Structural features and antiviral activity of sulphated fucans from the brown seaweed *Cystoseira indica*

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Natural compounds offer interesting pharmacological perspectives for antiviral drug development. In this study, we have analysed sulphated-fucan-containing fractions isolated from the brown seaweed *Cystoseira indica.* The crude water extract (CiWE) and the main fraction (CiF3) obtained by anion exchange chromatography had potent antiviral activity against herpes simplex virus types 1 (HSV-1) and 2 (HSV-2) without cytotoxicity for Vero cell cultures. Furthermore, they had no direct inactivating effect on virions in a virucidal assay, and lacked anticoagulant activity. The mode of action of these compounds could be mainly ascribed to an inhibitory effect on virus adsorption. Chemical, chromatographic and spectroscopic methods showed that the major polysaccharide had an apparent molecular mass of 35 kDa and contained a backbone of α-(1→3)-linked fucopyranosyl residues substituted at C-2 with fucopyranosyl and xylopyranosyl residues. This sulphated fucan, considered the active principle of the *C. indica* water extract, also contained variously linked xylose and galactose units and glucuronic acid residues. Sulphate groups, if present, are located mostly at C-4 of (1→3)-linked fucopyranosyl units, and appeared to be very important for the anti-herpetic activity of this polymer.

Keywords: antiviral, brown seaweed, *Cystoseira indica*, herpes simplex virus, sulphated fucan

Introduction

Sulphated fucans, polysaccharides containing substantial percentages of L-fucose and sulphated ester groups, are constituents of brown algae and some marine invertebrates (Berteau & Mulloy, 2003; Painter, 1983; Vilela-Silva *et al.,* 2002). They consist of (1→3)- and/or (1→4)-linked fucosyl backbones that are partially substituted at C-2 and/or C-4 with sulphate groups and fucosyl residues (Chevelot *et al.,* 1999; Kariya *et al.,* 2004; Patankar *et al.,* 1993). In some cases, algal sulphated fucans also contain galactose, glucose, mannose and glucuronic acid as minor components (Nishino *et al.,* 1994; Painter, 1983). These complex polysaccharides showed a wide variety of biological activities, such as anti-adhesive (Haroun-Bouhedja *et al.,* 2002; McCormick *et al.,* 2000), anti-proliferative (Patel *et al.,* 2002), anti-coagulant (Chevelot *et al.,* 1999; Pereira *et al.,* 1999), anti-thrombotic (Nishino *et al.,* 1999), and anti-platelet-aggregation properties (Alwayn *et al.,* 2000), among others. In particular, sulphated fucans were found to be inhibitors of human pathogenic enveloped viruses including HIV, herpes simplex virus (HSV) and human cytomegalovirus (HCMV) (Adhikari *et al.,* 2006; Baba *et al.,* 1988; Beress *et al.,* 1993; Feldman *et al.,* 1999; McClure *et al.,* 1991; Ponce *et al.,* 2003; Preeprame *et al.,* 2001).

However, the structural features of sulphated fucans responsible for these biological activities have not been determined. Most of the difficulties for these studies may be related to the reported diverse, complex and heterogeneous structures of this kind of polysaccharides (Berteau & Mulloy, 2003; Painter, 1983; Patankar *et al.,* 1993; Pereira *et al.,* 1999). Occasionally, the presence of a mixture of molecules with different structural types has been reported in fucan sulphates isolated from any brown seaweed (Duarte *et al.,* 2001; Ponce *et al.,* 2003). Additionally, the difficulty in isolating contaminant-free fucans with well defined structures to establish structure–activity relationships has hampered further studies of the biological properties of fucans. For example, in contrast to the well known and thoroughly studied antiviral properties of other types of sulphated seaweed polysaccharides such as galactans, agarans, dextrans and carrageenans (reviewed in Damonte *et al.,* 2004; Witvrouw *et al.,* 1994; Witvrouw & De Clercq, 1997), only the few reports mentioned above describe the virus-inhibitory

activities of fucans. In the present study, the structural characteristics and the anti-herpetic activities of sulphated fucans isolated from the brown seaweed *Cystoseira indica* are analysed.

Materials and methods

Chemistry

Plant material and preliminary treatments. Samples of *C. indica* 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 (Waring Products Inc., Torrington, CT, USA). Algal powder (130 g) was depigmented 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; 91 g).

