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Further studies on structure of fucoidan from brown alga *Saccharina gurjanovae*

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ABSTRACT

A sulfated galactofucan **SgF** (MW 123 kDa) was purified from the brown alga *Saccharina gurjanovae*. Polysaccharide was depolymerized by autohydrolysis at 25 and 60 °C, and products were studied by mass spectrometry and ^{13}C NMR spectroscopy. According to results of investigation, the main chain of this polysaccharide is built of a repeating units →3)-α-L-Fuc*p*-(2,4-OSO_3^-)-(1→. Fucose chains could be sometimes terminated by (1 → 3)-linked galactose residues. Shorter (1 →4)- and/or (1 → 6)-linked sulfated galactose chains are attached at positions C-2, C-3 of fucose residues. Sulfate groups can occupy positions C-2 and/or sometimes C-3 of Gal residues, but a sulfation at C-4 of the galactofucan could not be excluded. The **SgF-AH25-H** preparation (71 kDa) was obtained by autohydrolysis of **SgF** at 25 °C, which leaded to a selective desulfation at C-2 and, probably, to a cleavage of galactose chains, since structure of **SgF-AH25-H** represented a repeating unit →3)-α-L-Fuc*p*-(4-OSO_3^-)-(1→, which was definitely established by ^{13}C NMR spectroscopy. Galactofucan **SgF** and its derivative **SgF-AH25-H** exhibited no cytotoxic activity and leaded to about the same colony formation inhibition in colon cancer DLD-1 cells. Hence, structural simplification of **SgF** by lowering its molecular weight, desulfation at C-2 and removing of galactose residues by autohydrolysis at 25 °C did not decrease its anticancer activity. This procedure allows obtaining standardized products which can be used as medical.

1. Introduction

Fucoidans are water-soluble polysaccharides synthesized by brown algae which possess multiple biological activities (Anastyuk, Shevchenko, Ermakova, et al., 2012; Ermakova et al., 2011; Khil'chenko et al., 2011; Vishchuk, Ermakova, & Zvyagintseva, 2011). Fucoidans represent the family of sulfated homo- and heteropolysaccharides mainly build up α-L-fucopyranose residues, which may be partially acetylated. In addition, they can contain different amounts of other monosaccharides residues such as galactose, xylose, mannose, rhamnose and uronic acids (Kusaykin et al., 2008; Li, Lu, Wei, & Zhao, 2008).

Fucoidans have complex and diverse structure, which strongly depends of the life-stage of the seaweed (Anastyuk, Imbs, Shevchenko, Dmitrenok, & Zvyagintseva, 2012; Skriptsova, Shevchenko, Tarbeeva, & Zvyagintseva, 2012; Skriptsova, Shevchenko, Zvyagintseva, & Imbs, 2009). Therefore, in order to analyze fucoidans by spectroscopic techniques, such as ^{13}C NMR its structure must be modified in many cases (Bilan & Usov, 2008). At present time fucoidans are modified by chemical (Bilan et al., 2010, 2013) and enzymatic (Berteau & Mulloy, 2003; Kusaykin, Burtseva, Svetasheva, Sova, & Zvyagintseva, 2003; Silchenko et al., 2013, 2014) methods, and structures of simplified polysaccharides and/or oligosaccharides are investigated by NMR spectroscopy and mass spectrometry.

Since the enzymes as best instruments for the fucoidan analysis are not widely available (Kusaikin et al., 2004), mild acid hydrolysis of fucoidans is often employed (Chevolot, Mulloy, Ratiskol, Foucault, & Colliec-Jouault, 2001; Daniel et al., 2007; Duarte, Cardoso, Noseda, & Cerezo, 2001). The recent study on sulfated fucans from marine invertebrates, which have a repeating sulfation pattern (unlike fucoidans) leads not only to

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depolymerization of polysaccharides, but also to their selective desulfation at the position C-2 (Pomin, Valente, Pereira, & Mourao, 2005). The requirement for the selective 2-desulfation was the presence of an exclusively 2-sulfated fucose residues, linked to or preceded by a 4-sulfated fucose residue (Pomin et al., 2005).

An alternative to mild acid hydrolysis, method of autohydrolysis[1] was successfully used for depolymerization of fucoidans, because fragments (often invisible by ^{13}C NMR technique) of fucoidans could be directly analyzed by mass spectrometry even without purification (Anastyuk, Imbs, Dmitrenok, & Zvyagintseva, 2014; Anastyuk, Imbs, et al., 2012; Anastyuk, Shevchenko, Dmitrenok, & Zvyagintseva, 2012b; Anastyuk, Shevchenko, Ermakova, et al., 2012; Anastyuk, Shevchenko, Nazarenko, Dmitrenok, & Zvyagintseva, 2009; Anastyuk et al., 2010).

Recently, the polysaccharide and lipid composition of the brown alga *Saccharina* (ex name *Laminaria*) *gurjanovae* was reported (Shevchenko et al., 2007). It was found that the fucoidan fraction, extracted from *S. gurjanovae* is actually a galactofucan with a main chain, built up of fucose units. But, chains of galactose residues (with degree of polymerization DP up to 6) were also found to be incorporated into the structure of galactofucan. This information was extracted by observation of the products of partial decomposition of galactofucan by acid hydrolysis and autohydrolysis. The partially desulfated products of degradation were registered by MALDI-TOF mass spectrometry.

The present work is focused on further investigation by chemical and instrumental methods of structural features and biological activity of native and modified fucoidan fractions, extracted from *S. gurjanovae*.

2. Experimental

2.1. Materials

Organic solvents, inorganic acids and salts were commercial products (Vekton, Russia). $NaBH_4$ reagent was purchased from Sigma (USA).

Brown seaweed *S. gurjanovae* (**Sg**) was collected during the expedition of research vessel "Academician Oparin" in August, 2012 at the coast of island Big Shantar (Sea of Okhotsk). The algae species were identified by Dr. A. Skriptsova (A.V. Zhirmunsky Institute of Marine Biology FEB RAS, Russian Federation). 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) were used as standards for GC/GC–MS of alditol derivatives.

A MALDI-TOFMS matrix, 2,5-dihydroxybenzoic acid (DHB), was purchased from Sigma (USA). All experiments were performed using ultra-pure water, produced with Direct-Q equipment (Millipore, USA). Arabinoosazone matrix (phenylosazone of D-arabinose) was synthesized by V.I. Gorbuch as described (Chen, Baker, & Novotny, 1997).

MTS reagent – 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide was purchased from "Promega" (USA). Roswell Park Memorial Institute Medium (RPMI 1640), phosphate buffered saline (PBS), L-glutamine, penicillin–streptomycin solution, trypsine and fetal bovine serum (FBS), and sodium hydrocarbonate ($NaHCO_3$) were purchased from "Biolot" (Russian Federation).

