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Polysaccharides from *Padina tetrastromatica*: Structural features, chemical modification and antiviral activity

Paramita Karmakar [a], Carlos Alberto Pujol [b], Elsa Beatriz Damonte [b], Tuhin Ghosh [a], Bimalendu Ray [a,*]

[a] Natural Products Laboratory, Department of Chemistry, The University of Burdwan, Burdwan 713 104, West Bengal, India
[b] Laboratorio de Virología, Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales UBA, Ciudad Universitaria-Pabellón 2 Piso 4, 1428 Buenos Aires, Argentina

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ABSTRACT

Many viruses display affinity for cell surface heparan sulfate proteoglycans with biological relevance in virus entry. This raises the possibility of the application of sulfated polysaccharides in antiviral therapy. In this study, we have analyzed polysaccharide containing fractions derived from *Padina tetrastromatica*. A glucan containing α-(1 → 4)-linked glucopyranosyl residues, an alginate with apparent molecular mass of 50 kDa, and a fucoidan were isolated. Further sulfated derivatives of the fucoidan (S1–S3), which contained 0.8–1.2 sulfate groups per monomer unit showed inhibitory activity against herpes simplex virus (HSV) types 1 and 2. Their inhibitory concentration 50% (IC_{50}) values were in the range 0.30–1.05 μg/ml and they lacked cytotoxicity at concentrations up to 1000 μg/ml. The antiviral effect was exerted during virus adsorption to the cell. The degree of sulfation seemed to play an important role because further sulfation of the isolated macromolecules largely influences their in vitro anti-HSV activity.

1. Introduction

Herpes simplex viruses (HSV) 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). In particular, genital herpes (generally caused by HSV type 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. These viruses are circulated and amplified by transmission through direct contact with a lesion or the body fluid of an infected individual. Transmission may also occur through skin-to-skin contact during periods of asymptomatic shedding. The adsorption of viruses to the host cell surface is the initial and critical step for viral infection. HSV interaction with target cells involves cell surface heparan sulfate (HS) interacting with several viral envelope proteins, especially glycoproteins gB, gC, and gD (Adamiak, Ekblad, Bergstrom, Ferro, & Trybala, 2007). gD interacts with herpes virus entry mediator (HVEM), nectin-1, and specific sites on heparan sulfate generated by certain 3-*O*-sulfotransferases (Copeland et al., 2008; Geraghty, Krummenacher, Cohen, Eisenberg, & Spear, 1998; Montgomery, Warner, Lum, & Spear, 1996; Shukla et al., 1999).The uniquely distributed sulfation pattern of HS polysaccharide is believed to regulate its functional specificity (Gama et al., 2006; Liu & Pedersen, 2007). Accordingly, heparin (heparan sulfate analogue) and several sulfated carbohydrate polymers including dextran, fucoidans, galactans and xylomannans have been found to inhibit HSV because of their structural similarity to HS (Balzarini & Van Damme, 2007; Ghosh et al., 2009a; Pujol, Carlucci, Matulewicz, & Damonte, 2007; Witvrouw & De Clercq, 1997). To date, the performance of these macromolecules in efficacy trials has been disappointing (Cohen, 2008; Grant et al., 2008), but next-generation concepts now in or approaching clinical trials offer improved prospects for efficacy (Ghosh et al., 2009a; 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; Ghosh et al., 2009a; Liu, Lu, Neurath, & Jiang, 2005). The characterization of active polysaccharides from marine algae may identify macromolecules with superior efficacy. Structurally defined polysaccharides generated by chemical sulfation may also produce drug candidate with higher potency.

The present study reports isolation and chemical characterisation of polysaccharides present in the marine brown alga *Padina tetrastromatica*. Using chemical and chromatographic methods, and various forms of spectroscopy we have been able to deduce structural features of a neutral glucan, a fucoidan and alginic acid. The possibility to generate fucoidan derivatives by chemical sulfation in the *O*-positions along the polysaccharide chain has led to

* Corresponding author. Tel.: + 91 34 22 55 65 66; fax: + 91 34 22 63 42 00.
E-mail address: bimalendu_ray@yahoo.co.uk (B. Ray).

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the synthesis of various sulfated derivatives with different degrees of sulfation. With these tailored modifications a range of macromolecules have been generated that have potential anti-HSV activity and low cytotoxicity.

2. Experimental

2.1. Characterization and further sulfation of polysaccharides from P. tetrastromatica

2.1.1. Plant material and preliminary treatments

Samples of *P. tetrastromatica* (Dictyotaceae) were collected and depigmented as described (Karmakar et al., 2009).

2.1.2. Extraction with hydrochloric acid

Extraction of depigmented algal powder (DAP) (5 g) with 0.1 M HCl at a solute to solvent ratio of 1:100 (w:v) was conducted thrice at 25–30 °C for 4 h under constant stirring. Separation of the residue from the extract was performed by filtration through a G2 glass filter. The residue was briefly washed with additional distilled water and the wash was collected to maximize polysaccharide recovery. The combined extract was dialyzed extensively against distilled water and lyophilized to yield the acid extracted polymer, named A (200 mg).

2.1.3. Extraction with potassium carbonate

The residue left after extraction with HCl was extracted with 2% K_2CO_3 (w:v::1:100) at 25–30 °C for 5 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*, 15 min), washed with water and then dissolved by careful addition of NaOH. The 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, dialyzed and finally lyophilized to yield sodium alginate B (240 mg). The supernatant after precipitation with HCl was dialysed and lyophilized separately to give fraction C (130 mg).

