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Polysaccharides from the brown seaweed *Padina tetrastromatica*: Characterization of a sulfated fucan

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ABSTRACT

Sulfated fucans, the complex polysaccharides from brown seaweeds, possess various biological activities. To understand the structure activity relationship of sulfated fucans, we have investigated the structural features of one such polymer from *Padina tetrastromatica* using standard methods of carbohydrate structural analysis. We report a novel structural motif for this polymer. The average structure of this macromolecule that has a molecular mass of 25 kDa differs from the previous models in three respects. First, the core region of this macromolecule is composed primarily of α-(1 → 2)- and α-(1 → 3)-linked fucopyranosyl residues. Sulfate groups, when present are located at position 4 and 2 of fucosyl residues. Secondly, fucose and xylose is attached to this polymer to form branch points, one for every two residues within the chain. Finally, this macromolecule contained smaller amount of sulfate (0.21 mol of sulfate per mol of deoxyhexose).

1. Introduction

Sulfated fucans, polysaccharides containing substantial percentages of L-fucose and sulfated ester groups, are constituents of brown algae and some marine invertebrates (Berteau & Mulloy, 2003). These complex polysaccharides showed a wide variety of biological activities, such as anti-adhesive, (McCormick, Newbold, & Berendt, 2000), anti-coagulant, (Cumashi et al., 2007; Mourão, 2004) anti-complimentary, (Tissot & Daniel, 2003), anti-oxidant (Chew, Lim, Omar, & Khoo, 2008), anti-proliferative, (Patel, Mulloy, Gallagher, O'Brien, & Hughes, 2002), anti-thrombotic, (Nishino, Fukuda, Nagumo, Fujihara, & Kaji, 1999), anti-platelet aggregation, (Alwayn et al., 2000), anti-tumor, (Alekseyenko et al., 2007) and anti-viral properties (Ghosh et al., 2009).

Conchie and Percival (1950) described sulfated fucan from the common brown algae *Fucus vesiculosus* as a polysaccharide based on L-fucose with mainly α-(1 → 2) glycosidic bonds and sulfate groups at position 4. This structural model was accepted for over forty years. In 1993, Patankar and co-workers reinvestigated the structure of sulfated fucan of this alga and it was shown that the main chain of the polysaccharide contains (1 → 3)-linked fucopyranosyl residues. More recent studies have also shown that representatives of the orders *Chordariales* and *Laminariales* contain sulfated fucan with a backbone of α-(1 → 3)-linked L-fucopyranose residues (Chizhov et al., 1999; Nagaoka et al., 1999; Nishino, Nishioka, Ura, & Nagumo, 1994). This regular backbone is often masked by different substituents such as monosaccharides, acetyl groups and sulfate esters (Chizhov et al., 1999; Mandal et al., 2007; Nagaoka et al., 1999). In some cases, however, it is possible to observe the regular backbone. Sulfate group, when present, resides mostly at position 4 of the fucopyranosyl residues (Berteau & Mulloy, 2003), although in some cases it can be present at position 2 (Daniel et al., 2007).

In contrast, fucan sulfates from two representatives of the order Fucales, namely, *Ascophyllum nodosum* and *Fucus vesiculosus*, were shown to have a backbone built up of α-(1 → 3)- and α-(1 → 4)-linked fucopyranosyl residues (Chevelot, Mulloy, Ratiskol, Foucault, & Colliec-Jouault, 2001, 1999; Daniel, Berteau, Jozefonvicz, & Goasdoue, 1999). A repeating structure of alternating α-(1 → 3)- and α-(1 → 4)-linked fucopyranose residues was determined for oligosaccharides (of ~8–14 monosaccharide units) generated from the fucan sulfate of *A. nodosum* (Chevelot et al., 2001). Based on the finding that the characteristic ^{1}H NMR spectrum of this repeating disaccharide is the major feature in the ^{1}H NMR spectra of whole fucan sulfates from *F. vesiculosus* and *A. nodosum* (Pereira, Mulloy, & Mourão, 1999) it was proposed that the backbone of both fucan sulfate consists of this structure. More recent study suggests that fucan sulfate from *Fucus serratus* is branched, one branching point being present on average in every heptasaccharide fragment (Bilan, Grachev, Shashkov, Nifantiev, & Usov, 2006).

