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Sulfated polysaccharides from *Laminaria angustata*: Structural features and *in vitro* antiviral activities

Sudipta Saha[a,1], Mojdeh Heidary Navid[b,1], Shruti S. Bandyopadhyay[a], Paul Schnitzler[b], Bimalendu Ray[a,*]

[a] Natural Products Laboratory, Department of Chemistry, The University of Burdwan, West Bengal 713 104, India
[b] Department of Infectious Diseases, Virology, University of Heidelberg, Im Neuenheimer, Feld 324, 69120 Heidelberg, Germany

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ABSTRACT

Sulfated polysaccharides potently inhibit the infectivity of herpes simplex virus (HSV) in cultured cells. In this study, we have analyzed sulfated xylogalactofucan and alginic acid containing fractions generated from *Laminaria angustata*, a marine alga. The xylogalactofucan that has apparent molecular mass of 56 ± 5 kDa and unusually low sulfate content contains, *inter alia*, 1,3-, 1,4- and 1,2-linked fucopyranosyl residues. The algin (molecular mass: 32 ± 5 kDa) contains gulo- (55.5%) and mannuronic (44.5%) acid residues. Introduction of sulfate groups enhanced the macromolecules capability to inhibit the infection of cells by HSV-1. The 50% inhibitory concentration (IC_{50}) values of these macromolecules against HSV-1 were in the range of 0.2–25 $\mu g\,ml^{-1}$ and they lacked cytotoxicity at concentrations up to 1000 $\mu g\,ml^{-1}$. The sulfate content appeared to be an important hallmark of anti-HSV-1 activity. Our results suggest the feasibility of inhibiting HSV attachment to cells by direct interaction of polysaccharides with viral particles.

1. Introduction

Herpes simplex viruses (HSVs) cause various forms of disease such as lesions on the lips, eyes, or genitalia to encephalitis and even disseminated disease in immunocompromised individuals (Kleymann, 2005). The prevalence of herpes simplex virus type 1 (HSV-1) infection increases progressively from childhood, the seroprevalence being inversely related to socioeconomic background. Primary HSV-1 infections in children can give rise to mucocutaneous vesicular eruptions which might be reactivated frequently in adults (Fleming et al., 1997). Genital herpes, generally caused by herpes simplex virus type 2 (HSV-2), is characterized by a high seroprevalence worldwide (8–40%), with an increasing trend in the last 20 years in nearly all countries (Gupta, Warren, & Wald, 2007). Among the high-risk population, HSV-2 infection is a major public health problem in young adults. The use of mimetics of cellular receptors for viruses for the prevention and/or treatment of viral infections is a promising approach that has already resulted in the development of novel drugs against influenza virus (Von Itzstein, 2007). Another well known example of this approach are mimetics of cell surface heparan sulfate (HS) (Balzarini & Van Damme, 2007; Ghosh et al., 2009; Rusnati et al., 2009; Vaheri, 1964; Witvrouw & De Clercq, 1997), a molecule that serves as an initial receptor for many different viruses including herpes simplex virus (WuDunn & Spear, 1989). HS mimetics such as sulfated polysaccharides target the virus attachment/entry components, thus preventing adherence of viral particles to cells. The uniquely distributed sulfation pattern of HS polysaccharide is believed to regulate its functional specificity (Gama et al., 2006; Liu & Pedersen, 2007). To date, the performance of these macromolecules in efficacy trials has been disappointing (Cohen, 2008; Grant et al., 2008), but next-generation concepts offer improved prospects for efficacy (Ekblad et al., 2010; Klasse, Shattock, & Moore, 2008). The most plausible approach involves a combination of several drugs, preferentially targeting different steps in the viral infection process. Because sulfated polysaccharides are safe and acceptable (Bollen et al., 2008; Kilmarx et al., 2008), development of several second-generation combination formulation based on first generation lead candidates may be more effective (Brache et al., 2007; Liu, Lu, Neurath, & Jiang, 2005; Said et al., 2010). The identification of active polysaccharides from marine algae may identify macromolecules with superior efficacy. New antiviral agents from natural origin can have easy acceptability being non-toxic and inexpensive. Moreover, structurally defined polysaccharides obtained by chemical sulfation may also produce drug candidate with higher potency.

The present study reports isolation and chemical characterization of water soluble polysaccharides present in the marine alga *Laminaria angustata* (Phaeophyceae). Using chemical and chromatographic methods and various forms of spectroscopy we have

* Corresponding author. Tel.: +91 34 22 55 65 66; fax: +91 34 22 53 04 52.
E-mail address: bimalendu_ray@yahoo.co.uk (B. Ray).
[1] These authors equally contributed to this work.

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been able to deduce structural features of an alginic acid and a xylogalactofucan. The possibility to generate derivatives by chemical sulfation in the *O*-positions along the polysaccharide chain has led to the synthesis of sulfated derivatives with different charge densities. With these tailored modification selected macromolecules have been generated that have potential anti-HSV activities and low cytotoxicity.

2. Experimental

2.1. Characterization and sulfation of polysaccharides from L. angustata

2.1.1. General experimental procedures

The chemicals used were of an analytical grade or the best available. All experiments were conducted at least in duplicate. Evaporations were carried out under reduced pressure at around 50 °C (SB 1100 Rotary Evaporator; Eyela, Tokyo, Japan). Dialysis against distilled water was performed with continuous stirring, and toluene was being added to inhibit microbial growth. Total sugars were estimated as anhydroglucose by the phenol–sulfuric acid assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Uronic acids were assayed as anhydrogalacturonic acid using m-hydroxydiphenyl phenol colour reagent (Ahmed & Labavitch, 1977). Neutral sugars were analyzed after hydrolysis with 1 M sulfuric acid (3 h, 100 °C), preceded by 1 h with aqueous 72% (v/v) H_2SO_4 at room temperature for insoluble residues. Sugars were reduced, acetylated and analyzed as their alditol acetate (Blakeney, Harris, Henry, & Bruce, 1983) by gas–liquid chromatography (GLC; Shimadzu GC-17A; Shimadzu, Kyoto, Japan) on columns of 3% SP-2340 on Supelcoport 100–120 mesh, and DB-225 (J&W Scientific, Folsom, CA, USA) and by gas–liquid-chromatography–mass spectrometry (GLC–MS; Shimadzu QP 5050 A, Shimadzu). Myo-inositol was used as an internal standard. Sugars in the acid hydrolysate were also analyzed by thin-layer chromatography.