2.1.4. Extraction with hot water

The alkali insoluble residue was again extracted with water at 80 °C (twice) for 2 h and the combined extract was dialyzed, concentrated in vacuo, and treated with 10% hexadecyltrimethylammoniumbromide (CTAB) solution. The precipitate formed was centrifuged (30 min, 8000*g*), washed with water and stirred with 20% ethanolic KI solution (3 × 50 ml). After washing with ethanol the precipitate was dissolved in water, dialyzed exhaustively and lyophilised to give D (20 mg). The centrifugate was dialyzed, concentrated, and diluted with 4 volumes of ethanol. The precipitate formed was then dissolved in water and lyophilized to give a neutral polysaccharide containing fraction E (8 mg). The hot water insoluble residue was washed with ethanol, acetone, ether and dried to give the fraction (INS).

2.1.5. Size exclusion chromatography (SEC)

System a: Fractions (B and C) were chromatographed on a Sephacryl™ S-200 column (2.6 × 90 cm; Amersham Biosciences AB, Uppsala, Sweden) using 0.5-M sodium acetate buffer (pH 5.0) as eluent. The column was calibrated with standard dextrans (500, 70, 40 and 10 kDa).

System b: A solution (~2 ml) of fraction E in 500 mM sodium acetate buffer (pH 5.5), was loaded to a column (60 cm × 2.6 cm) of Superdex 30 prep grade (Pharmacia) 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. The column was calibrated with standard dextrans within a molecular-weight range of 1–30 kDa.

2.1.6. Sugar analysis

Total sugars and uronic acids were determined by the phenol–sulfuric acid (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and *m*-hydroxydiphenyl (Ahmed & Labavitch, 1977) assay, respectively. For the determination of sugar composition, the monosaccharide residues released by acid hydrolysis were converted into their alditol acetate (Blakeney, Harris, Henry, & Bruce, 1983) and analyzed by gas–liquid chromatography (GLC; Shimadzu GC-17A, Shimadzu, Kyoto, Japan). Monosaccharides were identified by thin-layer chromatography and gas–liquid chromatography–mass spectrometry (GLC–MS; Shimadzu QP 5050 A, Shimadzu, Kyoto, Japan) as described (Karmakar et al., 2009). Alternatively, TMS-derivatives of methyl glycosides were analyzed by GLC (York, Darvill, O'Neill, Stevenson, & Albersheim, 1985).

2.1.7. Sulfate analysis

Estimation of sulfate groups were estimated by modified barium chloride (Craigie, Wen, & vanderMeer, 1984) method and infrared spectrometry (Rochas, Lahaye, & Yaphe, 1986), and solvolytic desulfation by the method of Falshaw and Furneaux (1998) were carried out as described previously (Ghosh et al., 2009b).

2.1.8. Further sulfation

Further sulfation of the fucoidan fraction (C) was carried out as described (Ghosh et al., 2009b). Briefly, samples (40 mg each) and SO_3-pyridine (68 mg each) were dispersed in 5 ml dry *N*,*N*-dimethylformamide by sonication followed by addition of 50 μl of dry pyridine. The mixtures were heated in an oil bath at 90 °C in nitrogen atmosphere for 0.5, 1.0, and 1.5 h; the solution was subsequently neutralized with NaOH, dialyzed, and lyophilized to give the sulfated polysaccharides S1, S2, and S3, respectively.

2.1.9. Methylation analysis

The triethyl amine forms (Stevenson & Furneaux, 1991) of native (C), desulfated (C-D) and further sulfated (S3) fucoidans (~2 mg each) were subjected to two rounds of methylation (Blakeney & Stone, 1985). The methylated polysaccharide was hydrolyzed, and the liberated glycoses converted into their partially methylated alditol acetates and analysed by GLC and GLC/MS as described previously (Chattopadhyay, Adhikari, Lerouge, & Ray, 2007).

2.1.10. Spectroscopy

2.1.10.1. Fourier transform infra red (FT-IR). Recording of IR spectra were carried out as described previously (Ghosh et al., 2009b).

2.1.10.2. Nuclear magnetic resonance (NMR). The ^{1}H NMR spectra were recorded on a Bruker 500 (Bruker Biospin AG, Fallanden, Switzerland) and Bruker 600 spectrometer operating at 500 and 600 MHz, respectively. The sample of alginate (~10 mg) was heated (at 80 °C for 30 min) with water (1 ml), then 0.1 M HCl (at 80 °C for 60 min), dialysed and the resulting solution was lyophilized. The freeze-dried sample was deuterium-exchanged by lyophilization with D_2O and then examined in 99.96% D_2O.

2.2. Biological activities

2.2.1. Cells and viruses

Vero (African green monkey kidney) cells were grown in Eagle's minimum essential medium (MEM) supplemented with 5% fetal

calf serum. For maintenance medium (MM), the serum concentration was reduced to 1.5%.