In some cases, algal fucan sulfates also contain galactose, glucose, mannose, xylose and glucuronic acid (Nishino et al., 1994). To date, naturally occurring fucan with degree of sulfation (mol of sulfate per mol of monosaccharide) lower than 0.3 have never been reported.

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In the course of our studies on the structure and anti-viral activity relationship of sulfated polysaccharides from marine algae, we have investigated the structure of polysaccharide from Indian sample of the brown seaweed *Padina tetrastromatica* (Dictyotaceae). We found that the water extracted fraction of this alga consists mainly a novel sulfated fucan containing α-(1 → 2)- and α-(1 → 3)-linked L-fucopyranosyl together with galactopyranosyl and xylopyranosyl residues. Moreover, compared to the sulfated fucan of *Padina gymnospora* (Silva et al., 2005), the sulfate content of the fucan of present study is low (0.32 vs. 0.21) and its degree of branching is high (1 terminal for every 9 residues vs. 1 terminal for every 2 residues within the chain).

2. Experimental

2.1. General experimental procedures

Chemicals used were analytical grade or best available. All experiments were conducted at least in duplicate, the mean and standard deviation was directly calculated from the functions present in excel program. Evaporations were performed under diminished pressure at ~45 °C (bath) and small volume of aqueous solutions was lyophilized. Dialysis against distilled water was performed with continuous stirring, toluene being added to inhibit microbial growth. Moisture was determined by drying ground material in an air-circulated oven at 110 °C for 3 h. Recording of IR spectra and optical rotation measurements were carried out as described previously (Adhikari et al., 2006). Gas–liquid chromatography (GLC) was carried out on a Shimadzu GC-17A (Shimadzu, Kyoto, Japan) gas chromatograph equipped with FID, and nitrogen was used as a carrier gas. Gas–liquid chromatography–mass spectrometry (GLC–MS) analysis was carried out on a Shimadzu QP 5050 A, Shimadzu, and the ionization potential was 70 eV. The ^{1}H NMR spectra were recorded on a Bruker 600 and 500 spectrometer (Bruker Biospin AG, Fallanden, Switzerland) operating at 600 and 500 MHz, respectively for ^{1}H. ESI-MS experiments were performed on a Q-TOF Micro mass spectrometer (waters) equipped with electrospray interface. The analyses were carried out in positive mode. Dried samples were diluted in 1:1 MeOH–water and introduced into the mass spectrometer.

2.2. Plant material and preliminary treatments

Samples of *P. tetrastromatica* 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 Waring Blender. Depigmentation of 150 g of algal powder was done using sequential extraction with petroleum ether and acetone as solvent in a Soxhlet apparatus. The unextracted material was placed in a plastic beaker, and air-dried to yield depigmented algal powder (DAP, 108 g).

2.3. Extraction of the sulfated fucan

DAP (5 g) was extracted thrice with water (1:100) (pH 6.5–7) at 30–37 °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 liquid extract was dialyzed extensively against water and lyophilized. The recovered material was dissolved in water; the polysaccharides were precipitated twice with ethanol (4 vol.) and then collected by centrifugation. The final pellet was dissolved in water and lyophilized to yield the water-extracted polysaccharide, named PtWE (0.4 g).

2.4. Precipitation with calcium chloride

A solution of the water extracted material (PtWE) in water was diluted with 2% $CaCl_2$ and the suspension kept for overnight in cold condition (Bilan et al., 2004). The soluble fraction as obtained from centrifugation of the suspension was then dialyzed and lyophilized (PtWE-I). The insoluble fraction was treated with 100 mM HCl (30 mL, 3 times) and the residual material was then treated with 1-N NaOH, dialyzed, centrifuged, and the supernatant was lyophilized (PtWE-II). Another fraction named as PtWE-III was recovered from the alkali insoluble material after dialysis and lyophilisation.

2.5. Anion exchange chromatography

A solution (20 mL) of PtWE-I in 50 mM sodium acetate (pH 5.5) was applied to a column (2.6 × 25 cm) of DEAE-Sepharose FF (AcO^-). Thereafter, the column was eluted (0.6 mL min^{-1}) successively with 0.2-M (fraction F1), 0.7-M (fraction F2) and 2.0-M (fraction F3) NaOAc buffer in a stepwise manner. Appropriate fractions were pooled, dialyzed and lyophilized.