2.1.2. Algal material and preliminary treatments

Samples of *L. angustata* were collected from the Okha coast of Gujarat, India in August 1995. The seaweeds were washed thoroughly with tap water, dried by forced air circulation and pulverized in a blender (Warring Products Inc., Torrington, CT, USA). Algal powder (150 g) was depigmented sequentially with petroleum ether and acetone in a Soxhlet apparatus. The unextracted material was placed in a plastic beaker and air dried to yield de-pigmented algal powder (DAP; 101 g).

2.1.3. Extraction and purification of the xylogalactofucan

DAP (20 g) was extracted three times with water (1:100; pH 6.5–7) at 30–35 °C under constant stirring. Separation of the residue from the liquid extract was performed by centrifugation followed by filtration through glass filter (G-2). The residue was briefly washed with additional distilled water and the wash was collected to maximize polysaccharide recovery. The combined liquid extract was dialyzed extensively against water and lyophilized. The recovered material was dissolved in water, precipitated by the addition of ethanol (4 volumes) and then collected by centrifugation (repeated three times). The final pellet was dissolved in water and lyophilized to yield the water extracted polysaccharide, named WEP (0.9 g).

A solution (20 ml) of WEP in 50 mM sodium acetate (pH 5.5) was applied to a column (2.6 cm × 25 cm) of DEAE Sepharose Fast Flow (AcO^-; Amersham Biosciences AB, Uppsala, Sweden). Thereafter, the column was eluted (0.6 ml min^{-1}) successively with 0.05 M, 0.2 M (fraction F1), and 2 M (fraction F2) NaOAc buffer pH 5.5 in stepwise manner. Residual bound polysaccharides were washed from the column with 0.2 M NaOH (fraction F3). Appropriate fractions were pooled, dialyzed and lyophilized.

2.1.4. Isolation and purification of alginic acid

The residue left after extraction with water was extracted with 3% Na_2CO_3 using a solute to solvent ratio of 1:100 at 30–35 °C for 4 h under constant stirring (thrice). The combined extract was carefully acidified with HCl to pH ~1 and the precipitate formed was collected by centrifugation (10,000 × *g*, 20 min), washed with water and then dissolved by careful addition of NaOH. The slightly alkaline solution was dialyzed, concentrated and diluted with 4.0 M $CaCl_2$ solution to make a final concentration of 2% $CaCl_2$. The precipitate formed at this stage was isolated by centrifugation, washed with water and treated with 0.1 M HCl (4× 50 ml, stirring at room temperature for 2 h). Then it was dissolved in NaOH, the solution was dialyzed and finally lyophilized to yield the base extracted polysaccharide (BEP, 1.7 g).

2.1.5. Sulfation and sulfate estimation

Sulfation of the samples was carried out using fuming sulfuric acid. The dry polysaccharide (BEP, 50 mg) was suspended in dry *N*,*N*-dimethylformamide (DMF, 10 ml) and 1.5 mL reagent mixture of oleum–DMF (v:v:: 2:1) was mixed with the polysaccharide suspension in ice-cold condition. The reaction was carried out under inert atmosphere at 20 °C under different time intervals (24 and 46 h). The mixture was subsequently neutralized with NaOH and desalted using Sephadex G-25 column (2.6 cm × 90 cm; Amersham Pharmacia biotech AB, Uppsala, Sweden). Fractions eluted between K_{av} values 0 and 0.5 were collected and lyophilized to give the sodium salt of the sulfated alginic acids (S1 and S2). Similarly, xylogalactofucan fraction (F2) was also further sulfated with oleum–DMF mixture for different time intervals. The sulfated derivatives F2S1 (41 mg) and F2S2 (39 mg) obtained when the reaction times were 20 and 40 min, respectively.

Sulfate groups were estimated by IR-spectrometric (Rochas, Lahaye, & Yaphe, 1986) and modified barium chloride (Craigie, Wen, & vanderMeer, 1984) methods.

2.1.6. Size exclusion chromatography

In system A, solutions (3–5 ml) of purified xylogalactofucan (F2) in 200 mM sodium acetate buffer (pH 5.0) were loaded to a Sephacryl™ 200 column (2.6 cm × 90 cm; Amersham Pharmacia Biotech AB, Uppsala, Sweden) equilibrated with the same buffer. The column was eluted ascendingly with the same buffer at 15 ml h^{-1}, and the temperature was 30–35 °C. Fractions of 5 ml were collected and analyzed for their total sugar contents by the phenol–sulfuric-acid method using glucose as the standard. The elution of polymer was expressed as a function of the partition coefficient K_{av} ($K_{av} = [V_e - V_0]/[V_t - V_0]$ with V_t and V_0 being the total and void volume of the column determined using potassium hydrogen phthalate and dextran (500 kDa), respectively, and V_e is the elution volume of the sample. The column was calibrated with standard dextrans (100, 70, 40 and 1 kDa).

In system B, all sulfated derivatives were injected separately on a Sephadex™ G-25 column (2.6 cm × 90 cm; Amersham Pharmacia Biotech AB). The desalted materials were concentrated and then lyophilized.