HSV-1 strain F and HSV-2 strain MS were used as reference strains. B2006 is an HSV-1 thymidine kinase negative (TK^-) acyclovir-resistant strain obtained from Prof. Dr. E. De Clercq (Rega Institute, Belgium). The syncytial variant of HSV-1 1C3-syn 14-1 was obtained by serial passage in the presence of the *mu/nu*-carrageenan 1C3 as previously described (Carlucci, Scolaro, & Damonte, 2002). Virus stocks were propagated and titrated by plaque formation in Vero cells.

2.2.2. Cytotoxicity assay

Cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma–Aldrich) method (Mosmann, 1983). Confluent cultures in 96-well plates were exposed to different concentrations of the polysaccharides, with three wells for each concentration, using incubation conditions equivalent to those used in the antiviral assays. Then 10 μl of MM containing MTT (final concentration 0.5 mg/ml) was added to each well. After 2 h of incubation at 37 °C, the supernatant was removed and 200 μl of ethanol was added to each well to solubilize the formazan crystals. After vigorous shaking, absorbance was measured in a microplate reader at 595 nm. The cytotoxic concentration 50% (CC_{50}) was calculated as the compound concentration required to reduce cell viability by 50%.

2.2.3. Antiviral assay

Antiviral activity was evaluated by a virus plaque reduction assay. Vero cell monolayers grown in 24-well plates were infected with about 50 plaque-forming units (PFU)/well in the absence or presence of various concentrations of the compounds. After 1 h of adsorption at 37 °C, residual inoculum was replaced by plaquing medium (MM with 0.7% methylcellulose) containing the corresponding dose of each compound. Plaques were counted after 2 days of incubation at 37 °C. The inhibitory concentration 50% (IC_{50}) was calculated as the compound concentration required to reduce virus plaques by 50%. All determinations were performed in triplicate.

2.2.4. Virucidal assay

A virus suspension of HSV-1 containing 4×10^6 PFU was incubated with an equal volume of MM with or without various concentrations of the polysaccharides for 2 h at 37 °C. The samples were then diluted in cold MM to determine residual infectivity by plaque formation. The sample dilution effectively reduced the drug concentration to be incubated with the cells at least 100-fold to assess that titer reduction was only due to cell-free virion inactivation.

2.2.5. Effect of treatment period on the antiviral activity

Vero cells grown in 24-well plates were infected with 50 PFU of HSV-1 strain F following different treatment conditions, as follows:

Adsorption: Cells were exposed to about 50 PFU of HSV-1 in the presence of 2 μg/ml of the compound, and after 1 h at 4 °C, both compound and unadsorbed virus were removed, the cells were washed with cold PBS and overlaid with plaquing medium.

After adsorption: Cells were infected with HSV-1 in the absence of the compound and after adsorption at 4 °C, the unadsorbed virus was removed, the cells were washed two times with cold PBS and further incubated with plaquing medium containing 2 μg/ml of the compound.

Always: Cells were infected with HSV-1 in the presence of 2 μg/ml of the compound, and after 1 h at 4 °C, both compound and unadsorbed virus were removed, the cells were washed with cold PBS and overlaid with plaquing medium containing 2 μg/ml of compound.

3. Results and discussion

The objectives of this study were to analyze the polysaccharides present in brown alga *P. tetrastromatica* and to study the antiviral activity of selected fraction and its chemically modified derivatives. To achieve these goals, sequential extraction of polysaccharides were carried out as shown in Fig. 1. This procedure was based on the different solubilities of polysaccharides from brown seaweeds.

3.1. Chemical characterization and further sulfation of the alkali extracted fucoidan

3.1.1. Sugar composition

In an earlier study fucoidan from *P. tetrastromatica* was extracted with water (Karmakar et al., 2009), but this water soluble material contained alginic acid and other macromolecules. Purification of this fucoidan requires a number of steps including anion exchange and size exclusion chromatography. In the present study we have isolated pure fucoidan simply by using sequential extraction described by Usov (1998). However, the HCl extracted material (named as A, 4%; w/w) contains protein (12%; w/w) and other polymers. The potassium carbonate extract, after removal of alginic acid by sequential precipitation with HCl and $CaCl_2$, was dialysed and lyophilized to give a fucoidan rich fraction (C). Sugar compositional analysis revealed that fraction C consist mainly of fucose as the major neutral sugar together with smaller amount of galactose and xylose units (Table 1). The uronide content of this fucoidan fraction (6%; w/w) was higher than that of the water extracted fucoidan (Karmakar et al., 2009), but the former contained lesser amount of sulfate (degrees of sulfation: 0.15 vs. 0.21). Thin layer chromatographic analysis of the monosaccharides present in the hydrolysate indicates the presence of an uronic acid with an R_f value similar to that of glucuronic acid. GLC analysis of the TMS-derivatives of the derived methyl glycosides confirmed this result.