2.6. Size exclusion chromatography

Size exclusion chromatography of F3 on a Sephacryl S-300 column (89 × 2.6 cm; Amersham Biosciences AB) using 0.5-M sodium acetate buffer (pH 5.0) as eluant was done as described previously (Adhikari et al., 2006). The column was calibrated with standard dextrans (500, 70, 40 and 10 kDa).

2.7. Smith degradation

Smith degradation of the desulfated fucan (F3D) was carried out by the method of Fry (1988) as described by Mandal et al., 2008. The Smith degraded material which was desalted on Sephadex G-10 column was used for methylation and NMR analysis.

2.8. Chemical analysis

Total sugars and uronic acids were determined by the phenol-sulfuric acid (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and *m*-hydroxydiphenyl assay (Ahmed & Labavitch, 1977), 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 GLC. Monosaccharides were identified by thin-layer chromatography and GLC/MS. Alternatively, trimethylsilyl (TMS) derivatives of methyl glycosides were analyzed by gas chromatography (York, Darvill, O'Neill, Stevenson, & Albersheim, 1985). The triethylamine forms (Stevenson & Furneaux, 1991) of polysaccharides (F3, F3D and Sm-F3D) were subjected to two rounds of methylation (Blakeney & Stone, 1985). Permethylated samples were hydrolysed, converted into their partially methylated alditol acetates and analysed by GLC/MS as described (Mandal et al., 2008). Estimation of sulfate by the modified barium chloride method (Craigie, Wen, & van der Meer, 1984) and IR spectrometry (Rochas, Lahaye, & Yaphe, 1986), and desulfation by solvolytic (Falshaw & Furneaux, 1998), methanol–HCl and auto-hydrolysis (Rochas et al., 1986) method were carried out as described previously (Mandal et al., 2008).

3. Results and discussion

3.1. Isolation and purification of a sulfated fucan

The depigmented algal powder (DAP) from *P. tetrastromatica*, which contained fucose as one of the constituent sugar, was ex-

tracted with water and polysaccharides were isolated therefrom by repeated precipitation with ethanol. This water extracted fraction (named as PtWE) amounted to 8% of DAP dry weight and contained fucose as the major neutral sugar (Table 1). Like the sulfated fucans of other brown seaweeds the polysaccharides from *P. tetrastromatica* also contained galactose and xylose, but it did not contain any amino sugar. The uronide content of PtWE was 14%, possibly due to the presence of alginic acid. Sodium alginates are soluble in water but they form insoluble precipitates with calcium salts. Therefore, these macromolecules were removed from the water extract by precipitation with calcium chloride. Indeed, fractionation of PtWE with calcium chloride yielded three populations: a fucoidan containing fraction (Table 1) soluble in calcium chloride (PtWE-I) and two other fractions (PtWE-II and PtWE-III) derived from the calcium chloride insoluble material.

Sulfated fucans show potent activity against herpes simplex viruses (Ghosh et al., 2009). Moreover, anti-viral activity of a polymer depends upon its charge density (Ghosh et al., 2009). Therefore, one of the goal of this research was to isolate a sulfated fucan with high charge density and for this attempts have been made to purify the fucoidan present in fraction (PtWE-I) by chromatography. Anion exchange chromatography on DEAE Sepharose column and size exclusion chromatography on Sephacryl S-300 column were used to purify the fucoidan present in fraction PtWE-I as has been described previously for similar macromolecules from other algae (Adhikari et al., 2006; Mandal et al., 2007). These techniques were necessary in order to achieve complete separation of the sulfated fucan from the other macromolecules and to obtain a fraction with high charge density. Anion exchange chromatography separated the PtWE-I into three new fractions eluted with 0.2-M (F1), 0.7-M (F2) and 2.0-M (F3) NaOAc. The peak (F1) eluted at the beginning of the salt gradient, containing uronic acid, corresponds to a alginic acid containing fraction. It also contained a low level of sulfate (0.07 mol per mol of deoxyhexose). The second fraction (F2) contained relatively higher amount of sulfate (0.12 mol per mol of deoxyhexose). Fraction eluted with 2.0-M NaOAc (named as "F3") yielded 30% of the total carbohydrates recovered from the column. This fraction was further subjected to size exclusion chromatography on Sephacryl S-300 (Fig. 1). Only a single band was obtained after these steps of purifications, indicating that this fraction (F3) is essentially pure. Based on calibration with standard dextrans, the apparent molecular mass of F3 would be 25 kDa. The major neutral sugar of F3 is fucose (Table 1) and this fraction contained 4–5% (w/w) of uronic acid. Thin layer chromatographic analysis of the monosaccharides present in the hydrolysate indicates the presence of *inter alia*, an uronic acid with R_f value similar to that of glucuronic acid. GLC analysis of the TMS-derivatives of the derived methyl glycosides confirmed this result. Therefore, F3 is essentially a fucan that might contain sulfate groups, as indicated by its late