Human colon carcinoma DLD-1 (ATCC® no. CCL-221) cell line was obtained from the American Type Culture Collection (ATCC, USA).

2.2. Instruments

MALDI-TOF mass spectra were recorded with an Ultra Flex III MALDI-TOF/TOF mass spectrometer (Bruker, Germany) with SmartBeam laser (355 nm), reflector and potential LIFT tandem modes of operation.

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

GC–MS of alditol acetate derivatives of monosaccharides were performed using a Carlo Erba Fractovap 4200 (Italy) chromatograph equipped with Ultra-1 capillary column (25 m × 0.2 mm) with a temperature gradient of 150–230 °C at 5 °C/min.

NMR spectra were obtained on an Avance DPX-500 NMR spectrometer (Bruker, Germany) resonating at 75.5 MHz at 35 °C. The sample concentration was 20 mg of polysaccharide/mL of D_2O for ^1H and ^{13}C experiments.

Infrared spectra were recorded with a Vektor-22 FT-IR spectrometer (Bruker, Germany).

2.3. General methods

2.3.1. Analytical procedures

Total carbohydrates were quantified by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Monosaccharides composition after polysaccharide hydrolysis by 2 M TFA (6 h, 100 °C) and obtaining of alditol acetate derivatives was determined by GLC.

The content of proteins and polyphenols was determined by Bradford (1976) and Folin–Ciocalteau (Singleton & Rossi, 1965) methods, respectively. Sulfate group determination was carried out using the $BaCl_2$ gelatin method (Dodgson & Price, 1962).

2.3.2. Defatted algae

The algae were air-dried in well-ventilated place without direct sunlight at room temperature for three days and milled to powder. The dried powder (particle size = 3–4 mm) thalli of alga (620 g) were pretreated with 70% EtOH for 10 days (w/v = 1:1) to remove pigments and other low-molecular weight compounds. Then, the alga was air-dried at room temperature for two days.

2.3.3. Extraction of polysaccharides

Samples of defatted, dried, and powdered algal fronds (500 g) were extracted twice 2% $CaCl_2$ at 80 °C (ratio 1:20). The extracts were combined, concentrated to one-fifth of the volume, dialyzed against 10 volumes of distilled water, and lyophilized to obtain **SgFL** preparations of water-soluble polysaccharides. The yield of this fraction was 116.5 g.

2.3.4. Anion-exchange chromatography on DEAE-cellulose column

A solution of polysaccharide **SgFL** in 0.01 M HCl (3.2 g in 50 mL) was applied to a DEAE-cellulose column (Cl^- form, 21 cm × 3 cm) equilibrated with 0.01 M HCl. Laminaran was eluted with water, concentrated under a vacuum and lyophilized to get preparation **SgL** with yield of 1.8 g.

Then the column was successively eluted with 1.0 and 2.0 M NaCl solutions until the disappearance in eluate of positive reaction for carbohydrates by the phenol–sulfuric acid method (Dubois et al., 1956) in each case. The fractions were concentrated under a vacuum, dialyzed by ultrafiltration using a 5 kDa cut-off membrane

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

and lyophilized to get the polysaccharide preparations **SgF1** and **SgF2** with yields of 230 and 346 mg, respectively.

2.3.5. Anion-exchange chromatography on Macro-Prep DEAE

A solution of fucoidan **SgF2** in 0.1 M NaCl (346 mg in 10 mL) was applied to a Macro-Prep DEAE (Bio-Rad, USA) column (Cl^- form, 9 cm × 2.5 cm) that was equilibrated with 0.1 M NaCl. The column was then successively eluted with a linear gradient of NaCl (from 0.1 to 2 M) until the disappearance in the eluent of positive reaction for carbohydrates by the phenol–sulfuric acid method (Dubois et al., 1956). The corresponding polysaccharide fraction was concentrated under a vacuum, dialyzed against distilled water using a 5 kDa cut-off membrane and lyophilized to obtain fucoidan **SgF** (240 mg).

*2.3.6. Depolymerization of fucoidan **SgF** by autohydrolysis*

Autohydrolyses at 25 °C (Anastyuk et al., 2010) and 60 °C were employed to degrade fucoidan to oligosaccharides suitable for MS analyses: aliquots of 20 mL of fucoidan **SgF** (5 mg/mL) were turned to the H^+-form using a minicolumn of cation exchange (Amberlite CG-120, 200–400 mesh, Serva, Germany) and left for 72 h. Mixtures were neutralized with 5% NH_4OH solution in water and concentrated under a vacuum to 1 mL. Mixture (25 °C) was fractionated by MeOH (1:10) to obtain high-molecular weight (pellet **SgF-AH25-H**) and low-molecular weight (solution **SgF-AH25-L**) fractions.

2.3.7. Gel-filtration chromatography

Mixture of the oligosaccharides, obtained by autohydrolysis at 60 °C **SgF-AH60** (7 mg) was applied to a Bio-Gel P-2 (Bio-Rad, USA) column (120 cm × 1 cm, 60 °C), and eluted with 0.2 M NH_4HCO_3 buffer at a flow rate 80 μL/min. The fractions were concentrated under vacuum. A fraction **SgF-AH60-3**, which contained mixture of monosulfated trisaccharides, was found by a MALDI-TOFMS analysis.

2.3.8. Reduction of oligosaccharides

$NaBH_4$ reagent (50 μL, 0.05 M $NaBH_4$ in 0.01 M NaOH) was added to the dry oligosaccharides (~50 μg), and the reduction was carried out at 4 °C overnight as described (Kogelberg, Piskarev, Zhang, Lawson, & Chai, 2004). The solution was neutralized with 0.1 mL AcOH and evaporated. Boric acid was removed by repeated coevaporation with MeOH.

2.4. Mass spectrometric analysis of oligosaccharides

Instrument settings are as follows: accelerating voltage, 25 kV; laser power, 30 μJ; pulse width, 6 ns; number of shots, 100; laser shot rate, 66 Hz. Sample preparation is as follows: a mixture containing 1 μL of sample (0.1 mg/mL) and either 1 μL of 0.5 M DHB (positive-ion experiment) or 0.5 M arabinoosazone matrix (negative-ion experiment) solution in MeOH for DHB and acetone for arabinoosazone was introduced onto the sample plate and air dried. A negative-ion experiment required further 10-fold dilution of a sample in water.

2.5. 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-620 173 HQ (7.5 mm × 300 mm) at 50 °C with elution by 0.5 M NH_4HCO_3 (0.8 mL/min). Columns were calibrated using standard pullulans of MWs from 180 to 667 kDa (Polymer Laboratories, USA) and blue dextran (Amersham, 177 Sweden).

2.6. Biological activity

2.6.1. Cell culture

Human colon carcinoma cells DLD-1 were cultured in RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS), 200 mM L-glutamine and penicillin–streptomycin solution.