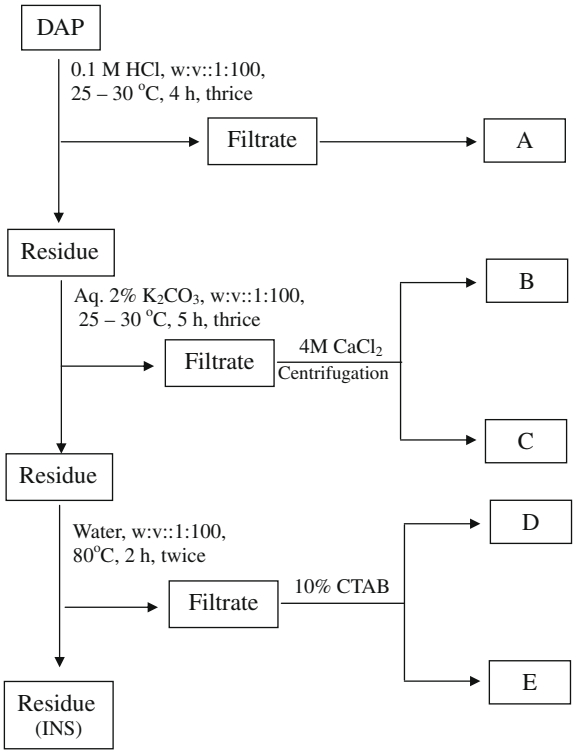

Fig. 1. Flow chart describing the process to obtain fraction starting from depigmented algal power (DAP) of *P. tetrastromatica*.

Table 1
Sugar composition (mol.%) of fractions[a] obtained from *P. tetrastromatica*.

Fractions	DAP	A	B	C	E	C-D	S1	S2	S3
Yields[b]		40	48	26	2				
TS[c]	32	34	Nd	45	56	59	38	33	31
UA[c]	5	5	56	5	Nd	6	4	4	3
Rhamnose[d]	2	Tr	Nd	Tr	Tr	Tr	Tr	Tr	Tr
Fucose[d]	34	68	Nd	73	Tr	72	75	74	71
Xylose[d]	11	16	Nd	16	Tr	17	15	15	17
Mannose[d]	44	5	Nd	Nd	Tr	Nd	Nd	Nd	Nd
Galactose[d]	4	9	Nd	11	Tr	11	10	11	12
Glucose[d]	6	2	Nd	Tr	100	Tr	Tr	Tr	Tr

TS, total sugar; UA, uronic acid; Nd, not detected; Tr, trace.
[a] See text for the identification of fractions.
[b] mg quantities per gm of depigmented algal powder (DAP).
[c] Percent weight of fraction dry weight.
[d] mol percent of neutral sugars.

3.1.2. FT-IR

The FT-IR spectrum of C showed an absorption band at 1252 cm^{-1} related to a >S=O stretching vibration of the sulfate group (Fig. 2). An additional weak sulfate absorption band at 848 cm^{-1} (C–O–S, secondary axial sulfate) indicated that the sulfate group is located at position 4 of the fucopyranosyl residue (Chizhov et al., 1999; Patankar, Oehninger, Barnett, Williams, & Clark, 1993).

3.1.3. Molecular mass

Fraction C was subjected to further chemical analysis. First, the apparent molecular mass was determined by size exclusion chromatography. Based on calibration with standard dextrans, the apparent molecular mass of C would be 20 kDa. Notably, the molecular mass of the fucoidan of present study is lower than that of water extracted one (Karmakar et al., 2009). But the extraction conditions used in these studies are different. Here the polymer was extracted with K_2CO_3, whereas in the earlier study water was used as extracting solvent. The higher molecular mass of the fucoidan extracted with water may be due to the possible co-precipitation of protein and alginate with the fucoidan.

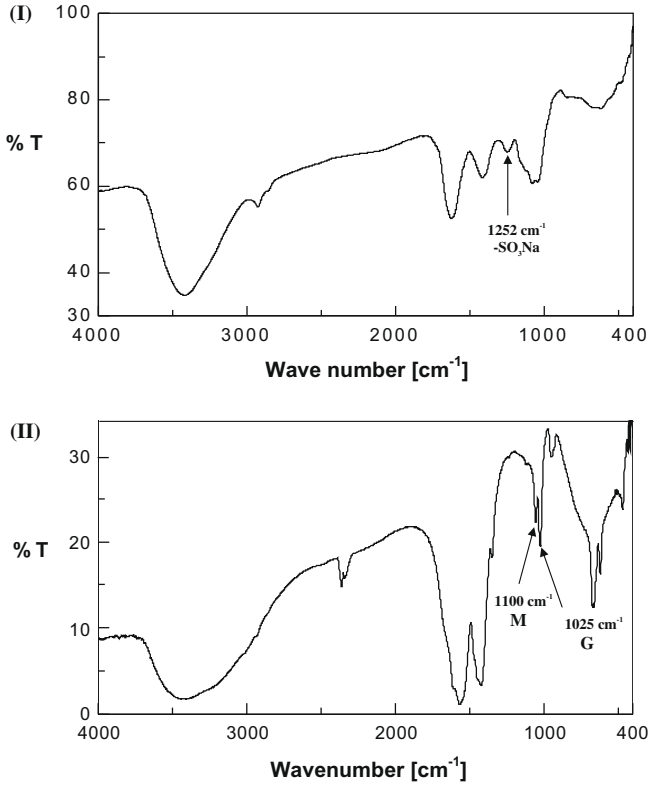

Fig. 2. FT-IR spectra of the (**I**) fucoidan (C) and (**II**) sodium alginate (B) isolated from the brown seaweed *P. tetrastromatica*. Absorption band at 1252 cm^{-1} in (**I**) arises from the sulfate group of the fucoidan whereas bands at 1025 and 1100 cm^{-1} in (**II**) arise from guluronic (G) and mannuronic (M) acid residues of sodium alginate, respectively.