Extraction and fractionation of sulphated fucans. DAP (10 g) was extracted three times with water (1:100; pH 6.5–7) at 20–25°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 dialysed extensively against water and lyophilized. The recovered material was dissolved in water; the polysaccharides were precipitated twice with ethanol (4 volumes) and then collected by centrifugation. The final pellet was dissolved in water and lyophilized to yield the water-extracted polysaccharide, named CiWE (1.8 g).

A solution (20 ml) of CiWE in 50 mM sodium acetate (pH 5.5) was applied to a column (2.6×25 cm) of DEAE-Sepharose Fast Flow (AcO^-; Amersham Biosciences AB, Uppsala, Sweden). Thereafter, the column was eluted (0.6 ml/min) successively with 0.05 M, 0.2 M (fraction CiF1), 0.7 M (fraction CiF2) and 2 M (fraction CiF3) NaOAc buffer pH 5.5 in a stepwise manner. Residual bound polysaccharides were washed from the column with 0.2 M NaOH (fraction CiF4). Appropriate fractions were pooled, dialysed and lyophilized.

Size exclusion chromatography. Size exclusion chromatography of CiF3 on a Sephacryl S-300 column (90×2.6 cm; Amersham Biosciences AB) using 0.5 M sodium acetate buffer (pH 5.0) as eluant was done as described (Adhikari *et al.,* 2006). The column was calibrated with standard dextrans (500, 70, 40 and 10 kDa).

Chemical analysis. Total sugars and uronic acids were determined by the phenol sulphuric acid (Dubois *et al.,* 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 *et al.,* 1983) and analysed by gas–liquid chromatography (GLC; Shimadzu GC-17A, Shimadzu, Kyoto, Japan). Monosaccharides were identified by thin-layer chromatography and gas–liquid chromatography–mass spectrometry (Shimadzu QP 5050 A, Shimadzu) as described (Mazumder *et al.,* 2005). Alternatively, trimethylsilyl (TMS) derivatives of methyl glycosides were analysed by gas chromatography (York *et al.,* 1985).

Estimation of sulphate by the modified barium chloride method (Craigie *et al.,* 1984) and IR spectrometry (Rochas *et al.,* 1986), and desulphation by solvolytic (Falshaw & Furneaux, 1998), methanol–HCl and auto-hydrolysis (Percival & Wold, 1963) method were carried out as described (Ghosh *et al.,* 2004).

The triethylamine form (Stevenson & Furneaux, 1991) of native and desulphated fucan was subjected to two rounds of methylation (Blakeney & Stone, 1985). Permethylated samples were hydrolysed, converted into their partially methylated alditol acetates and analysed by GLC and GLC/mass spectometry (Shimadzu QP 5050 A, Shimadzu) as described (Ray & Lahaye, 1995).

Spectroscopy. Recording of IR spectra and optical rotation measurements were carried out as described previously (Ray, 2006). The ^{1}H-NMR spectra of the native and desulphated fucan were recorded on a Bruker 600 spectrometer (Bruker Biospin AG, Fallanden, Switzerland) operating at 600 MHz for ^{1}H. The sulphated fucan was converted into its sodium salt by passage through a column (7ml, Bio-Rad, Hercules, CA, USA) of Amberlite IR 120 (H^+), and all samples were deuterium-exchanged by lyophilization with D_2O and then examined as 1% solutions in 99.8% D_2O.

Virology

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%. Human diploid foreskin fibroblast cells were obtained from Dr G Carballal (CEMIC, Buenos Aires, Argentina) and propagated in MEM supplemented with 10% fetal calf serum.

HSV-1 strain F and HSV-2 strain MS were used as reference strains. B2006 and Fields are HSV-1 thymidine-kinase-negative (TK^-) acyclovir-resistant strains obtained from Prof. Dr E De Clercq (Rega Institute, Leuven, Belgium). The syncytial variants of HSV-1 1C3-syn 13-8 and 1C3-syn 14-1 were obtained by serial passage in the presence of the μ/ν-carrageenan 1C3 as previously described

(Carlucci *et al.,* 2002). Virus stocks were propagated and titrated by plaque formation in Vero cells.

Cytotoxicity assay. Cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma-Aldrich, St. Louis, MO, USA) method. 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 50% cytotoxic concentration (CC_{50}) was calculated as the compound concentration required to reduce cell viability by 50%.