Human colon carcinoma DLD-1 cells were cultured in RPMI-1640/10% FBS media. The cell cultures were maintained at 37 °C in humidified atmosphere containing 5% CO_2. The cells were grown for 3–4 days and after reaching 90% of confluence were harvested by exposure to 0.25% trypsin–EDTA solution and then passed into new T-75 tissue culture flasks.

2.6.2. In vitro cell proliferation assay

The effects fucoidan (**SgF**) and its derivative (**SgF-AH25-H**) from the brown alga *S. gurjanovae* on cells viability was determined colorimetrically using a CellTiter 96® aqueous non-radioactive cell proliferation assay (Promega, Madison, WI), which is based on the cleavage of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2H-tetrazolium (MTS reagent) into a formazan product soluble in tissue culture medium (Berridge & Tan, 1993). Briefly, DLD-1 cells (0.8×10^4) were cultured in 200 μL of complete RPMI-1640/10% FBS medium, for 24 h at 37 °C in 5% CO_2 incubator. The cell monolayer was washed with phosphate buffered saline (PBS) to remove unattached cells. The attached cells were incubated with fresh medium containing various concentrations of **SgF** and **SgF-AH25-H** (0–800 μg/mL) for 24, 48, and 72 h. Subsequently, the cells were incubated with 15 μL MTS reagent for 3 h, and the absorbance of each well was measured at 490/630 nm using microplate reader (Power Wave XS, USA). All the experiments were performed three times, and the mean absorbance values were calculated. The results are expressed as the percentage of inhibition that produced a reduction in absorbance by polysaccharides treatment compared to the non-treated cells. Soft agar assay (Colburn, Wendel, & Abruzzo, 1981) DLD-1 cells (3×10^4) were seeded in six-well plate and treated without (control) or with **SgF** and **SgF-AH25-H** (0–800 μg/mL) in 1 mL of 0.3% Basal Medium Eagle (BME) agar containing 10% FBS, 2 mM L-glutamine, and 25 μg/mL gentamicin. The cultures were maintained at 37 °C, in a 5% CO_2 incubator for 14 days, and the cell's colonies were scored using a microscope "Motic AE 20" (China) и and the Motic Image Plus software.

2.6.3. Statistical analysis

Results are expressed as the mean ± standard deviation (SD). Student's *t*-test was used to evaluate the data with the following significance levels: $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. All assays were performed in at least three independent experiments.

3. Results and discussion

3.1. Isolation and characterization of fucoidan from S. gurjanovae

Water-soluble polysaccharides **SgFL** were extracted from the dry defatted brown alga, *S. gurjanovae* (**Sg**) with 2% $CaCl_2$. Analysis of the monosaccharide composition of **SgFL** indicated a mixture of laminaran and fucoidan because the fraction contained mainly residues of glucose, fucose and galactose (76:19:5).

The separation of neutral and charged polysaccharides, as well as the fractionation of fucoidans was carried out using anion-exchange chromatography on DEAE-cellulose of the water-soluble polysaccharide fraction **SgFL**. As the result, laminaran **SgL** and two fucoidan fractions **SgF1** and **SgF2** (13.1, 1.7 and 2.5% of the dry defatted alga weight, respectively) were obtained. Fucoidan fractions contained no proteins and/or polyphenols. Fraction **SgF2** was chosen for further investigation.

Table 1
Yields and compositions of fucoidan fractions from *S. gurjanovae*.

Fucoidan[a] fraction	Eluent [NaCl] (M)	Yield (%)[b]	MW (kDa)	SO_3Na (%)[c]	Monosaccharide composition (mol%)			
					Fuc	Gal	Man	Xyl
SgF1	1.0	7.2	n.d.	9.5	64.1	27.4	5.7	2.8
SgF2	2.0	10.8	n.d.	23.8	75.9.7	21.2	1.8	1.1
SgF	1.3–1.6	7.5	123	25.1	76.3	23.7	0	0

[a] Fractions **SgF1** and **SgF2** were obtained by separation of water-soluble polysaccharides fraction **SgFL** on DEAE cellulose; fraction **SgF** was obtained by purification of **SgF2** on Macro-Prep DEAE.
[b] % of water-soluble polysaccharides preparation **SgFL**.
[c] % of sample weight.
n.d.: not determined.

Fucoidan **SgF2** was purified additionally by anion-exchange chromatography on Macro-Prep DEAE (see supplementary data) to obtain preparation **SgF**. An analysis of the monosaccharide composition of **SgF** indicated that fucoidan contained only fucose and galactose (Table 1). Hence, **SgF** was a highly sulfated galactofucan (25.1% sulfate content). Molecular weight of **SgF** was 123 kDa.

The infrared spectra of sulfated polysaccharides of fraction **SgF** were obtained (data not shown). A strong adsorption band of S=O stretching vibration at 1240 cm^{-1} indicated the presence of ester sulfate. A moderate band near 820 cm^{-1} showed equatorial position of sulfate groups, while the shoulder at 844 cm^{-1} demonstrated axial locations.

Like many native algal fucoidans, the fucoidan **SgF** had complex ^{13}C NMR spectrum that was difficult to elucidate completely. The spectrum contained a group of signals in the anomeric region (97.3–101.0 ppm) and intensive signals in the high-field region (16.2–17.5 ppm), typical to the C-6 of α-L-fucopyranosides. A signal of low intensity at 62.1 ppm was attributed to the free C-6 of β-D-galactose residues. The signals at 21–22 ppm (CH_3) and 175–176 ppm (C=O) were indicative to the presence of acetyl groups.

Brown algae of the genus *Saccharina* belongs to the family Laminariaceae of the order Laminariales. Fucoidans isolated from these algae were sulfated fucans and galactofucans differing content of fucose and galactose residues, sulfate groups and presence or absence of trace amounts of mannose, xylose, rhamnose, glucose, uronic acids and acetyl groups. Structural characteristics of fucoidans from algae of the genus *Saccharina* (ex name *Laminaria*) were studied by many groups. The fucan chain, build up of (1 → 3)-linked 2,4-disulfated α-L-fucose residues, was found to be specie-specific for Laminariales (Cumashi et al., 2007). However, Laminariales often contain galactose in the monosaccharide composition from minor to significant. It was recently shown that brown alga *Saccharina latissima*, along with others types of polysaccharides, synthesized fucogalactan having a backbone of 6-linked β-D-galactopyranose residues branched mainly at C-4 and containing both terminal galactose and fucose residues (Bilan et al., 2010). Also, the structure of galactofucan from the brown alga *Saccharina japonica* was described (Wang, Zhang, Zhang, Zhang, & Niu, 2010). It is interesting to note, that the backbone of fucoidan contained primarily 75% of (1 → 3)- and 25% of (1 → 4)-linked α-L-fucopyranose residues. The branch points were at C-4 of (1 → 3)-linked α-L-fucose residues as β-D-galactopyranose units (35%, molar ratio) or at C-2 of (1 → 3)-linked fucose residues as non-reducing terminal fucose units (65%, molar ratio). Sulfate groups occupied at position C-4 and/or C-2 of fucose, and C-3 and/or C-4 of galactose residues.