2.1.7. Linkage analysis

The triethylamine form (Stevenson & Furneaux, 1991) of the native xylogalactofucan was methylated by lithium dimethylsulfinyl anion and iodomethane (Blakeney & Stone, 1985). The permethylated polysaccharide was hydrolyzed with 2.5 M trifloro acetic acid at 120 °C for 75 min, reduced with 1 M $NaBD_4$ in 2 M NH_4OH for 3 h at room temperature and acetylated using perchloric acid as a catalyst. The partially methylated alditol acetates

(PMAA) were analyzed by GLC and GLC–MS using a DB-225 column as previously described (Ghosh, Auerochs, Saha, Ray, & Marschall, 2010). The mass spectra were recorded with a Shimadzu QP 5050A GLC–MS instrument (Shimadzu) at 70 eV. The partially methylated alditol acetates were identified by (i) the measurement of relative retention times, (ii) methoxyl substitution pattern as obtained from GLC–MS, and (iii) carbohydrate composition of the non-methylated polymers.

2.1.8. Spectroscopy

Infra red (IR) spectra were recorded on a Fourier transform (FT) spectrophotometer (Spectrum RX1; PerkinElmer, CITY, ST, Singapore) using KBr discs containing finely powdered samples. The ^{1}H-NMR spectra of samples were recorded on a Bruker 500 spectrometer (Bruker Biospin AG, Fallanden, Switzerland) operating at 500 MHz for ^{1}H. The alginic acid (10 mg) was heated (80 °C, 30 min) with water (1 ml), then with dilute (0.1 M) hydrochloric acid (80 °C, 60 min), neutralized with sodium hydroxide, dialyzed and lyophilized. The sulfated xylogalactofucan was converted into its sodium salt by passage through a column (7 ml; Bio-Rad, Hercules, CA, USA) of Amberlite IR 120 (H^+) followed by neutralization using 50 mM NaOH solution and dialysis. All samples were deuterium-exchanged by lyophilization with D_2O (Cambridge Isotope, Ltd, Andover, ST, USA) and then examined as 1% solutions in D_2O (99.96 atom% D).

2.2. Biological activities of anionic polysaccharides

2.2.1. Cell culture and herpes simplex virus type 1 (HSV-1)

Anti-viral experiments were performed on RC-37 cells (African green monkey kidney cells). Cells were grown in monolayer culture with Dulbecco's modified Eagle's medium (DMEM; Gibco Invitrogen Corporation; Karlsruhe, Germany) supplemented with 5% fetal calf serum, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. Herpes simplex virus type 1 (HSV-1) strain KOS was used for all experiments (Schnitzler, Schneider, Stintzing, Carle, & Reichling, 2008). Acyclovir and heparin were purchased from GlaxoSmithKline (Bad Oldesloe, Germany) and Sigma–Aldrich (Schnelldorf, Germany), respectively, and were used as anti-viral inhibitor controls.

2.2.2. Cytotoxicity assay

For cytotoxicity assays, cells were seeded into 96-well plates and incubated for 24 h at 37 °C. The medium was removed and fresh DMEM containing serial dilutions of tested polysaccharides were added onto subconfluent cells in eight replicates for each concentration of the drugs. After 3 days of incubation, the growth medium was removed and viability of the drug-treated cells was determined in a standard neutral red assay. Neutral red dye uptake was determined by measuring the optical density (OD) of the eluted neutral red at 540 nm in a spectrophotometer. The mean OD of the untreated control wells was assigned a value of 100%. The cytotoxic concentration of the drug which reduced viable cell number by 50% (CC_{50}) was determined from dose–response curves. Additionally the maximum non-cytotoxic concentration of each drug was determined as described previously (Schnitzler, Koch, & Reichling, 2007).

2.2.3. Anti-viral activity

Polysaccharides were tested for anti-viral activity against HSV-1 using a plaque reduction assay with monolayer cultures of RC-37 cells. Usually 2×10^3 plaque forming units (pfu) were incubated with different concentrations of drugs for 1 h at room temperature. Virus was allowed to adsorb to the cells for 1 h at 37 °C. The residual inoculum was then discarded and infected cells were overlaid with medium containing 0.5% methylcellulose. Each concentration was performed in three replicates; heparin and the synthetic anti-herpetic drug acyclovir was used as controls. Monolayers were fixed with 10% formalin, stained with 1% crystal violet and subsequently plaques were counted. By reference to the number of plaques observed in virus control monolayers (untreated cultures), the concentration of test compound which inhibited plaque numbers by 50% (IC_{50}) was determined from dose–response curves (Nolkemper, Reichling, Stintzing, Carle, & Schnitzler, 2006).

2.2.4. Time-on-addition assay

To identify the step at which the viral infection might be inhibited by polysaccharides, cells and viruses were incubated with drugs at different times. The cell pretreatment assay was performed with cells being treated with compounds at indicated concentrations for 1 h at 37 °C before viral infection. For the virus pretreatment assay, the virus suspension was incubated with compounds at indicated concentrations for 1 h at room temperature before adding to the cell culture for plaque assay. For comparative purposes, heparin and acyclovir were used as control compounds for positive anti-viral effect. For analysis of an anti-herpetic effect during intracellular viral replication, cells were infected with HSV and incubated at 37 °C 1 h. The infected cell monolayer was then washed three times with PBS and overlaid with medium containing 0.5% methylcellulose and maximum non-cytotoxic concentration of the sulfated polysaccharides (Schnitzler et al., 2010).

