1 → 3)-type of linkage to hydrolyze faster than (1 → 4)-type of linkage in selected conditions. Fucoidan **FeF1** has been shown to exert anticancer activities in both SK-MEL-28 and SK-MEL-5 cell lines. In contrast, oligosaccharide mixture **FeF1-AHL**, obtained by autohydrolysis of **FeF1**, slightly inhibited cell proliferation and colony formation of melanoma cell lines SK-MEL-5. But, it exhibited almost the same activity as **FeF1** fucoidan against colony formation of melanoma cell lines SK-MEL-28. Probably, the inhibition activity for SK-MEL-28 cell lines depended on the presence of sulfates and (1 → 4)-linked α-L-Fuc*p* residues in the main chain of fucoidan/oligosaccharides. The results clearly showed that fucoidans and their low-molecular-fragments play an inhibitory role of colony formation of human melanoma cells and may be effective antitumor agents.

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 Sciences.

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