Sulfated galactofucan was isolated from the *Saccharina longicruris*, and its structure was studied by methylation. This polysaccharide contained (1 → 3)-linked fucopyranose 4-sulfate and (1 → 6)-linked galactopyranose 3-sulfate moieties (Rioux, Turgeon, & Beaulieu, 2010).

The fucoidan from *S. gurjanovae* (collected in July, 2003) was studied by our group earlier. It was found to be a highly sulfated partially acetylated galactofucan (Fuc:Gal is ~1:1). The oligosaccharide products of desulfation and partial acidic hydrolysis of galactofucan were studied by MALDI-TOFMS; they were found to be fuco-, galacto- and mixed fucogalactooligosaccharides (Shevchenko et al., 2007). In a present investigation galactofucan fraction contained lower amount of galactose. Possibly, the monosaccharide composition of fucoidans depended on a season of harvesting of algae (Anastyuk, Imbs, et al., 2012; Skriptsova et al., 2009, 2012).

3.2. NMR analysis of the fraction *SgF-AH25-H*

Depolymerization by autohydrolysis was carried out to simplify the structure of fucoidan **SgF**. As a result, HMW and LMW fractions, **SgF-AH25-H** and **SgF-AH25-L**, respectively, were obtained (Section 2.3.6). An analysis of the monosaccharide composition of **SgF-AH25-H** indicated that the fraction contained fucose only. Molecular weight of **SgF-AH25-H** was 71 kDa. Thus, **SgF-AH25-H** fraction could be represented the linear main chain of the native fucoidan, while **SgF-AH25-L**, probably, contained branches, including short chains, built up of Gal residues with DP 1–5, or, probably, more, which were found in our former work (Shevchenko et al., 2007).

The ^{13}C NMR spectrum for **SgF-AH25-H** (see supplementary data) contained six intensive signals with chemical shifts of 99.7 (C-1), 67.9 (C-2), 77.5 (C-3), 81.0 (C-4), 68.4 (C-5), and 16.8 (C-6) ppm. These signals corresponded to the residues of 4-sulfated fucopyranose substituted in the C-3 positions. It must be noted that this spectrum was practically identical to that observed for a similarly obtained (T = 37 °C) fucan HMW fraction from *Saccharina cichorioides*. The structure of a fucan from *S. cichorioides* was typical for Laminariales: (1 → 3)-linked 2,4-sulfated α-L-fucan (Anastyuk et al., 2010; Zvyagintseva et al., 2003). See supplementary data for both spectra. Probably, similarly to mild acid hydrolysis (Pomin et al., 2005), a selective desulfation at C-2 could also happen in the process of autohydrolysis. Thus, fucan backbone of **SgF** galactofucan consisted of (1 → 3)-linked 2,4-sulfated fucopyranoside residues.

3.3. Mass spectrometric analysis of the oligosaccharide fraction *SgF-AH25-L*

Since the direct analysis of fucoidans by mass spectrometry is impossible by now, the polysaccharide should be softly (and, preferable, specifically) decomposed to fragments, suitable by MW and sulfate-groups-per-molecule parameters. The autohydrolysis technique allows obtaining both HMW and LMW fractions of polysaccharide. The LMW fraction cannot be directly analyzed by NMR technique due to high heterogeneity, but mass spectrometry, especially MALDI-TOFMS allows working with complex mixtures (Anastyuk, Shevchenko, Ermakova, et al., 2012).

The elucidation of the structural features of sulfated oligosaccharides by a negative-ion tandem mass-spectrometry was carried out using the following observations, estimated in the former

studies on glucosaminoglycans (Saad & Leary, 2004), sulfated galactans (Goncalves, Ducatti, Grindley, Duarte, & Noseda, 2010; Minamisawa & Hirabayashi, 2005; Yu et al., 2006) and sulfated fucan-derived fragments (Daniel et al., 2007; Tissot, Salpin, Martinez, Gaigeot, & Daniel, 2006). It was shown, that the product ion spectra of $[M-Na]^-$ (where M represents the sodium salt of oligosaccharides) featured an extensive series of B- and C-type (according to nomenclature, suggested by Domon & Costello, 1988) glycosidic cleavages, while the Y-type cleavage occurred mainly at the sulfated at C-4 residues that was shown for carrageenans and glucosaminoglycans (Saad & Leary, 2004; Yu et al., 2006). C-type cleavages were rare for fucoidans, but B-type cleavages were intensive, since glycosidic cleavages were, probably, induced by sulfation at C-2 (Anastyuk, Imbs, et al., 2012; Anastyuk, Shevchenko, Ermakova, et al., 2012; Anastyuk et al., 2014; Goncalves et al., 2010). Fragment ions from cross-ring cleavages of $^{0,2}X/^{0,2}A$ – type were observed only for mono/oligosaccharides that were not 3-linked (Anastyuk et al., 2009; Daniel, Laiko, Doroshenko, & Zenobi, 2005; Minamisawa & Hirabayashi, 2005; Saad & Leary, 2004; Tissot et al., 2006; Yu et al., 2006). As recently shown by ESIMS/MS and computational chemistry methods on fucose residues, sulfated at C-2, C-3 and C-4 (Tissot et al., 2006), the mechanism of formation of the $^{0,2}X/^{0,2}A$ fragment ions involved unsubstituted proton on the C-3 hydroxyl group. Hence, in case of 3-linked sulfated fucans/galactofucans from Laminariales (Anastyuk, Imbs, et al., 2012; Anastyuk et al., 2010; Jin, Wang, Ren, Song, & Zhang, 2012) and enzymatically released oligosaccharides from carrageenan (Anastyuk et al., 2011), when all protons of the C-3 hydroxyl groups are substituted, no $^{0,2}X/^{0,2}A$ – type ions could be produced during CID MS/MS.