3.1.4. Further sulfation and desulfation

Degree of sulfation (DS) affects the antiviral activity of polysaccharides (Ghosh et al., 2009a; Witvrouw & De Clercq, 1997). In general, for a particular class of polysaccharide, the higher the charge density, the better is its activity. In addition to the well documented DS dependence, the specific position of the sulfate ester group also appears to be additionally important for the antiviral activity of sulfated polysaccharide (Copeland et al., 2008; Ghosh et al., 2009a). To study the effect of sulfate groups, we have sulfated the fucose containing fraction (C) under various conditions as given in Section 2 to yield sulfated derivatives S1, S2, and S3. Their degrees of sulfation are 0.8, 1.0, and 1.2, respectively. Notably, the sugar composition of further sulfated fractions is similar to their parent fraction (C). The FT-IR spectra of these sulfated fucoidans (S1–S3) showed a band around 1240–1260 cm^{-1} related to >S=O stretching vibration of the sulfate group (Lloyd, Dodgson, Price, & Rose, 1961). We have also desulfated the fucoidan (C) by the method of Falshaw and Furneaux (1998). Preliminary experiments (data not shown) showed that this method gives lowest sulfate content compared to auto-desulfation and methanol–HCl method (Percival & Wold, 1963). Desulfated derivative of C (named as C-D) had a recovery yield of 39%. The sugar composition of C and its desulfated derivative C-D, respectively, were close (Table 1) indicating that sugar backbone remained almost unaffected by chemical modification.

3.1.5. Glycosidic linkage analyses

The results of methylation analysis of the desulfated fucoidan (C-D) suggest that it contains (1 → 2)- and (1 → 3)-linked fucopyranosyl residues. This data is in accordance to the result obtained from the water extracted fucoidan from the same alga (Karmakar et al., 2009). The further sulfated fucoidan (S3), on the other hand, gave five different partially methylated alditol acetates (PMAA) indicating that this polymer is highly sulfated (Table 2). The composition of the PMAA remains same even after another two rounds of methylation. It should, however, be noted that complete methylation of the sulfated polysaccharide, because of the steric hindrance by the sulfate esters, is very difficult (Patankar et al., 1993; Pereira, Mulloy, & Mourão, 1999). 4-*O*-Methyl fucitol, unmethylated fucitol, xylitol and 4-*O*-galactitol were the abundant products of methylation anal-

Table 2
Partially methylated alditol acetates derived from further sulfated (S3) and desulfated fucoidan (C-D) of *P. tetrastromatica*.

Methylation products[a]	Peak area[b]	
	S3	C-D
2,3,4-Xyl	Nd	13
3,4-Xyl	4	4
Xyl	19	3
2,3,4-Fuc	Nd	25
2,4-Fuc	Nd	13
2,3-Fuc	Nd	4
3,4-Fuc	Nd	23
4-Fuc	5	4
Fuc	67	3
4-Gal	5	7

Nd, not detected.
[a] 2,3,4-Xyl denotes 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-xylitol, etc.
[b] Percentage of total area of the identified peaks.

ysis of this further sulfated polymer (S3). Although it is clear both xylose and fucose residues were sulfated, but it was not possible to locate the exact position of sulfate groups.

3.1.6. NMR analysis

The ^{1}H NMR spectrum of the desulfated fucoidan is very complex (Fig. 3) due to its complex structure. A number of spin systems attributable to anomeric protons of α-fucose residues were distinguishable in the spectrum of this macromolecule. It also include resonance characteristic of fucoidan such as signals from ring protons (H2–H5) between 3.14 and 4.63 ppm, and intense signals from the methyl protons H6 between 1.17 and 1.39 ppm. Broad signals between 1.82 and 2.70 ppm, and at about 0.86 ppm, can be attributed to a small proportion of protein molecule in the sample.

3.2. Chemical characterisation of the alginic acid

Sodium alginates form insoluble precipitates at acidic pH and with calcium salts, but they are stable in solution between pH 6 and 9. Therefore, these macromolecules were extracted with K_2CO_3.

3.2.1. Molecular mass

This fraction (B) was subjected to further chemical analysis. First, the apparent molecular mass was determined by size exclusion chromatography. Based on calibration with standard dextrans the apparent molecular mass of fraction B would be 50 kDa.

3.2.2. IR analysis

The FT-IR spectrum of this fraction contains band at 3400 (OH stretching), 2925 (CH stretching), 1675 and 1420 (COO^- stretching) cm^{-1} characteristic of alginate (Fig. 2). Moreover two bands at approximately 1100 and 1025 cm^{-1} responsible for mannuronic (M) and guluronic (G) units respectively, were observed (Pereira, Sousa, Coelho, Amado, & Ribeiro-Calro, 2003).