Antiviral assays. Antitiviral 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 MM containing 0.7% methylcellulose and 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 twice and each in duplicate.

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 ≥100-fold, to confirm that titre reduction was only due to cell-free virion inactivation. The 50% virucidal concentration (VC_{50}), defined as the concentration required to inactivate virions by 50%, was then calculated.

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 given below.

Adsorption: cells were exposed to HSV-1 in the presence of 10 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 phosphate-buffered saline (PBS) and overlaid with plaquing medium (MM containing 0.7% methylcellulose).

Internalization: cells were infected with HSV-1 in the absence of compound and after 1 h of adsorption at 4°C the cells were washed twice with cold PBS and further incubated for 1 h at 37°C in MM containing 10 μg/ml of the compound. Then, cells were washed with cold PBS, and 0.1 ml of citric buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl, pH 3.0) was added to remove adsorbed, non-internalized virus. After 1 min, cells were washed again with PBS, and incubated with plaquing medium without compound.

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 and the cells were washed twice with cold PBS and further incubated with plaquing medium containing 10 μg/ml of the compounds.

Always: the compound was present during HSV-1 adsorption at 4°C and in the plaquing medium added after adsorption.

For all treatments, virus plaques were counted after 2 days of incubation at 37°C and results were expressed as % inhibition for each treatment with respect to untreated infected cell control.

Assay for anticoagulant activity. Anticoagulant activity of the fucans was determined using the activated partial thromboplastine time (APTT) assay. Briefly, 30 μl of test solutions were added to 100 μl of pooled human plasma and 100 μl of APTT reagent (Wiener lab, Rosario, Argentina). The mixture was incubated for 1 min at 37°C. After the incubation, 70 μl of $CaCl_2$ 0.025 M was added and the time to clot formation was recorded.

Results

Chemical characterization of sulphated fucans from *C. indica*

Isolation and purification. The depigmented algal powder (DAP) from *C. indica*, which contained fucose as dominant monosaccharide (Table 1), was extracted with water and polysaccharides were isolated by repeated precipitation with ethanol. This water-extracted fraction CiWE amounted to 9% of DAP dry weight, with a total sugar content of 36%, of which the major sugar was fucose (Table 1). Like the sulphated fucans of *Fucus vesiculosus* (Nishino *et al.,* 1994) the polysaccharides from *C. indica* contained galactose and xylose, but it did not contain any amino sugars. Thin layer chromatographic analysis of the monosaccharides present in the hydrolysate indicates the presence of, *inter alia*, a 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

Table 1. Sugar composition of fractions obtained from *Cystoseira indica*

Fractions	DAP	CiWE	CiWED	CiF1	CiF2	CiF3	CiF4	CiF3D
Neutral sugar *	23	36	46	21	29	38	–†	49
Glucuronic acid*	3	4	6	–†	–†	2	–†	2
Sulphate*	–	8	Tr	–†	–†	9	–†	Tr
Fucose‡	83	75	76	77	76	84	61	89
Xylose‡	7	14	9	14	15	7	Tr	5
Mannose‡	1	Tr	Tr	Tr	Tr	4	9	3
Galactose‡	8	11	15	8	9	5	30	3
Glucose‡	1	Tr	Nd	1	Tr	Nd	Nd	Nd

For a description of the fractions obtained see 'Materials and methods'. *Percent weight of fraction dry weight. †Not determined. ‡Mol percent of neutral sugars. Nd, not detected; Tr, trace.

result. The presence of uronic acid has already been reported in sulphated fucans of *F. vesiculosus* (Nishino *et al.,* 1994). The uronide content of CiWE was 4% and this fraction also contained sulphate groups (Table 1).

Anion exchange chromatography on a DEAE Sepharose column separated CiWE into four fractions (CiF1, CiF2, CiF3 and CiF4). CiF1, which accounted for 7% of the total polymers recovered from the anion exchanger, was the minor component of CiWE fraction. It consisted mostly of fucose together with smaller amount of galactose, xylose and traces of glucose (Table 1). The sugar composition of CiF2 was similar to CiF1, although its yield (15%) was higher. The difference in elution between CiF1 and CiF2 was probably due to the level of uronic acid and sulphate. CiF3 was the major fraction, amounting to 55% of the total polymers recovered from the column. Fucose accounted for more than 84% of the neutral sugars of CiF3, which also contained 2% (w/w) of uronic acid. Therefore, CiF3 is essentially a fucan that might contain a high number of sulphate groups, as indicated by its late elution. Indeed, the high charge density of this polysaccharide was confirmed by its high sulphate content (9%, w/w). This purified sulphated fucan had negative specific rotation $[\alpha]_D^{32}$ -91° (*c* 0.2, H_2O), revealing that fucose in CiF3 belongs to the L-series, like other sulphated fucans from brown seaweeds (Berteau & Mulloy, 2003; Kariya *et al.,* 2004 Painter, 1983; Patankar *et al.,* 1993).