2.2.5. Virucidal assay

To determine the effect of polysaccharides on direct inactivation of virus particles, HSV-1 (2×10^7 pfu 50 μl^{-1}) was incubated with different concentrations of polysaccharides for 1 h at 37 °C. The samples were then diluted below the IC_{50} concentration and added to cell monolayers for 1 h at 37 °C. Cell monolayers were overlaid with medium containing 0.5% methylcellulose for plaque assay (Ogura, Hayashi, Lee, Kanekiyo, & Hayashi, 2010).

2.2.6. Attachment assay

Cell monolayers grown in 6 well plates were pre-chilled at 4 °C for 1 h and infected with HSV-1 (200 PFU ml^{-1}) for 3 h at 4 °C in the presence or absence of different concentrations of polysaccharides. Unabsorbed viruses were washed with PBS buffer and cells overlaid with medium.

3. Results and discussion

The objectives of this study were to analyze the polysaccharides generated from the brown alga *L. angustata* and to study their anti-HSV activities. The de-pigmented algal powder (DAP) contained 61% (w/w) polysaccharides composed of, *inter alia*, fucose and mannuronic acid residues. Therefore, it contained fucoidan and alginic acid and was extracted sequentially with various inorganic solvents as described in Section 2.

3.1. Isolation, chemical characterization and sulfation of the xylogalactofucan

3.1.1. Sugar composition

The water extracted material after dialysis and lyophilization gave a xylogalactofucan fraction (WEP). Sugar compositional analysis revealed that fraction WEP consists of fucose as the major neutral sugar together with smaller amount of galactose and xylose units (Table 1). The uronide content of this fraction is 7% and it contained 3% sulfate. Thin layer chromatographic analysis of the monosaccharide present in the hydrolysate indicates the presence of uronic acid with R_f value similar to that of mannuronic acid.

Anion exchange chromatography on a DEAE–Sepharose FF column chromatography separated the crude polymers into three

Table 1
Sugar composition of the crude (WEP), purified (F2) and further sulfated (F2S1 and F2S2) xylogalactofucan from *Laminaria angustata*.

	WEP	F2	F2S1	F2S2
Total sugar[a]	51	49	33	29
Sulfate[a]	3	4.2	6.7	7.3
Rhamnose[b]	3	Nd	Nd	Nd
Fucose[b]	59	82	79	77
Xylose[b]	14	8	5	5
Mannose[b]	2	Tr	1	1
Galactose[b]	15	9	13	15
Glucose[b]	6	1	2	3

Nd, not detected; Tr, trace.
[a] Percent weight of fraction dry weight.
[b] Percentage mol of neutral sugars.

fractions (F1, F2 and F3). F2 was the major fraction, amounting to 65% of the total polysaccharides recovered from the column. Fucose, galactose and xylose accounted for 99% of the neutral sugars of F2, which also contained 3% (w/w) of uronic acid. Therefore, F2 is essentially a xylogalactofucan that might contain sulfate groups, as indicated by its late elution. Indeed, the sulfate content of this polysaccharide is 4.2% (w/w), which is low compared to other fucoidans, suggests the presence of a novel structural motif. This xylogalactofucan (F2), which had negative specific rotation $[\alpha]_D^{32}$ −81° (*c* 0.2, H_2O), was subjected to structural analysis. The high negative rotation of the polysaccharide revealed that fucose belongs to the L-series, like in other fucoidans from brown seaweed.

3.1.2. Molecular mass

The elution profile of this macromolecule on size exclusion chromatography suggests that this polymer is homogeneous. Based on calibration with standard dextrans, the apparent molecular mass of F2 would be 56 ± 5 kDa. Notably, the macromolecule molecular mass obtained in the present study is high compared to the one from fucoidans collected from *Cystoseira*, *Padina*, *Sargassum* and *Stoechospermum* (Adhikari et al., 2006; Karmakar et al., 2009; Mandal et al., 2007; Sinha, Astani, Ghosh, Schnitzler, & Ray, 2010).

3.1.3. Chemical modification and FT-IR analyses

Sulfate content affects the anti-viral activity of polysaccharides (Witvrouw & De Clercq, 1997). In general, for a particular class of polysaccharide, the higher the charge density, the higher is its anti-herpetic activity (Ghosh et al., 2009). To study the effect of sulfate groups, we have chemically modified the xylogalactofucan (F2) to yield further sulfated derivatives (F2S1 and F2S2). These sulfated polysaccharides were purified by size exclution chromatography on a Sephadex™ G25 column. Fractions eluted at K_{av} values 0.0–0.6 were pooled and lyophilized. The yield and sulfate content of F2S1 and F2S2 were 82% and 78%, and 6.7% and 7.3% (w/w), respectively. The IR spectra of the native xylogalactofucan (F2) and its C-sulfated derivative F2S1 (Fig. 1) strongly suggest the conversion of hydroxyl groups to C-sulfate groups. The intensity of the absorbances at 1250 cm^{-1} and 830–850 cm^{-1} attributed to the stretching of >S=O bond and C–O–S bonds, respectively, are increased by sulfation. Assignments of IR absorption bands at 1251 cm^{-1} were based on the principle originally published by Orr (1954), and Lloyd, Dodgson, Price, and Rose (1961). The band at 847 cm^{-1} was ascribed to C–O–S stretching of axial sulfate groups of sugar residues based on reports by Lloyd and Dodgson (1961), and Lloyd et al. (1961). Among the constituent sugars present, only fucose and galactose residues possess axial hydroxyl groups at C4 position and hence, C4 may be the position of the sulfate group.

Table 2
Partially methylated alditol acetates derived from the native xylogalactofucan (F2) of *Laminaria angustata*.