3.3.1. *Preliminary analysis of the oligosaccharides mixture **SgF-AH25-L***

SgF-AH25-L fraction, rich in galactose, was obtained by autohydrolysis at 25 °C (Section 2.3.6). MALDI-TOFMS analysis (Fig. 1) revealed that mixture contained a set of mono- and disaccharides with 1–3 sulfate groups per molecule. It must be noted that by lowering the temperature from 37 to 25 °C, due to MALDI-TOFMS data, it was possible to increase the relative intensity/yield of a doubly sulfated ion at *m/z* 345 in comparison to *S. cichorioides* (Anastyuk et al., 2010). Despite of the monosaccharide composition data, galactose-containing trisaccharide was found only as a doubly sulfated $[Fuc_2Gal(SO_3Na)_2{}^-Na]^-$ ion. However, the obtained results are in accord with mass spectrometric data on the analysis of fragments from other Laminariales – *Costaria. costata* (Anastyuk, Imbs, et al., 2012) and *S. cichorioides* (Anastyuk et al., 2010), obtained earlier. But, as it was found for galactofucan from *C. costata*, galactose-containing fragments were observed only as disaccharides GalFuc, where position of Gal residues was predominantly terminal. This fact was also observed in Laminariales by other groups of researchers (Bilan et al., 2010; Rioux et al., 2010), which could be explained by the selected conditions for polysaccharide destruction. The acid hydrolysis in rather stressed (1 N TFA at 60 °C) conditions, which was employed in the last work on *S. gurjanovae* gave better results and Gal-containing fragments were observed by MALDI-TOFMS (Shevchenko et al., 2007). However, this approach failed with *C. costata* (Anastyuk, Imbs, et al., 2012).

3.3.2. *MALDI-LIFT TOF/TOF analysis of **SgF-AH25-L***

A negative-ion MALDI-TOF/TOFMS of fragments, built up of fucose (not shown) were similar to that observed earlier for *S. cichorioides* (Anastyuk et al., 2010) and *C. costata* (Anastyuk, Imbs, et al., 2012). The ions from cross-ring cleavages, suggesting (1 → 4)- or (1 → 2)-type of linkages were not observed. It was concluded that fragments of the LMW fraction under study are linked by (1 → 3)-type of linkages, similarly to the HMW fraction (see above). However, sulfation pattern of LMW fragments was closer to typical to fucans from Laminariales, since 2,4-disulfated fucose building unit (Cumashi et al., 2007) was found as a main component of LMW mixture.

A tandem negative-ion MALDI-TOFMS of the triply-sulfated fucobiose ion at *m/z* 593.0 (Fig. 2) in comparison to a spectrum, recorded for the similar fragment from *Fucus evanescens* (Anastyuk, Shevchenko, Ermakova, et al., 2012), obtained in close conditions, had only 5 signals and showed no evidence of the fragment ions from cross-ring cleavages. Hence, the analysis of a

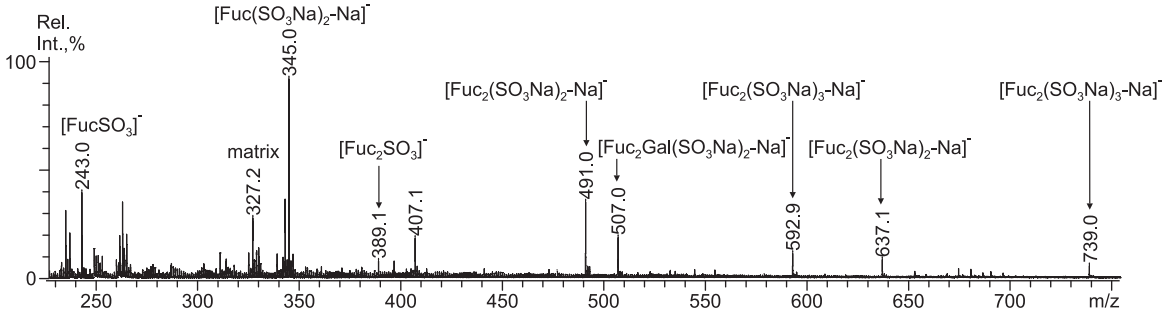

Fig. 1. Negative-ion MALDI-TOFMS of oligosaccharides fraction, obtained by autohydrolysis (25 °C) of fucoidan **SgF**.

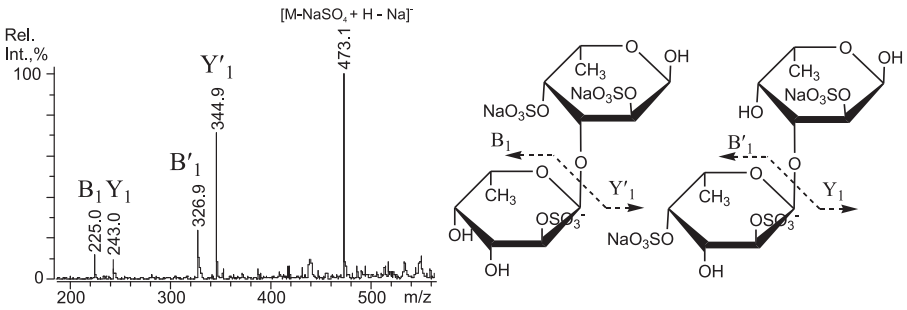

Fig. 2. Tandem negative-ion MALDI-TOFMS of the $[Fuc_2(SO_3Na)_3{}^-Na]^-$ ion at *m/z* 593.0.

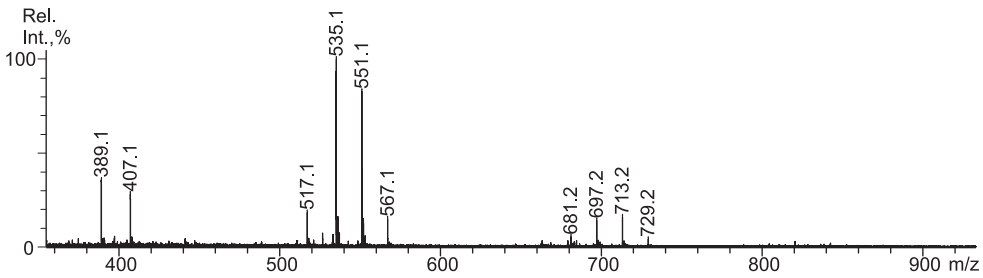

Fig. 3. Negative-ion MALDI-TOFMS of the trisaccharide fraction, obtained by autohydrolysis (60 °C) of fucoidan **SgF**. Separation was performed on BioGel P-2 in 0.2 M NH_4HCO_3 buffer.

mass spectrum suggested the fragment to have two variants: Fuc-2-SO_3^--α-(1 → 3)-Fuc-2,4-di-SO_3^- and, probably, less abundant Fuc-2,4-di-SO_3^--α-(1 → 3)-Fuc-2-SO_3^-. The absolute configuration was suggested by ^{13}C NMR data on **SgF-AH25-H** fraction. The high intensity of Y_1' ion at *m*/*z* 344.9 suggested structural variant Fuc-2-SO_3^--α-(1 → 3)-Fuc-2,4-di-SO_3^- to be predominant, since sulfation at C-4 of the reducing Fuc residue induced the cleavage of the spatially closer glycosidic bond (Yu et al., 2006).