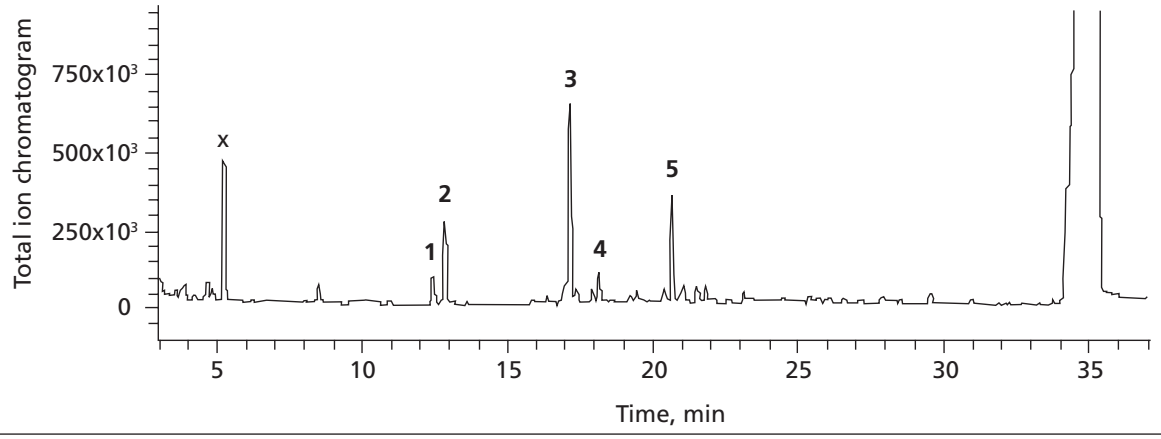
Molecular mass. CiF3 was subjected to further chemical analysis. First, the apparent molecular mass was determined by size exclusion chromatography. The elution profile of this macromolecule on Sephacryl S-300 suggests that the sulphated fucan is homogeneous. Based on calibration with standard dextrans, the apparent molecular weight of CiF3 would be 35 kDa.

Desulphation and glycosidic linkage analysis. To investigate the effect of sulphate group on biological properties of fucan sulphates from *C. indica* and to determine the location of this group, we have desulphated the macromolecule. Both the crude extract CiWE and the purified CiF3 were desulphated by solvolysis in dimethyl sulphoxide (Falshaw & Furneaux, 1998). Preliminary experiments (data not shown) showed that this method gave better recovery compared to methanol–HCl and auto-desulphation method (Percival & Wold, 1963). Desulphation of CiWE and CiF3 fractions had a recovery yield of 45% and 40%, respectively.

The fourier transform-IR spectrum of CiF3 showed an intense absorption band in the region of 1,254 cm^{-1} related to a >S=O stretching vibration of the sulphate group (Lloyd *et al.,* 1961). An additional sulphate absorption band at 848 cm^{-1} (C-O-S, secondary axial sulphate) indicated that the sulphate group is located at position 4 of the fucopyranose residue (Chizhov *et al.,* 1999; Patankar *et al.,* 1993). Desulphation of CiF3 resulted in the disappearance of these absorbances, demonstrating that they were associated with sulphate groups.