Methylation products[a]	*m/z* values	Peak area[b] F2
2,3,4-Xyl	43, 101, 102, 117, 118, 161 and 162	1
3,4-Xyl	43, 101, 117, 130 and 190	8
2,3,4-Fuc	43, 102, 118, 131, 162 and 175	1
2,4-Fuc	43, 118, 131, 173, 174 and 234	35
2,3-Fuc	43, 102, 118, 143, 162 and 203	7
3,4-Fuc	43, 115, 130, 131,175, 190 and 234	7
2-Fuc	43, 118 and 275	14
4-Fuc	43, 131, 202 and 262	9
Fuc	43, 103, 145, 71, 187, 218, 260 and 290	4
2,4,6-Gal	43, 45, 101, 118, 129, 161 and 234	14

[a] 2,3,4-Xyl denotes 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylxylitol, etc.
[b] Percentage of total area of the identified peaks.

3.1.4. Glycosidic linkage analyses indicate 1,3-, 1,4- and 1,2-linkages

Glycosyl linkage analysis of the xylogalactofucan (F2) gave products arising mainly from 1,3-, 1,2,3- and 1,3,4-linked fucopyranosyl residues (Table 2). Significant amount of 1,4- and 1,2-linked fucopyranosyl residues were also found. Fucose residues constitute ~77% of all sugar derivatives. Galactose was found in one main methylated product arising from 1,3-linked galactopyranosyl residues. Small amounts of terminal and 1,2-linked xylose residues were also present. So far, fucose residues in algal fucoidans are either 1,2-, or 1,3-, or 1,2- and 1,3-, or 1,3- and 1,4-linked (Berteau & Mulloy, 2003; Chevelot et al., 1999; Karmakar et al., 2009; Patankar, Oehninger, Barnett, Williams, & Clark, 1993). Overall, the results of methylation analyses suggest that the macromolecule of present study possesses a structural motif that was not found in other fucoidan.

3.1.5. Nuclear magnetic resonance (NMR) analysis

^1H NMR spectrum of the xylogalactofucan (F2) of *L. angustata* is very complex (Fig. 2). A number of separate spin systems (5.1–5.5 ppm) attributable to anomeric protons of α-L-fucose residues were distinguishable in the spectrum of this polysaccharide. It also include resonances characteristic of xylogalactofucan such as signals from ring protons (H2–H5) between 3.6 and 4.5 ppm, and intense signals from the methyl protons H6, one at about 1.5 ppm (minor) and a major envelope of signals at around 1.3 ppm. The residues with H6 signals at 1.3 ppm may be attributed to (1,3)-linked fucose (Kariya et al., 2004) whereas signal appearing at 4.5 ppm can be assigned to the H4 of 4-*O*-sulfated galactose residues (Bilan et al., 2004; Kariya et al., 2004; Mandal et al., 2007; Pereira, Mulloy, & Mourão, 1999). It can be safely said that the ^1H NMR spectrum of this novel polysaccharide is complex, overlapping, and inconclusive for structural information as observed for fucoidans from other marine brown algae (Adhikari et al., 2006; Kariya et al., 2004; Mandal et al., 2007; Mulloy, Ribeiro, Alves, Vieira, & Mourão, 1994; Pereira et al., 1999).

3.2. Isolation, chemical characterization and sulfation of the alginic acid

Sodium alginate forms insoluble precipitates at acidic pH and with calcium salts, but soluble in water as its sodium salt. Therefore, this macromolecule was extracted with K_2CO_3 and purified by precipitation with calcium chloride. The fractional product (BEP) was soluble in water.

3.2.1. Molecular mass

The elution profile of this macromolecule on size exclusion chromatography suggests that this polymer is homogeneous. Based on