3.3.3. *MALDI-LIFT TOF/TOF analysis of autohydrolysis products at 60 °C*

As already mentioned above, the recent study on galactofucan from *C. costata* (Laminariales) by ESIMS technique has shown that it was impossible to obtain abundant LMW galactose-containing fragments using autohydrolysis at 37 °C and (Anastyuk, Imbs, et al., 2012) in comparison to former studies on *S. gurjanovae* (Shevchenko et al., 2007) where strong acid hydrolysis was employed. Due to that fact the temperature was increased up to 60 °C to enhance the yield of galactose-containing fragments. The complex mixture **SgF-AH60** was purified by gel-filtration on a BioGel P-2 in 0.2 M NH_4HCO_3 buffer. The MALDI-TOFMS analysis (not shown) revealed that oligosaccharides were both sulfated and unsulfated. However, it was possible to isolate informative fraction of sulfated galactose-containing oligosaccharides using negative-ion MALDI-TOFMS (Fig. 3).

The tandem (MALDI-TOF/TOFMS) mass spectrometric technique was employed to analyze the structural features of the components. Fragments, built up with fucose only ($[Fuc_2SO_3]^-$ at *m*/*z* 389.0 and $[Fuc_3SO_3]^-$ at *m*/*z* 535.1) were found (MS/MS data not shown) to be linear and 3-linked, confirming ^{13}C NMR data, data on autohydrolysis at 25 °C (see above) and the literature data on Laminariales (Anastyuk et al., 2010; Cumashi et al., 2007; Zvyagintseva et al., 2003).

The tandem MALDI-TOFMS (Fig. 4A) of "mixed" fragment at *m*/*z* (405.0), made up in comparison with a similar fragment (which had one sulfate group more, though, *m*/*z* 507.0, Fig. 4B), found in oligosaccharide mixture of autohydrolysis at 25 °C (Fig. 1) was more interesting.

Galactose residue in the disaccharide under study (autohydrolysis at 60 °C; Fig. 4A) resided mostly on the non-reducing end and had a sulfate group at C-2 due to intensive B-type ion at *m*/*z* 241.0, (Anastyuk, Imbs, et al., 2012; Goncalves et al., 2010). Galactose residue (autohydrolysis at 25 °C; Fig. 4B) resided in the same position, but, surprisingly, it did not show any B-type ions. The sulfated fucose residue, on the other hand, exhibited abundant Y-type ion at *m*/*z* 243.0 like in oligosaccharides from κ-carrageenan, sulfated at C-4 (Anastyuk et al., 2011; Yu et al., 2006). Oligosaccharides, obtained from fucoidans usually rich of B-type ions, instead (Anastyuk, Imbs, et al., 2012Anastyuk et al., 2010, 2014; Daniel et al., 2007). The lack of B-type ion from galactose residue could be explained by the location of the sulfate group at C-3 of Gal

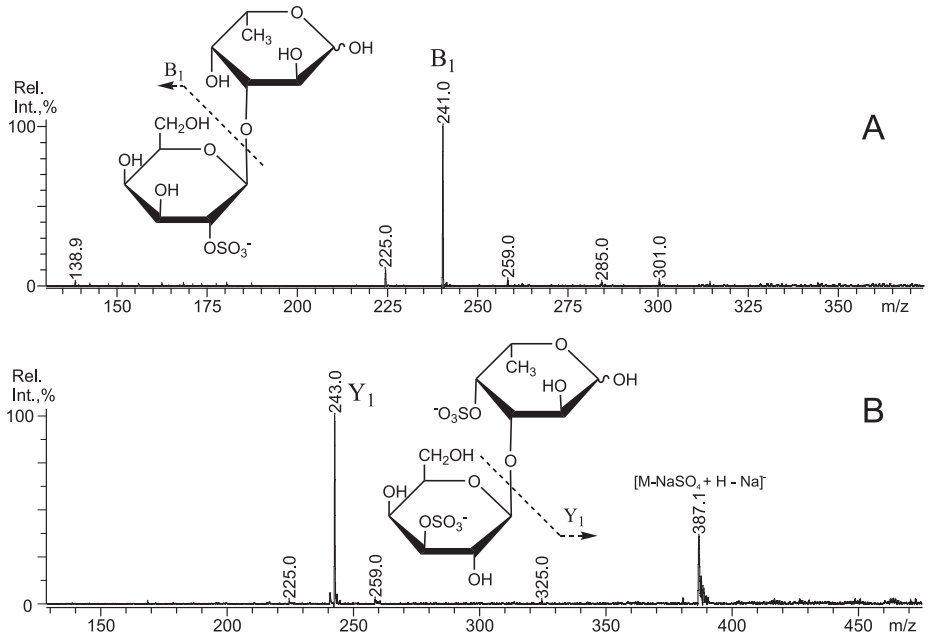

Fig. 4. Tandem negative-ion MALDI-TOFMS of the $[GalFucSO_3]^-$ ion at *m*/*z* 405.0 (A) and $[GalFuc(SO_3Na)_2{-}Na]^-$ ion at *m*/*z* 507.0 (B) from autohydrolysis in "exhaustive" (60 °C) conditions and mild conditions (25 °C), respectively.

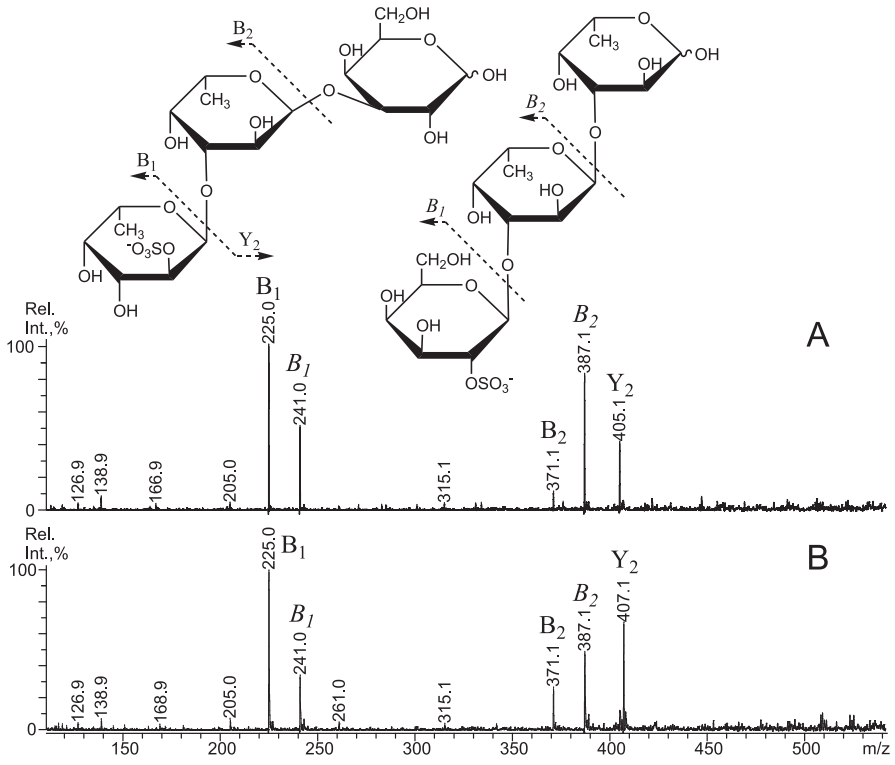

Fig. 5. Tandem negative-ion MALDI-TOFMS of the $[GalFuc_2SO_3]^-$ ion at *m/z* 551.1 (A) and its reduced variant (B).