Glycosyl linkage analysis of the desulphated fucan sulphate gave, *inter alia*, 2,3,4-tri-*O*-methylfucitol, 2,4-di-*O*-methylfucitol and 4-*O*-methylfucitol residues, suggesting that the fucan sulphate has a (1→3)-linked backbone together with branches at C-2 (Figure 1). The presence of terminal xylopyranosyl residues was also indicated. Linkage analysis of the native fucan sulphate CiF3 yielded a variety of monomethylated, dimethylated and trimethylated products (Table 2) indicating that the structure of this polymer is highly complex. 2-*O*-methyl fucitol, 4-*O*-methyl fucitol and unmethylated fucitol were the abundant products of methylation analysis of the native polymer. The increase in the proportions of 2,4-di-*O*-methyl fucitol after desulphation, as a consequence of decreased proportions of 2-*O*-methyl fucitol residues, suggests that sulphate esters, when present, are located at C-4 of the (1→3)-linked fucosyl residues. This result also confirms the prediction made by IR analysis

Figure 1. Total ion chromatogram of partially methylated alditol acetates (PMAAs) derived from the desulphated fucans (Ci F3D) of the brown alga *Cystoseira indica*



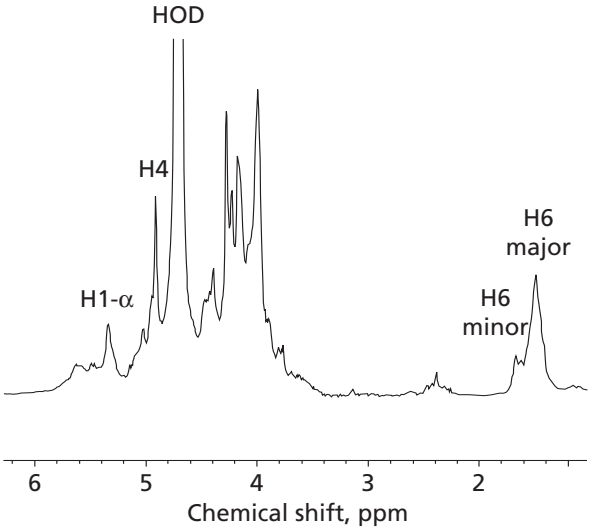
The desulphated fucans were completely methylated and then hydrolysed and reduced, and the alditol acetates were converted into their PMAA as described under 'Materials and methods'. **1:** 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylxylitol; **2:** 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylfucitol; **3:** 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methylfucitol; **4:**1,3,5-tri-*O*-acetyl-2,4-di-*O*-methylfucitol; **5:**1,2,3,5-tetra-*O*-acetyl-4-*O*-methylfucitol. X, impurity.

Table 2. Partially methylated alditol acetates derived from sulphated fucan (CiF3) of *Cystoseira indica* and its desulphated derivative (CiF3D)

Methylation products	Peak area*	
	CiF3	CiF3D
2,3,4-Fuc†	7	19
3,4-Fuc	6	Nd
2,3-Fuc	5	5
2,4-Fuc	8	47
4-Fuc	16	25
3-Fuc	7	Nd
2-Fuc	29	Nd
Fuc	11	Nd
2,3,4-Xyl	4	4
2,4,6-Gal	5	Tr

*Percentage of total area of the identified peaks. †2,3,4-Fuc denotes 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylfucitol; 2,3,4-Xyl, 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylxylitol; 2,4,6-Gal, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl galactitol; etc. Nd, not detected; Tr, trace.

Figure 2. ^{1}H NMR spectrum at 600 MHz of the sulphated fucan (CiF3) of *Cystoseira indica*



The spectrum was recorded at 60°C for sample in D_2O solution. Chemical shifts are relative to external trimethylsilylpropionic acid at 0 ppm. H1, H4 and H6 refer to signals of anomeric protons, H-4 of 4-*O*-sulphated residues and methyl protons of fucose residues, respectively. The signal for the residual water was designated as HOD.

that sulphate groups are located at C-4 of fucosyl residues. Small amounts of (1→4)-linked fucopyranosyl residues was also present.

NMR spectroscopy. NMR spectroscopy is a convenient method to obtain valuable structural information about polysaccharides. We employed NMR analysis to determine the anomeric configuration and the sulphation pattern of CiF3. The native polysaccharide has a very complex ^{1}H NMR spectrum (Figure 2) because of its heterogeneous sulphation pattern. A number of separate spin systems (5.03–5.63 ppm) attributable to anomeric protons of α-fucose residues were distinguishable in the spectrum of the pure sulphated fucan. It also included resonances characteristic of sulphated α-fucans, such as signals from ring protons (H-2 to H-5) between 3.4 and 4.9 ppm, and

Table 3. Antiviral and cytotoxic activities of sulphated fucans from *Cystoseira indica*