3.2.3. NMR analysis

To evaluate the content of G, M, and G–G linkage, we have investigated the ^{1}H NMR spectrum of sodium alginate (Fig. 3) using procedures as described previously (Chattopadhyay et al., 2010; Grasdalen, 1983). The relative areas of anomeric protons G1 (H1 of G) and M1 (H1 of M) correspond to the mole fractions of G

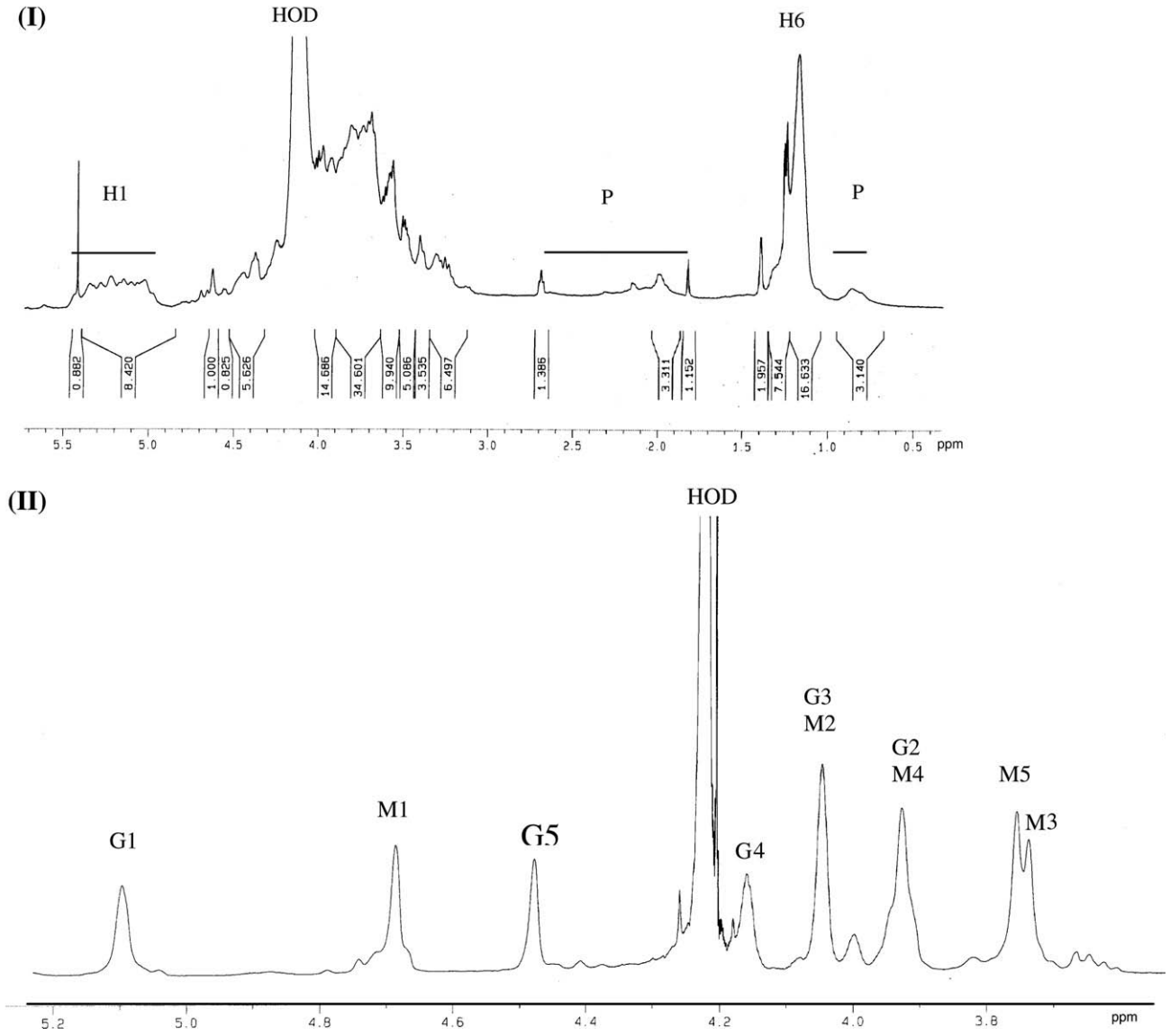

Fig. 3. ^{1}H NMR spectra at 500 MHz of (**I**) desulfated fucoidan (C-D) and (**II**) sodium alginate of *P. tetrastromatica*. These spectra were recorded at 80 °C for samples in D_2O solution. **I**, H1 and H6 refer to signals of anomeric protons and methyl protons of fucose residues, respectively. The signals for the residual water and protein were designated as HOD and P, respectively. **II**, G's and M's refer to the signals arising from the protons of guluronic acid and mannuronic acid, respectively.

and M, respectively. The peak areas of G5 (H5 resonances of G) give the distribution of G in G-block and GM-block, and the algebraic sum of their intensities accounts for the total G content, i.e., the relative area of G5 is equal to that of G1. From the measurement of peak areas of A (δ_H 5.1 (G1)), B (δ_H 4.69–4.74 (G5 in GM-block and M1)), and C (δ_H 4.45–4.48 (G5 in G-block)), the guluronic acid content (G%) and G–G diad frequency (GG%) calculated using Eqs. 1 and 2 were found to be 46.4 and 43.3, respectively.

$$G\%(\%) = \frac{\text{area of A}}{(\text{area of B}) + (\text{area of C})} \times 100 \quad (1)$$

$$GG\%(\%) = \frac{\text{area of C}}{(\text{area of B}) + (\text{area of C})} \times 100 \quad (2)$$