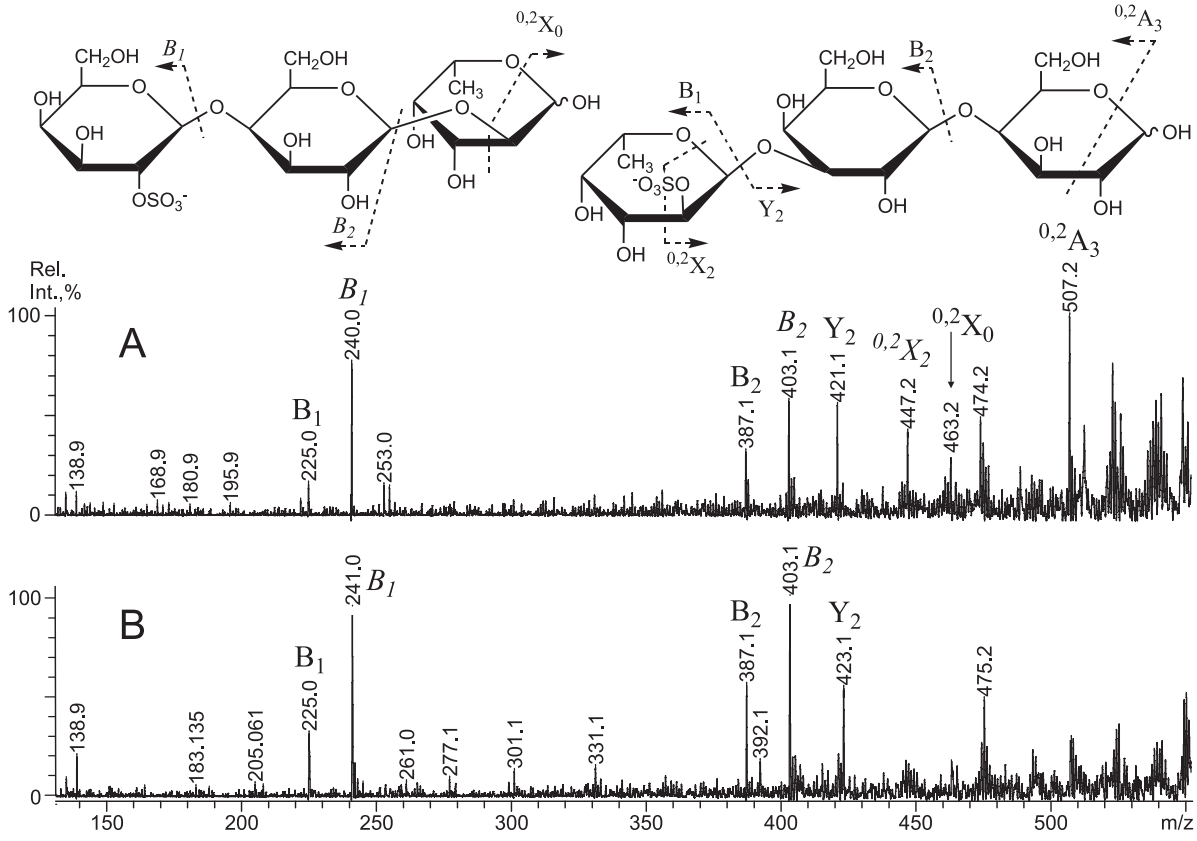

Fig. 6. Tandem negative-ion MALDI-TOFMS of the $[Gal_2FucSO_3]^-$ ion at *m/z* 567.1 (A) and its reduced variant (B).

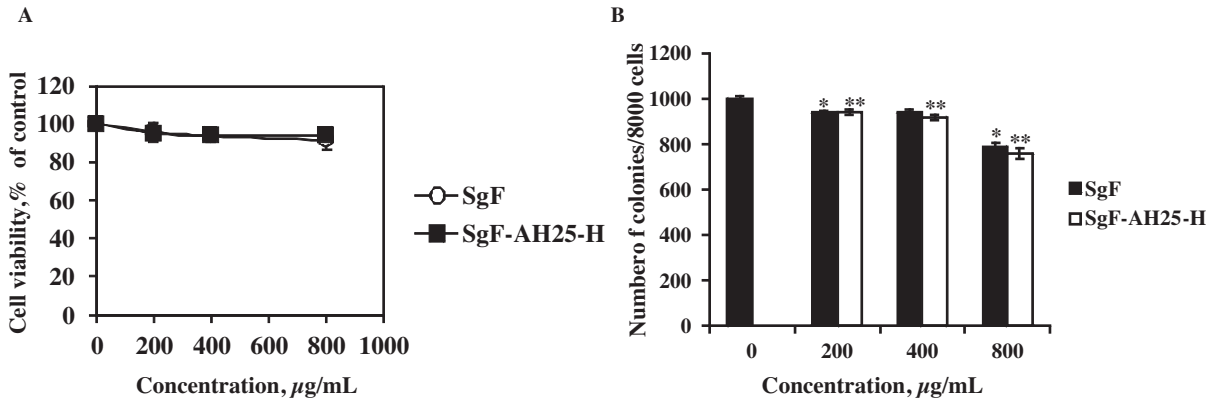

Fig. 7. The effect of **SgF** and **SgF-AH25-H** on the cell viability and colony formation of human colon carcinoma cells DLD-1. (A) DLD-cells were seeded (0.8×10^4 cells/200 μL of complete RPMI-1640/10% FBS) in 96-well plates, after 24 h they were treated with **SgF** and **SgF-AH25-H** (0–800 μg/mL) for 24, 48, and 72 h. The cell viability was estimated using the MTS assay. Data are represented as the means ± SD as determined from triplicate experiments. (B) DLD-1 cells (3.0×10^4) were either treated or not treated with **SgF** and **SgF-AH25-H** (0–800 μg/mL) in 1 mL of 0.3% Basal Medium Eagle (BME) agar containing 10% FBS, 2 mM L-glutamine, and 25 μg/mL gentamicin. The cultures were maintained at 37 °C, in a 5% CO_2 incubator for 14 days and the cell's colonies were scored using a microscope "Motic AE 20" (China) and the Motic Image Plus computer program. All assays were performed in at least three independent experiments. Results are expressed as the mean ± standard deviation (SD). Student's *t*-test was used to evaluate the data with the following significance levels: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

residue (Tissot et al., 2006), since A-type ion at *m*/*z* 180.9 and/or 198.9 from sulfation at C-4/C-6 (even at low intensities) were not found (Anastyuk et al., 2009; Kravchenko et al., 2014; Minamisawa & Hirabayashi, 2005). Since there were no X-type ions from cross-ring cleavages, fragment under study were assumed to be 3-linked.