Compound	CC_{50}†, μg/ml	IC_{50}*, μg/ml		SI (CC_{50}/IC_{50})	
		HSV-1	HSV-2	HSV-1	HSV-2
CiWE	>1,000	2.1 ±0.9	0.50 ±0.03	>476	>2,000
CiWED	>1,000	34.7 ±4.4	19.5 ±0.4	>29	>51
CiF3	>1,000	2.8 ±0.8	1.3	>357	>769
CiF3D	>1,000	>100	ND	Inactive	ND
DS8000	>1,000	2.1 ±0.4	0.57 ±0.01	>476	>1,754
Heparin	>1,000	1.2 ±0.5	0.5 ±0.1	>833	>2,000

*Inhibitory concentration 50% (IC_{50}), defined as compound concentration required to reduce virus plaques by 50%. Values are the mean from duplicate independent tests ±SD. †Cytotoxic concentration (CC_{50}), defined as compound concentration required to reduce cell viability by 50%, determined by MTT method. Values are the mean from duplicate independent tests ±SD. ND, not determined; SI, selectivity index.

intense signals from the methyl protons H-6, one at ~1.63–1.67 ppm (minor) and a major envelope of signals at ~1.49 ppm. The residues with H-6 signals at 1.49 ppm may be 3-linked (Kariya *et al.,* 2004). The signal appearing at 4.91 ppm, attributed to the H-4 of 4-*O*-sulphated residues (Bilan *et al.,* 2004; Kariya *et al.,* 2004; Pereira *et al.,* 1999), confirmed the results of methylation analysis. Interestingly, even after desulphation at least five prominent signals appeared in the anomeric region of the ^{1}H-NMR spectrum of the desulphated fucan (CiF3D), each consistent with α-fucosyl residues. Therefore, it is apparent that the NMR spectrum of this polysaccharide is complex, as observed for sulphated fucans from other marine brown algae (Kariya *et al.,* 2004; Mourao *et al.,* 1996; Patankar *et al.,* 1993; Pereira *et al.,* 1999; Vilela-Silva *et al.,* 2002).

Antiviral activities of the sulphated fucans from *C. indica*

The crude water extract from *C. indica*, CiWE, and the major purified fucan sulphate CiF3 were initially evaluated for cytotoxicity by assessing their effects on Vero cell viability. For comparative purposes, dextran sulphate and heparin were simultaneously assayed as known reference polysaccharides. No effect on cell viability was observed with any of these compounds at concentrations up to 1,000 μg/ml (Table 3). Additionally, cultures of human diploid foreskin fibroblasts were assayed for cytotoxicity and no morphological alterations nor reduction in cell viability were detected after 48 h of treatment with 1,000 μg/ml of CiWE and CiF3.

Thereafter, the polysaccharides were screened for antiviral activity against two reference strains of HSV, HSV-1 strain F and HSV-2 strain MS, by a virus plaque reduction assay on Vero cells. As shown in Table 3 both fucan samples CiWE and CiF3 exhibited potent *in vitro* antiherpetic activity with IC_{50} values in the range 0.50–2.8 μg/ml. The antiviral effectiveness of the fucans was similar to that shown by heparin and dextran sulphate. Given the lack of cytotoxicity exhibited by the fucans, the selectivity indices (ratio CC_{50}/IC_{50}) was >350–2,000 for the crude and pure preparations, indicating the specificity of the inhibitory effect of this type of polysaccharides against herpes viruses.

Table 4. Spectrum of antiherpetic activity of sulphated fucans from *Cystoseira indica*

HSV-1 strain	IC_{50}*, μg/ml		
	CiWE	DS8000	Heparin
F	2.1 ±0.9	2.1 ±0.4	1.2 ±0.5
TK$^-$ B2006	1.9 ±0.1	2.5 ±0.1	4.3 ±1.0
TK$^-$ Field	2.0 ±0.4	2.2 ±0.7	4.1 ±1.3
1C3-syn 13-8	>40	3.6 ±1.8	7.4 ±0.5
1C3-syn 14-1	>40	10.9 ±3.8	9.1 ±2.8

*Inhibitory concentration 50% (IC_{50}), defined as compound concentration required to reduce virus plaques by 50%. Values are the mean from duplicate independent tests ±SD. SDS8000, dextran sulphate 8000; HSV, herpes simplex virus

The antiviral activity of diverse classes of polysaccharides has been reported to be linked to the anionic features of the macromolecules (Damonte *et al.,* 2004; Witvrouw & De Clercq, 1997). To investigate the effect of sulphate group on the biological properties of the fucan sulphates, we evaluated the cytotoxicity and antiviral activity of the respective desulphated samples CiWED and CiF3D in comparison with the original ones. Whereas the lack of toxicity remained constant, the antiviral activity was reduced drastically after the desulphation process (IC_{50} values from 19.5 to >100 μg/ml).