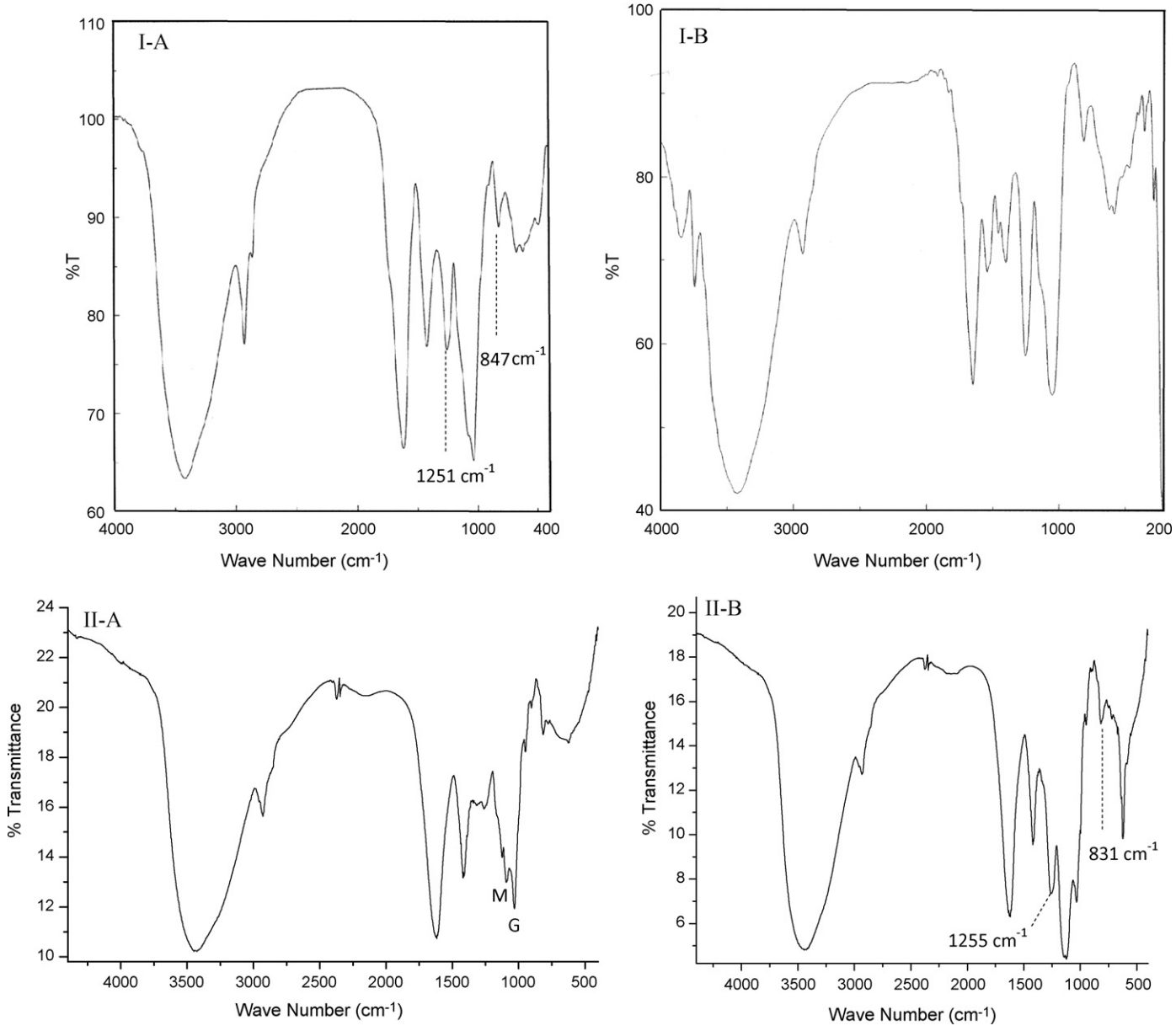

Fig. 1. FT-IR spectra of the (I-A) purified (F2) and (I-B) further sulfated (F2S1) xylogalactofucan, and (II-A) native (BEP) and (II-B) sulfated (S1) sodium alginate of the brown seaweed *Laminaria angustata*.

calibration with standard pullulans the apparent molecular mass of fraction BEP would be 32 ± 5 kDa.

3.2.2. NMR

To evaluate the contents of guluronic and mannuronic we have investigated the ^{1}H NMR spectrum (Fig. 2) of sodium alginate (BEP) using procedures as described previously (Chattopadhyay et al., 2010; Grasdalen, 1983). The relative areas of anomeric protons G1 (H1 of guluronic acid) and M1 (H1 of mannuronic acid) correspond to the mole fractions of G and M, and the values obtained are 55.5 and 44.5, respectively.

3.2.3. Chemical modification and FT-IR analyses of algin

The purified algin was chemically modified at two different time intervals to yield sulfated derivatives S1 (42%) and S2 (31%) having 7% and 5% sulfates, respectively. The IR spectrum of sodium alginate contains band at 1420 cm^{-1} (COO^- stretching) related to alginate and two bands at approximately 1100 and 1025 cm^{-1} responsible for mannuronic (M) and guluronic (G) units, respectively, were also observed (Fig. 1). The IR spectrum of S1 showed, in addition, an absorption band at 1255 cm^{-1} related to a >S═O stretching vibration of the sulfate group (Fig. 1). Another sulfate absorption band at 831 cm^{-1} (C–*O*–S, secondary equatorial sulfate) indicated the presence of sulfate group at C2/3 of the uronic acid residue (Lloyd et al., 1961). Notably, the intensity of band at 1025 cm^{-1} decreases considerably after chemical modification suggesting that the macromolecule becomes mannuronic acid rich after chemical sulfation.

3.3. Biological activities of anionic polysaccharides from L. angustata

3.3.1. Cytotoxicity assay

The 50% cytotoxic concentration (CC_{50}) of heparin, acyclovir and the anionic polysaccharides extracted from *L. angustata* and their chemically modified derivatives against RC37-cells were