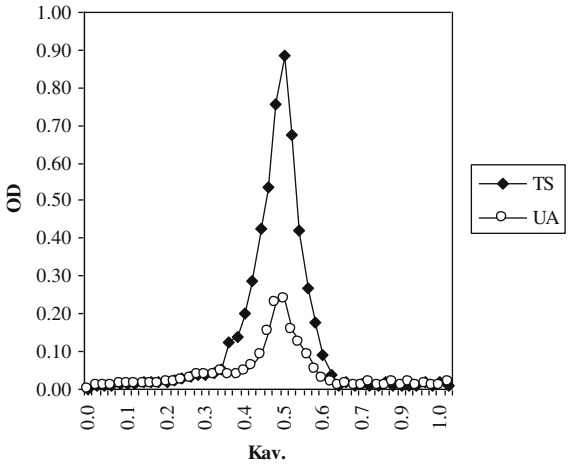

Fig. 1. Elution profile of the sulfated fucan (F3) of *Padina tetrastromatica* on Sephacryl S-300 column with 500 mM sodium acetate buffer (pH 4.0) at 20 mL/h. TS, Total sugar; UA, Uronic acid and OD, Optical density.

elution. Indeed, the relatively higher charge density of this polysaccharide was confirmed by its sulfate content (0.21 mol per mol of deoxyhexose). This purified fucan had negative specific rotation $[\alpha]_D^{32}$ −113° (*c* 0.2, H_2O), revealing that fucose in F3 belongs to the L-series, like in other sulfated fucans from brown seaweeds (Berteau & Mulloy, 2003; Kariya et al., 2004; Patankar, Oehninger, Barnett, Williams, & Clark, 1993). The Fourier transform-IR spectrum of this pure fucoidan (F3) showed an absorption band in the region of 1250–1255 cm^{-1} related to a >S=O stretching vibration of the sulfate group (Lloyd, Dodgson, Price, & Rose, 1961) (Fig. 2). An additional sulfate absorption band at 845–850 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 et al., 1993). Solvolytic desulfation (Falshaw & Furneaux, 1998) of the purified sulfated fucan (F3) (as pyridinium salt) produces a desulfated derivative (named as "F3D"). Preliminary experiments (data not shown) showed a higher recovery with this method compared to methanol–HCl and auto-desulfation methods (Percival & Wold, 1963). Notably the sugar composition of the pure fucoidan (F3) and its desulfated derivative (F3D) were comparable (Table 1). In the IR spectrum of desulfated derivative (F3D) these absorbances disappeared, demonstrating that they were associated with sulfate groups.

3.2. Methylation analysis indicates (1 → 2)- and (1 → 3)-linkages

To investigate the glycosidic linkage positions, we have permethylated the sulfated fucan (F3) and its desulfated derivative (F3D).

Table 1
Neutral sugar composition (mol%) of fractions[a] obtained from *Padina tetrastromatica*.

	PtWE	PtWE-I	F3	F3D
TS[b]	38	39	42	59
UA[b]	14	9	4.5	–
Rhamnose[c]	2	5	Tr	Tr
Fucose[c]	54	59	72	70
Xylose[c]	18	23	25	24
Mannose[c]	9	3	Nd	Nd
Galactose[c]	9	10	3	6
Glucose[c]	9	Tr	Nd	Nd

[a] See text for the identification of fractions.
[b] Percent weight of fraction dry weight.
[c] Percentage mol. –, not determined; TS, Total sugar; UA, Uronic acid; Nd, not detected and Tr, trace.