3.3. Structural features of the glucan

Algal glucans are water soluble, but their solubility depends on the temperature of the medium. The less branched glucans are soluble in warm water (60–80 °C), but are usually neutral. On the other hand the anionic polysaccharides form insoluble salt with detergents such as CTAB. Therefore, attempts have been made to separate glucan from other anionic polysaccharides present in the hot water by taking advantage of their differential solubility. Indeed, CTAB separates the crude hot water extracted polymers into two fractions D and E. The neutral fraction E consisted of glucose as the only neutral sugar. Based on calibration with standard dextrans, the apparent molecular-weight of this glucan would be 5 kDa. This glucan containing fraction (E) does not produce blue coloration with iodine. Moreover ^{1}H NMR spectrum of this polymer clearly shows the presence of one anomeric signal at 5.369 ppm indicative of α-configuration (Fig. 4). Finally, methylation analysis of this glucan suggest that it contained (1 → 4)-linked glucopyranosyl residues. The α-(1 → 4)-linked glucan from *Caulerpa racemosa* (Chattopadhyay et al., 2007) also exhibited similar type of backbone. On the basis of the glycosidic linkage and ^{1}H NMR analysis it may be concluded that the glucan of *P. tetrastromatica* is a linear polysaccharide and contained α-(1 → 4)-linked glucopyranosyl residues.

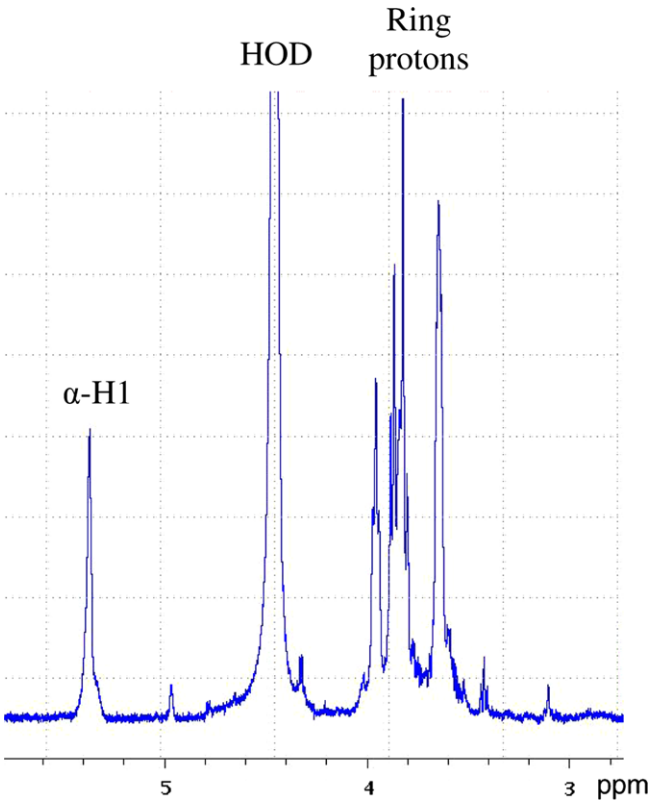

Fig. 4. ^{1}H NMR spectrum at 600 MHz of the glucan of *P. tetrastromatica*. The spectrum was recorded at 60 °C for sample in D_2O solution. α-H1 refers to anomeric signal of α-linked glucopyranosyl residue. The signal for the residual water was designated as HOD.

Table 3
Cytotoxicity and antiviral activity of desulfated (C–D) and further sulfated (S1–S3) fucoidans generated from *P. tetrastromatica*, and of heparin.

Fraction	CC_{50} (μg/ml)[a]	IC_{50} (μg/ml)[b]	
		HSV-1 F	HSV-2 MS
S1	>1000	1.05 ± 0.09	0.39 ± 0.11
S2	>1000	0.76 ± 0.19	0.31 ± 0.04
S3	>1000	0.74 ± 0.06	0.30 ± 0.01
C-D	>1000	>100	50 ± 5.0
Heparin	>1000	1.3 ± 0.1	0.5 ± 0.1

[a] Cytotoxic concentration 50% (CC_{50}): compound concentration required to reduce cell viability by 50%, as determined by MTT method.
[b] Inhibitory concentration 50% (IC_{50}): compound concentration required to reduce virus plaques by 50%. Values are the mean of triplicate tests ± standard deviation.

3.4. Biological activities of further sulfated fucoidan

In a recent review it is has been pointed out that the antiviral activity of anionic polysaccharides is not merely a function of high charge density, but has distinct structural specificities of which nature of anionic groups is undoubtedly important (Ghosh et al., 2009a). Sulfates are required for activity, whereas polymers containing carboxyl groups show less potency. Therefore, the antiviral activity of only the fucoidan derived fractions (S1–S3) was tested. These three further sulfated derivatives were initially evaluated for cytotoxicity by assessing their effects on Vero cell viability. For comparative purposes, heparin was simultaneously assayed as known reference polysaccharide. No effect on cell viability was observed with any of these compounds at concentrations up to 1000 μg/ml (Table 3).

Thereafter, the polysaccharides were screened for antiviral activity against two reference strains of herpes simplex virus, HSV-1 strain F and HSV-2 strain MS, by a virus plaque reduction assay on Vero cells. As shown in Table 3 the three fucoidan sulfated samples S1, S2 and S3 exhibited potent in vitro antiherpetic activity with IC_{50} values in the range 0.74–1.05 and 0.30–0.39 μg/ml against HSV-1 and HSV-2, respectively. Given the lack of cytotoxicity exhibited by the fucoidans, the selectivity indices (ratio CC_{50}/IC_{50}) were higher than 952–3333 for the three fucoidans against both viruses, indicating the specificity of the inhibitory effect of this type of polysaccharides against herpes viruses. Additionally, the sulfated fucoidans were more effective herpetic inhibitors than the reference compound heparin.