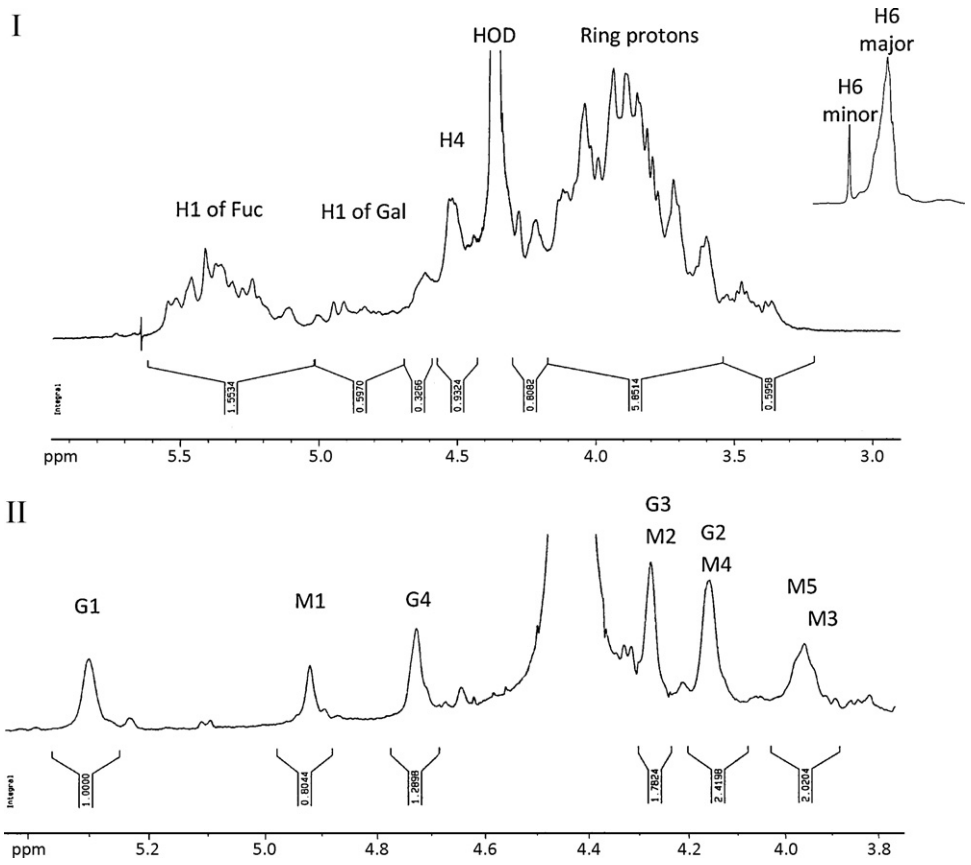

Fig. 2. ^{1}H NMR spectra at 500 MHz of the (I) purified (F2) xylogalactofucan, and (II) native sodium alginate (BEP) of *Laminaria angustata*. The spectrum was recorded for sample in D_2O solution. H6 refer to signals of methyl protons of fucose residues, whereas H4 for proton of galactose residue. G1–G4 refer to the signals of the protons of guluronic acid and M's refer to the signals of the corresponding protons of mannuronic acid residues. The signal for deuterated water was designated as HOD. The 1.53–1.29 ppm region of the ^{1}H NMR spectrum of the purified (F2) xylogalactofucan is shown in the inset.

determined with the neutral red assay on subconfluent monolayers. No effect on cell viability was detected for all tested polysaccharides at concentrations up to 1000 μg ml^{-1} (Table 3). The maximum non-cytotoxic concentration used for heparin and tested polysaccharides are 1000 μg ml^{-1}. Acyclovir was used in all experiments at 100 μM. Thus a very low or no toxicity can be assumed for these compounds.

3.3.2. Anti-viral activity

The potential anti-viral effect of the analyzed polysaccharides listed in Table 3 against HSV-1 was evaluated by standard plaque reduction assays. HSV-1 was incubated for 1 h at room temperature with various concentrations of compounds, and afterwards subjected to plaque assay. The 50% inhibitory concentrations (IC_{50}) of the tested anionic compounds for HSV-1 were determined in a range between 0.2 and 25 μg ml^{-1} and showed a dose-dependent anti-viral activity (Fig. 3). Their selectivity indices (SI), which are calculated as the CC_{50}/IC_{50} ratio, are shown in Table 3.

3.3.3. Mechanism of anti-viral action

A time-on-addition experiment was performed to investigate the effect of *L. angustata*-derived polysaccharides on virus infection. Cells were pretreated with 1000 μg ml^{-1} of anionic polysaccharides before viral infection; viruses were incubated with drugs before cell infection or after penetration of the virus into the host cells. When the host cells were pretreated with polysaccharides prior

Table 3
Cytotoxicity, anti-HSV-1 activity, and selectivity index of xylogalactofucan (F2) and sodium alginate (BEP) isolated from *Laminaria angustata* and their chemically sulfated derivatives (F2S1, F2S2, and S1, S2, respectively).

Polysaccharides	CC_{50} (μg ml^{-1})[a]	IC_{50} (μg ml^{-1})[b]	SI (CC_{50}/IC_{50})[c]
F2	>1000	0.65	>1538
F2S1	>1000	0.32	>3125
F2S2	>1000	0.21	>4761
BEP	>1000	25	>40
S1	>1000	0.31	>3225
S2	>1000	1.4	>714

[a] 50% cytotoxic concentration (CC_{50}), defined as compound concentration required to reduce cell viability by 50%, as determined from dose–response curves.
[b] 50% inhibitory concentration (IC_{50}), defined as compound concentration required to reduce virus plaques by 50%.
[c] Selectivity index (SI) is the ratio between CC_{50} and IC_{50}.

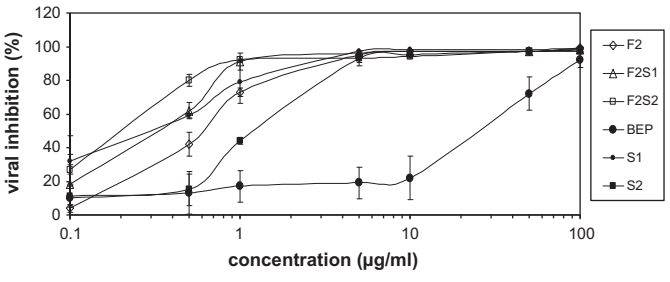

Fig. 3. Antiviral effect of serial dilutions of anionic polysaccharides against herpes simplex virus type1. Results represent the mean of three independent experiments. Purified xylogalactofucan (F2) and its sulfated derivatives (F2S1 and F2S2), sodium alginate (BEP) and its chemically sulfated derivatives (S1 and S2) were analyzed for their antiviral activity against HSV-1.