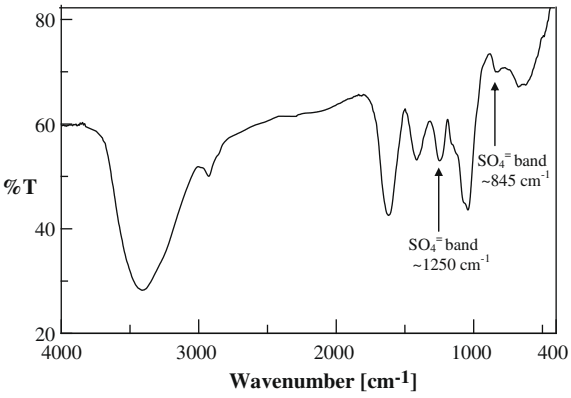

Fig. 2. FT-IR spectrum of the sulfated fucan (F3) of *Padina tetrastromatica*.

Partially methylated alditol acetates (PMAAs) derived from desulfated fucan (F3D) were subjected to GLC–MS analysis for unequivocal structural confirmation (Fig. 3). The identity of each PMAA was confirmed by relative retention times and or mass spectral fragmentation pattern (Table 2). Xylose (10%) and fucose (25%) as terminal units indicate that this sulfated fucan is a highly branched polysaccharide with one terminal for every two residues within the chain. Interestingly, 30–31% of the total fucose residues are (1 → 2)-linked whereas 17–18% are (1 → 3)-linked, the rest being nonreducing terminal (Table 2). So far, fucose residues in algal sulfated fucans are either (1 → 2)-, or (1 → 3)-, or (1 → 3)- and (1 → 4)-linked (Chevelot et al., 1999; Patankar et al., 1993; Percival & McDowell, 1967) (reviewed by Berteau & Mulloy, 2003). Even the heterofucan from *P. gymnospora* contained (1 → 3)- and (1 → 4)-linked fucose residues in addition to the minor amounts of (1 → 4)-linked fucose (Silva et al., 2005). The galactofucan from *Spatoglossum schroederi* contained mostly (1 → 4)-linked fucose residues together with smaller amount of (1 → 3)-linked ones (Rocha et al., 2005). Linkage analysis of the native sulfated fucan (F3) yielded a variety of monomethylated, dimethylated and trimethylated products (Table 2) indicating that the structure of this polymer is highly complex. 2-*O*-methyl, 3-*O*-methyl and unmethylated fucitol were the abundant products of methylation analysis of the native polymer. The increase in the proportions of terminal-, 2,4-di-*O*-methyl and 3,4-di-*O*-methyl fucitol after desulfation, and decrease in the proportions of 2-*O*-methyl, 3-*O*-methyl and nonmethylated fucitol residues, suggests that sulfate esters, when present, are located at position 4 and 2 of the fucosyl residues. Overall, the results of methylation analysis suggest that the said macromolecule of present study possesses a structural motif that was not found in other sulfated fucans.

Table 2
Partially methylated alditol acetates derived from sulfated fucan (F3), desulfated fucan (F3D) and the Smith-degraded material (Sm-F3D) of *Padina tetrastromatica*.

Methylation products[a]	Peak area[b]		
	F3	F3D	Sm-F3D
2,3,4-Xyl	7	10	Tr
3,4-Xyl	7	6	–
2,3-Xyl	–	–	5
Xyl	12	8	–
2,3,4-Fuc	5	25	12
2,4-Fuc	1	12	33
2,3-Fuc	3	3	2
3,4- Fuc	2	21	–
2-Fuc	18	3	7
3-Fuc	24	2	–
4-Fuc	2	2	–
Fuc	13	–	–
2,3,4,6-Gal	–	Tr	10
3,4,6-Gal	–	–	27
4,6-Gal	–	1	2
4-Gal	5	6	2