To determine the spectrum of antiherpetic activity of the sulphated fucans, CiWE was evaluated against other strains of HSV-1. CiWE was an effective inhibitor of two acyclovir-resistant TK$^-$ strains of HSV-1, with IC_{50} values in the range 1.9–2.5 μg/ml, similar to those obtained against the reference strains (Tables 3 and 4). On the other hand, CiWE was no longer active against the syncytial strains $1C_3$-syn 13-8 and $1C_3$-syn 14-1 up to the highest

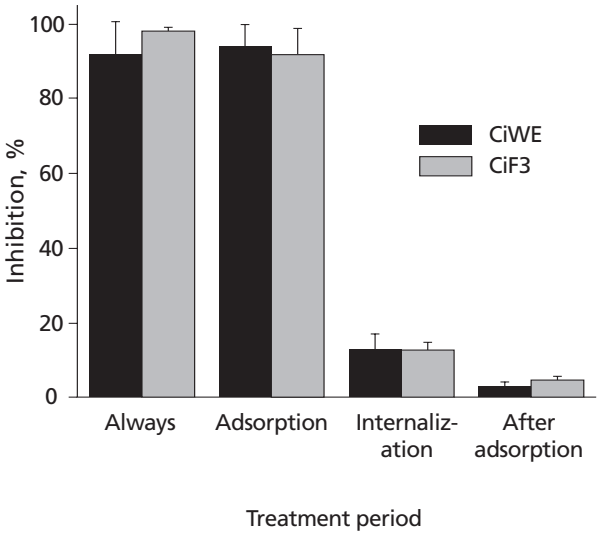
concentration evaluated (40 μg/ml). For comparative purposes, commercial samples of dextran sulphate 8000 and heparin were simultaneously evaluated against these HSV-1 strains. In general, as seen in Tables 3 and 4, the fucans obtained from *C. indica* had activity similar to heparin and dextran sulphate 8000, with the exception of the syncytial strains. The spectrum of antiherpetic activity shown by CiF3 was similar to CiWE (data not shown).

In order to analyse the possibility that these polysaccharides act directly on the virus particle, leading to infectivity inactivation, a virucidal assay against HSV-1 strain F virions was carried out. CiWE and CiF3 were unable to inactivate HSV-1 virions at the maximum concentration tested (40 μg/ml). This concentration is far from the antiviral IC_{50}, indicating that the inhibitory effect detected by the plaque reduction assay was indeed due to interference with some step of the HSV-1 multiplication cycle.

As an initial approach to understanding the mode of action of these polysaccharides against herpes viruses, a virus plaque reduction assay was performed under different treatment conditions. The compound was exposed to the cells in one of the following periods: during the virus adsorption period only; during the virus internalization period only; after virus adsorption only; or both during adsorption and throughout the incubation period after adsorption. As can be concluded from the data presented in Figure 3, both CiWE and CiF3 lost virtually all significant antiviral activity when not present during the virus adsorption period. The presence of the polysaccharides only during the virus adsorption was as effective as their presence throughout the whole incubation period. Furthermore, a lack of inhibitory action of the compounds in the internalization process, immediately after adsorption, was also demonstrated.

Furthermore no anticoagulant action was observed with CiWE and CiF3 at concentrations that highly exceeded the IC_{50} (data not shown).

Figure 3. Influence of various treatment periods on the anti-herpesvirus (HSV)-1 activity of CiWE and CiF3



Vero cells were infected with 50 plaque-forming units of HSV-1 under different treatment conditions with 10 μg/ml of CiWE or CiF3: the compounds present only during virus adsorption at 4°C (adsorption), only during virus internalization at 37°C (internalization), only after adsorption in the plaquing medium (after adsorption) or during and after adsorption (always). After 2 days of incubation at 37°C, plaques were counted and % inhibition for each treatment with respect to untreated infected cultures was determined. Each value represents the mean of duplicate assays.