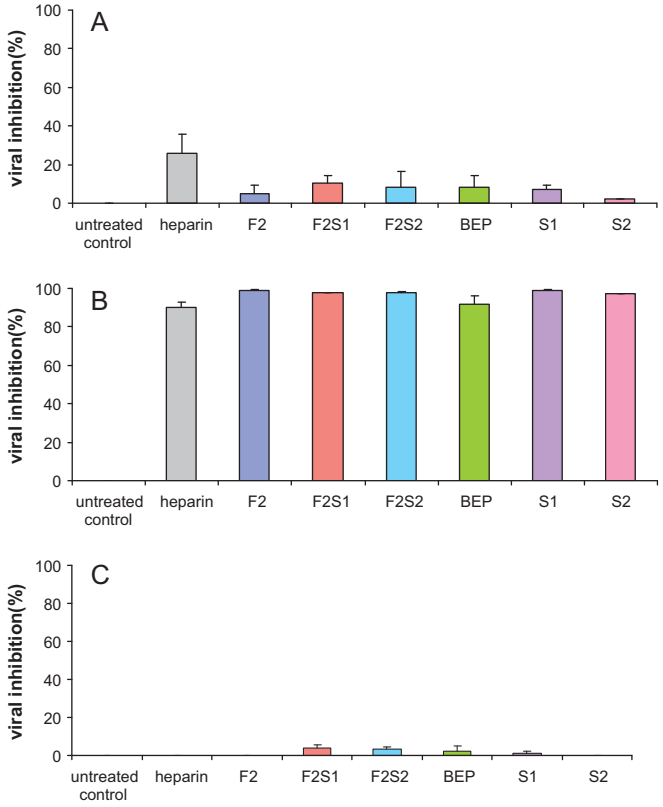

Fig. 4. Mode of inhibitory effect of sulfated polysaccharides and heparin against herpes simplex virus during different periods of the viral replication cycle. Viruses and cells were treated with the maximum non-cytotoxic concentration of compounds: (A) cells were treated with drugs for 1 h prior to infection with HSV, (B) HSV was preincubated with drugs for 1 h prior to infection of cells and (C) HSV-infected cells were drug-treated for 3 days during viral replication in the presence of the drugs. Experiments were repeated independently and performed in triplicate assays.

to infection, all anionic polysaccharides and acyclovir showed no significant effect on viral infection (Fig. 4A). On the other hand, pretreatment of HSV-1 with the drugs for 1 h prior to infection caused a significant reduction in plaque formation for F2, F2S1, F2S2, BEP, S1 and S2. Infectivity was reduced by >95% for F2, F2S1, F2S2, BEP, S1 and S2 (Fig. 4B). Acyclovir showed the highest anti-viral activity when added during the replication period with inhibition of the viral replication of 98.6% (Fig. 4C). This drug inhibits viral DNA replication when new viral DNA is synthesized. In contrast, when the anionic polysaccharides were added to the overlay medium after penetration of the viruses into the host cells, plaque formation was not significantly reduced (Fig. 4C). These data suggest that the anti-HSV activity of the tested macromolecules was exerted directly by interfering with virion particles or masking viral structures which are necessary for adsorption or entry into host cells as had been shown previously for plant derived extracts and isolated compounds (Astani, Reichling, & Schnitzler, 2010; Schnitzler et al., 2008).

To distinguish between direct inactivation of virus particles and interference in virus entry to cells, a virucidal assay was performed by incubation of virus with different concentrations of the polysaccharides for 1 h at 37 °C. These samples were then diluted in order to assure that the polysaccharide concentration is below the anti-viral value (IC_{50}) and the remaining infectivity of these mixtures was detected by plaque formation in cells. Heparin and all tested polysaccharides did not reveal any significant virucidal activity (data not shown). When cell monolayers were infected in the presence or absence of different concentrations of the polysaccharides and after 1 h of infection at 37 °C the cells were

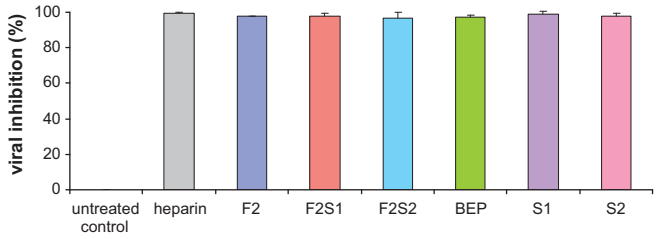

Fig. 5. Mode of inhibitory effect of sulfated polysaccharides and heparin against herpes simplex virus determined by the attachment assay. Cells were initially infected at 4 °C in the presence or absence of polysaccharides, where only adsorption takes place. Afterwards infected cells were incubated at 37 °C. Experiments were repeated independently and performed in triplicate assays.

overlaid with medium containing methylcellulose, heparin and all tested polysaccharides revealed a high anti-viral activity. Since no direct inactivating effect on cell-free HSV virions was detectable, an inhibitory effect on virus cell entry must be assumed. In order to establish more precisely at which step these compounds are effective, cells were infected at 4 °C where only adsorption/attachment takes place, and afterwards infected cells were shifted to 37 °C. Results of the attachment assay are shown in Fig. 5, where maximum non-cytotoxic contractions of all tested compounds were used. Heparin and all polysaccharides directly interfered with virus particles and inhibited viral adsorption/attachment to cells.

4. Conclusions

In conclusion, the findings of this study highlight several novel and important aspects of the brown algae derived polysaccharides with regard to their structures and anti-viral properties: (i) two series of water soluble sulfated polysaccharides having different structures could be generated from the marine alga *L. angustata*, (ii) the xylogalactofucan contains a structural motif that was not found earlier, whereas the sulfated algin is made up of mannuronic and guluronic acid residues, (iii) sulfated xylogalactofucan (F2S2) has higher potency than the sulfated alginate (S1), (iv) these substances exerted biological activity which could be analyzed in cell culture-based assay systems at a low level of cytotoxicity (undetectable up to the concentration of 1000 $\mu g\,ml^{-1}$), (v) strong anti-viral effect was demonstrated for HSV (IC_{50} of F2S2 = 0.21 $\mu g\,ml^{-1}$), (vi) all the polysaccharides directly interfered with virus particles and inhibited viral adsorption/attachment to cells, (vii) the sulfate content seemed to be important hallmark of their anti-HSV activity, and (viii) among the macromolecules investigated, the anti-viral activity of F2S2 was the highest. Given the interesting chemical characteristics of the sulfated fucoidan and the promising *in vitro* anti-herpetic properties reported here, this macromolecule might be useful in the prevention of herpetic infections and represents a good candidate for further anti-viral research.

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