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

3.3. Smith degradation

Smith degradation of the desulfated fucan (F3D) was used to simplify its structure. The periodate oxidized polymer was reduced with sodium borohydride and subjected to mild acid hydrolysis according to the usual Smith degradation condition. After removal of salts, the Smith-degraded material (Sm-F3D) was analysed by NMR and ESI-MS. We have also carried out the methylation analysis of the Smith-degraded material (Sm-F3D). The decrease in the proportion of xylose and fucose and consequent increase in the proportion of galactose residues after Smith degradation (Table 2) suggest that most of the fucose and xylose residues were degraded during periodate oxidation. So far we have very little information on the branch points, but from the results of the methylation analysis of the Smith degraded material some conclusions may be drawn. For example, appearance of (1 → 2)-linked galactopyranosyl residues in the Smith degraded material suggests that galactoses are branched through position 3 and 6. Similarly, the appearance of (1 → 4)-linked xylopyranosyl residues and consequent decrease in the proportion of 1,2,3,4-linked residues indicates that the (1 → 4)-linked xylopyranosyl residues are probably branched through position 2 and 3.

3.4. NMR spectroscopic analysis

NMR spectroscopy is a convenient method that produces valuable structural information of polysaccharides. The sulfated fucan (F3) of *P. tetrastromatica* has a very complex ^{1}H NMR spectrum (Fig. 4). A number of separate spin systems (5.03–5.63 ppm) attrib-

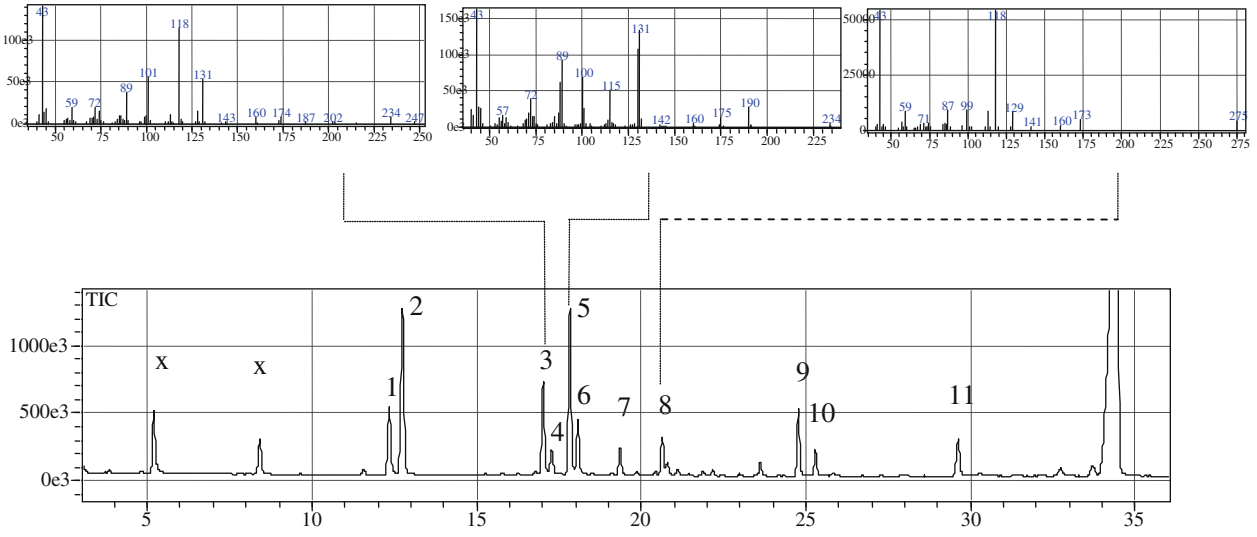

Fig. 3. GLC–MS analysis of the partially methylated alditol acetates (PMAA) derived from the desulfated fucan (F3D) of the brown alga *Padina tetrastromatica*. 1, T-Xyl; 2, T-Fuc; 3, 1,3-Fuc; 4, 1,4-Fuc; 5, 1,2-Fuc; 6, 1,2-Xyl; 7, 1,2,3- & 1,2,4-Fuc; 8, 1,3,4-Fuc; 9, Xyl; 10, 1,2,3-Gal and 11, 1,2,3,6-Gal. x = impurity.