The antiviral effectiveness of the fucoidans was directly dependent to the degree of the sulfation, with the highest activity for S3, the highly sulfated polysaccharide (Table 3). This finding is in accordance with previous reports about the link between the antiviral activity of diverse classes of polysaccharides and the anionic features of the macromolecules (Damonte, Matulewicz, & Cerezo, 2004; Witvrouw & De Clercq, 1997). To further confirm the effect of sulfate group on the biological properties of the fucoidan sulfates, we evaluated the cytotoxicity and antiviral activity of the respective desulfated sample C-D. The lack of toxicity remained constant in the desulfated compound, but the antiviral activity was reduced drastically after the desulfation process (IC_{50} values from 50 to >100 μg/ml).

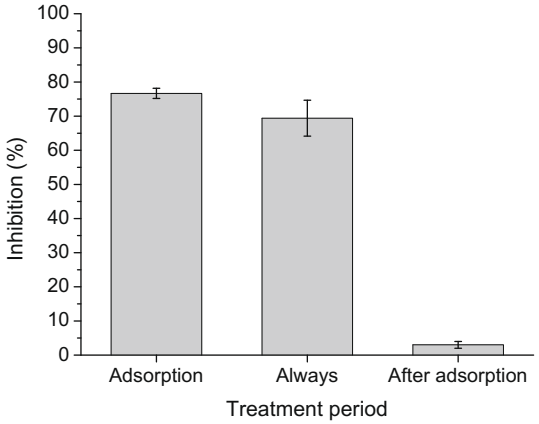

Fig. 5. Influence of the treatment period on the anti-HSV-1 activity of S3. Vero cells were infected with 50 PFU of HSV-1 strain F under different treatment conditions with 2 μg/ml of S3: the compound present only during virus adsorption (adsorption), only after adsorption (after adsorption) or during and after adsorption (always). After 2 days of incubation, plaques were counted and % inhibition for each treatment with respect to untreated infected cultures was determined. Each value represents the mean of triplicate assays.

To determine the spectrum of antiherpetic activity of the most active sulfated fucoidan, S3 was evaluated against other strains of HSV-1. S3 was effective inhibitor of the TK^- strain of HSV-1 resistant to acyclovir named B2006, with an IC_{50} value of 0.60 ± 0.14 μg/ml, similar to those obtained against the reference strains (Table 3). Similarly, S3 was also active against the syncytial strain $1C_3$-syn 14-1, with an IC_{50} value of 0.92 ± 0.05 μg/ml.

In order to analyze the possibility that these polysaccharides may act directly on the virus particle leading to infectivity inactivation, a virucidal assay against HSV-1, strain F, virions was carried out. S1, S2 and S3 were unable to inactivate HSV-1 virions at the maximum concentration tested of 50 μg/ml. This concentration is far from the antiviral IC_{50}, indicating that the inhibitory effect detected by the plaque reduction assay was really due to interference with some step of the HSV-1 multiplication cycle.

To understand the mode of action of these polysaccharides against herpes viruses, a virus plaque reduction assay was performed under different treatment conditions. The compound S3 was exposed to the cells either: during the virus adsorption period only; after virus adsorption only; or both during adsorption and all the incubation period after adsorption. As can be concluded from the data presented in Fig. 5, S3 lost virtually all significant antiviral activity when not present during the virus adsorption period. The presence of the fucoidan only during virus adsorption was as effective as their presence always during the whole incubation period.

4. Conclusions

In conclusion, the marine alga *P. tetrastromatica* contains at least three different types of polysaccharides. The alkali extracted fucoidan is branched, sulfated and contains, inter alia, (1 → 2)-linked fucopyranosyl residues. The presence of alginic acid and glucan were also indicated. This glucan is linear and contained α-(1 → 4)-linked glucopyranosyl residues. The complete structure of the alginic acid isolated with alkali was not obtained, but some important structural features were established. We came to know that the guluronic acid content (G%) and G–G diad frequency (GG%) are 46.4 and 43.3, respectively. Polysaccharides containing uronic acid are, in general, less active than sulfated one. But, the presence of a homoguluronan rich alginate with anti-HSV activity has recently been reported. Therefore, pharmacological studies of the alginate of present study will be of interest. Moreover, we found that the further sulfated fucoidans derived from *P. tetrastromatica* exhibited potent inhibitory activity against HSV-1 and HSV-2. The extent of inhibition produced by these sulfated polysaccharides was similar to that of a standard antiherpetic polysulfate such as heparin. In addition, the inhibition of in vitro HSV replication was observed at polymer concentrations, which do not have any effect on the cell viability. Furthermore, the DS appeared to be an important hallmark of anti-HSV activity.

Because the polysaccharides tested in this study were basically prepared without toxic chemical reagents, it can be assumed to be potentially useful as a safe antiviral agent. Moreover, as the generation of these polysaccharides involves a few inexpensive and easy steps it will be an additional point of interest. Given the interesting chemical characteristics of the sulfated fucoidans derived from *P. tetrastromatica* and the promising in vitro antiherpetic properties reported here, these macromolecules could be promising candidates for further thorough pharmacological and clinical studies.

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