Discussion

In this study, the active anti-HSV principle was isolated from the brown seaweed *C. indica* by extraction with water. The major constituent of *C. indica* water extract CiWE is a sulphated fucan with complex structure and a molecular mass of 35 kDa named CiF3. Given the results obtained in biological assays with CiWE and CiF3, the latter fucan can be considered as the active principle mainly responsible for the antiviral activity. Recent studies showed that sulphated fucans from common Fucales possesses large proportions of both (1→3)- and (1→4)-glycosidic linkages (Berteau & Mulloy, 2003; Bilan *et al.,* 2006; Chevelot *et al.,* 1999; Daniel *et al.,* 2001). A repeating structure of alternating (1→3)- and (1→4)-glycosidic bonds was determined for oligosaccharides prepared from sulphated fucans of *Ascophyllum nodosum* (Chevelot *et al.,* 2001). The polysaccharide of the present study, on the other hand, has a backbone of α-(1→3)-linked L-fucopyranosyl residues. In contrast to sulphated fucans from other brown seaweed such as *Chorda filum* and *F. vesiculosus*, which contain (1→3)-linked fucosyl backbones partially substituted with fucosyl residues (Berteau & Mulloy, 2003; Chizhov *et al.,* 1999; Patankar *et al.,* 1993), the backbone of this polysaccharide is substituted with both fucopyranosyl and xylopyranosyl residues. Moreover, it is highly branched, an average of 24 branching points being present in every hundred fucosyl residues. Therefore, the structure of the polysaccharide described in the present paper is different from these known sulphated fucans.

The anti-HSV activity of these sulphated fucans might be mainly attributed to their polyanionic character. Polysaccharides are known to affect the growth of animal viruses (Baba *et al.,* 1988; Franz *et al.,* 2000; McClure *et al.,* 1991; Rechter *et al.,* 2006). In particular, anionic polysaccharides, such as carrageenans, dextran sulphate, pentosan sulphate, fucoidan and galactans, are potent inhibitors of herpes virus binding to host cells (Damonte *et al.,* 2004; Gunay & Linhardt, 1999; Witvrouw & De Clercq, 1997). These polysaccharides are competitors of receptors to viral glycoproteins. Herold *et al.* (1996) showed that N-sulphation

and the presence of carboxyl groups on heparin are key determinants for HSV interactions with host cells, since N-desulphation and carboxyl reduction abolishes heparin's antiviral activity. Hence, our findings are consistent with the literature. The polysaccharides from *C. indica* drastically lost activity when they were desulphated. Furthermore, CiWE and CiF3 exhibited a potent inhibitory effect on HSV attachment, lacking any significant antiviral action when added after adsorption.

Because of poor oral absorption, sulphated polysaccharides failed to reach clinical trials as an oral drug (Lorentsen *et al.,* 1989; Hartman *et al.,* 1990). However, earlier studies demonstrated that sulphated polysaccharides can be used as a vaginal antiviral formulation without disturbing the function of the vaginal epithelial cells and normal bacterial flora (Pearce-Pratt & Phillips 1993; Stafford *et al.,* 1997). The polysaccharides of the present study exhibited potent inhibitory action against reference strains of HSV-1 and HSV-2 as well as against TK^- acyclovir-resistant strains. The extent of inhibition produced by the natural fucans was similar to that shown by known antiherpetic polysulphates such as heparin and dextran sulphate, but the former polymers, at concentrations much higher than IC_{50}, possesses no detectable anticoagulant activity. In addition, the inhibition of *in vitro* HSV replication was observed at fucan concentrations which do not have any effect on the cell viability. Therefore, the fucans of the present study could be considered as candidates for anti-HSV formulation. The duration and contact between virus and drug is short upon topical application and this may prevent the emergence of drug-resistant HSV.

Notably, among all the evaluated HSV-1 strains, only the two syncytial variants 1C3-syn 13-8 and 1C3-syn 14-1 were not inhibited by the fucans (Table 4). These two viral variants were derived by prolonged passage in presence of 1C3, a natural μ/ν-carrageenan from the seaweed *Gigartina skottsbergii*. The different resistance profile shown by these two variants against the fucans compared with heparin and dextran sulphate is perhaps attributable to more structural similarities between the carrageenan and the fucan compared with heparin and dextran.

Given the interesting chemical characteristics of the sulphated fucans from *C. indica* and the promising *in vitro* antiherpetic properties reported here, these compounds represent good candidates for further antiviral research.

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