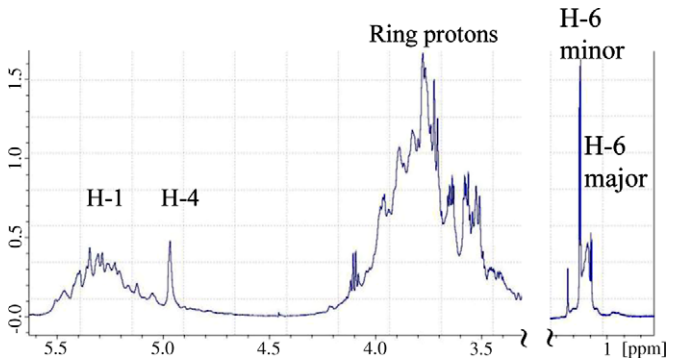

Fig. 4. ^{1}H NMR spectrum at 600 MHz of the sulfated fucan (F3) of *Padina tetrastromatica*. H-1, H-4 and H-6 refer to signals of anomeric, H-4 and methyl protons of fucopyranosyl residues, respectively.

utable to anomeric protons of α-fucose residues were distinguishable in the spectrum of this pure polysaccharide. It also include resonances characteristic of sulfated fucan such as signals from ring protons (H-2–H-5) between 3.41 and 4.91 ppm, and intense signals from the methyl protons H-6, one at about 1.62 ppm (minor) and a major envelope of signals at around 1.51 ppm. The residues with H-6 signals at 1.51 ppm may be (1 → 3)-linked (Kariya et al., 2004) where as signal appearing at 4.91 ppm can be attributed to the H-4 of 4-*O*-sulfated residues (Bilan et al., 2004; Kariya et al., 2004; Mandal et al., 2007; Pereira et al., 1999). This result also confirms the prediction made by IR analysis that sulfate groups are located at position 4 of fucosyl residues. The high proportion of xylose and galactose residues must be responsible for some of the signals in the spectrum, but is not possible to assign any particular signals to these residues. It can be safely said that the ^{1}H NMR spectrum of this novel polysaccharide is complex, overlapping, and inconclusive for structural information as observed for sulfated fucans from other marine brown algae (Mandal et al., 2007; Adhikari et al., 2006; Kariya et al., 2004; Mulloy, Ribeiro, Alves, Vieira, & Mourão, 1994; Pereira et al., 1999). Attempts to record 2D NMR spectra for this macromolecule gave no useful result.

Interestingly, even after Smith degradation at least three prominent signals appeared in the anomeric region of the ^{1}H NMR spectrum of the Sm-F3D, each consistent with α-fucopyranosyl residues.

4. Concluding remarks

The central goal of this study was the structural characterization of sulfated fucan present in brown alga *P. tetrastromatica*. Water extracted fucans was separated into fractions with different sulfate content by anion exchange chromatography. Glycosidic linkage analysis and NMR spectroscopic analysis of the purified fucan (F3) revealed that the sequence of sugar residues and the distribution of sulfate along polymer backbone are extremely heterogeneous. We have found no evidence for a regular repeating structure, although there is evidence for the presence of α(1 → 2)- and α(1 → 3)-linked fucopyranosyl residues with sulfates at position 4 and 2. In addition, this purified fucan contains significant amount of xylose and galactose residues as branch points. To the best of our knowledge, this is a type of polysaccharide that has not been previously described.

To date, it has not been possible to trace a relationship between the structures of various fucans and their biological activities. Most of the difficulties for these studies arise from the fact that many of the hitherto used sulfated fucans contained a number of other polysaccharides. These later macromolecules may have their own biological activities, and or at least dilute the sulfated fucan itself. The purified sulfated fucan fraction described in this work may allow more definitive conclusions to be drawn about the biological activities of fucans in systems with possible therapeutic potential.

Sulfated fucan was of particular interest to us because of its ability to inhibit herpes simplex viruses (Adhikari et al., 2006; Mandal et al., 2007). But, the mechanisms by which this macromolecule exerts its anti-viral activity remain controversial. The novel structural motif of the sulfated fucan of present study, which will add new tool in the hand of researchers, may be of help to explain the structure activity relationship in a better way. Finally, it will be of interest to study the potential of this macromolecule as therapeutic agents against virus and research in this direction is in progress.

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