The tandem MALDI-TOFMS (Fig. 5) of a next "mixed" fragment $[GalFuc_2SO_3]^-$ at *m*/*z* (551.1) was rather simple.

The reduction (Section 2.3.8) which was performed to differentiate the fragment ions, arising from the reducing and non-reducing termini (Fig. 5B) has shown that mass shift +2 Da has only Y_2-type ion at *m*/*z* 407.1 and, probably, sulfation of C-4 of the internal Fuc residues prompted the cleavage (Yu et al., 2006). The others were B-type ion. The analysis of MS revealed that Gal residue was either located at the reducing or non-reducing terminus, but it was not found internal, like in *Sargassum mcclurei* (Thinh et al., 2013). It must be noted that due to strong signals of B-type, the sulfate groups occupied mostly C-2 position, but, monosaccharide residues at the reducing terminus were unsulfated.

The analysis of a tandem MALDI-TOFMS of $[Gal_2FucSO_3]^-$ ion at *m*/*z* 567.1 (Fig. 6A) and its reduced variant (Fig. 6B) has confirmed Gal residues to form side chains.

The former study has shown that DP of Gal chains could be 5 or, probably, more. The presence of $^{0,2}A_3$ ion at *m*/*z* 507.2 suggested (1 → 4)- or (1 → 6)-type of linkage between Gal residues. The other fragment ions from cross-ring cleavage were $^{0,2}X$ at *m*/*z* 447.2 and 463.2. The literature on ESIMS/MS studies of oligosaccharides, derived from *Ascophyllum nodosum* suggested these signals to arise from non-reducing end. However, mass spectra from both Fucales and Laminariales did not enlighten the exact origin of these signals. Even the ion under study, being reduced, did not show any evidence of cross-ring cleavages. This fact could be explained that the mobile proton from the reducing terminus plays essential role in ion-molecular reactions, resulting in cross-ring cleavages, which carry structural information. Probably, $^{0,2}X$-type ions could arise from side chains, being most intensive when 2-linked to the reducing Fuc residue. This observation is supported by literature (Anastyuk et al., 2010; Cumashi et al., 2007; Zvyagintseva et al., 2003).

3.4. *The effect of **SgF** and **SgF-AH25-H** on the cell viability and colony formation of human colon carcinoma cells*

The antiproliferative activity of the fucoidans from different species of brown algae has been reported in recent years. These natural compounds have been shown to suppress viability of a wide variety of tumor cell lines, including HTLV-1-infected T-cell lines and primary ATL cells (Haneji et al., 2005), lymphoma cells (Aisa et al., 2005), and colon cancer cells (Kim, Park, Lee, & Park, 2010).

In this study, the effect of algal fucoidan and it derivative on proliferation of colon carcinoma cells was investigated at the first time. The fucoidan from brown alga *S. gurjanovae* and its derivative **SgF-AH25-H** did not result in any growth inhibition of DLD-1 cells at the concentration range up to 800 μg/mL even in 3 days of treatment (Fig. 7A). The percent of inhibition of colon cancer cells growth was less than 10% for both investigated polysaccharides. These results indicated that sulfated polysaccharides have no cytotoxicity *in vitro*.

Next we examined the effect of the fucoidans **SgF** and **SgF-AH25-H** on the colony formation of cancer cells using soft agar assay. The soft agar assay for tumor colony formation has applicability on a broad scope of human tumors and has proved valuable in studies of biology, clinical course, and chemosensitivity of human cancers (Combes et al., 1999).

Our results showed that **SgF** suppressed colony formation of DLD-1 cells on 6%, 6%, and 21%, at concentrations 200, 400, 800 μg/mL, respectively, and **SgF-AH25-H** at the same concentrations inhibited colony formation of cancer cells on 6%, 8%, and 24%, respectively (Fig. 7B).

4. Conclusion

The highly purified heavily sulfated galactofucan **SgF** (MW 123 kDa) was obtained from the brown alga *S. gurjanovae*. The polysaccharide was depolymerized by autohydrolysis. Sulfated polysaccharide fraction **SgF-AH25-H** (71 kDa) and low-molecular-weight fraction **SgF-LMW** were obtained by autohydrolysis at 25 °C. ^{13}C NMR analysis of **SgF-AH25-H** indicated that the polysaccharide was built up of the repeating unit →3)-α-L-Fuc*p*-(4-OSO_3^-)-(1→. It was shown, that autohydrolysis at 25 °C leaded to a selective desulfation of fucoidan at C-2 and removing of galactose residues. A MALDI-TOF MS/MS analysis of **SgF-AH25-L** fraction revealed the presence of α-L-Fuc*p*-2,4-di-OSO_3^- residues as main component and disaccharides, including triply-sulfated one: Fuc-2-SO_3^--α-(1 → 3)-Fuc-2,4-di-SO_3^-. Galactose residues in **SgF-AH25-L** mixture of oligosaccharides were found as part of disaccharides Gal-(1 → 3)-Fuc, where Gal residue was terminal. As an attempt to obtain other more high-molecular galactose-containing oligosaccharides, an autohydrolysis at 60 °C was employed. Thus

obtained **SgF-AH60** mixture was purified by gel-filtration on Bio-Gel P2 in 0.2 M ammonium bicarbonate buffer and a fraction of the galactose-containing trisaccharides was isolated. MALDI-TOFMS/MS analysis of fragments revealed the following. The galactose residues were found to be linked by (1 → 4)- and/or (1 → 6)-type of linkage and, due to the results of former studies formed chains with DP = 1–5, or, probably, more. Sulfate groups, probably, occupied positions C-2 and/or sometimes C-3 of Gal residues. Since only residual sulfate groups were observed, a sulfation at C-4 of the galactofucan could not be excluded. Presumably a main chain of galactofucan was represented by a repeating unit →3)-α-L-Fuc*p*-(2,4-OSO_3^-)-(1→, characteristic to Laminariales. Shorter (1 → 4)- and/or (1 → 6)-linked sulfated Gal chains were attached at positions C-2, C-3 of fucose residues. Single Gal residues were also terminal to Fuc chain and linked by (1 → 3)-type of linkage.

We have shown that fucoidan **SgF** and its derivative **SgF-AH25-H** had no cytotoxicity *in vitro* and suppressed colony formation of DLD-1 cells. The lowering of molecular weight from 123 to 71 kDa, selective desulfation at C-2 and removing of galactose residues by autohydrolysis at 25 °C did not lower its anticancer activity; a slight increase was detected instead.

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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.2014.12.042.

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