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ORIGINAL ARTICLE

Structure and antiviral activity of the galactofucan sulfates extracted from *Undaria pinnatifida* (Phaeophyta)

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Abstract The galactofucan sulfate extract (GFS) obtained from the brown seaweed *Undaria pinnatifida* by extraction with dilute acid is a potent inhibitor of the herpes viruses HSV-1, HSV-2 and HCMV, with IC_{50} values determined *in vitro* of 1.1, 0.2 and 0.5 μgmL^{-1}, respectively. Fractionation of GFS by anion exchange chromatography gave three fractions which differed in their uronic acid and sulfate contents and in their antiviral activity, as well as in having somewhat reduced molecular weights compared to GFS. The low uronic acid/high sulfate fraction (F2M), obtained in 63% yield, had similar molar proportions of galactopyranosyl and fucopyranosyl residues, little associated protein and was equipotent with GFS (IC_{50} values of 1.1, 0.1 and 0.5 μgmL^{-1}, respectively). The high uronic acid/low sulfate fraction (F1M), obtained in 18% yield, had a much lower proportion of galactopyranosyl residues and was less active (IC_{50} values of 4.6, 1.0 and 4.0 μgmL^{-1}, respectively). The minor low uronic acid/high sulfate fraction (F4M) had a significant amount of associated protein and was also less active (IC_{50} = 3.1, 1.0 and 2.0 μgmL^{-1}, respectively). The structure of the major fraction (F2M) was shown to be complex by glycosyl linkage analysis before and after solvolytic desulfation, with many component sugar residues being identified, although 3-linked fucopyranosyl 2,4-disulfate residues were a prominent feature.

Keywords Anti-herpes · Brown alga · Galactofucans · Structure

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Introduction

The brown alga *Undaria pinnatifida* (Wakame), both wild and cultured, has a long history of food use in Japan. This species is slowly spreading internationally and is now established along some regions of the coastlines of Australia and New Zealand. Marine algae contain sulfated polysaccharides. The potential of such polysaccharides as antiviral drugs was reviewed by Witvrouw and De Clercq (1997). Subsequently, there have been a number of studies on the sulfated fucans (fucoidans) from brown algae, which report a whole spectrum of biological activities and these have been outlined in a recent review by Berteau and Mulloy (2003). The structure of the sulfated fucans is also complex (Berteau & Mulloy, 2003). Unlike the majority of red algal galactans, even the linkage pattern in the sugar backbone is variable. Fucans from species in some orders (eg Laminariales) may have a predominantly (1→3)-linked backbone of α-L-fucopyranosyl residues, while fucans from species in other orders (eg Fucales) may have a backbone of alternating

(1→3)- and (1→4)-α-linked residues (Bilan et al., 2002, 2004). Substitution with galactopyranosyl residues can be significant (Majczak et al., 2003; Mori et al., 1982) and acetyl groups may be present (Chizhov et al., 1999). Fractionation of extracts has yielded fractions differing in uronic acid and sulfate ester content, while glycosyl linkage/substitution analysis has shown the presence of a variety of substitution patterns (Duarte et al., 2001; Ponce et al., 2003). The more biologically active fractions are generally the ones with higher levels of sulfate ester substituents and lower levels of uronic acid substituents (Majczak et al., 2003; Mori et al., 1982; Nishino et al., 1994; Ponce et al., 2003).

The extraction of sulfated fucans (galactofucan sulfates) from the sporophyll of *Undaria pinnatifida* was reported by Mori et al. (1982). Fractionation of the crude extract yielded fractions differing in uronic acid and sulfate ester content and in anticoagulant and lipoprotein clearance activity. More recently, Katsube et al. (2003) reported isolation and purification of a hyaluronidase-inhibiting galactofucan sulfate from the sporophyll of *U. pinnatifida*, Lee et al. (2004) described the characterisation and antiviral activity of a highly purified, low molecular weight galactofucan sulfate, also derived from the sporophyll and Thompson and Drager (2004) described the antiviral activity of an extract from blade plus sporophyll. In this paper we describe extraction and fractionation of galactofucan sulfates from *U. pinnatifida* (blade plus sporophyll), characterisation of the fractions and determination of their antiviral activities against herpes viruses HSV-1, HSV-2 and HCMV.

Materials and methods

Undaria pinnatifida (Harvey) Suringar was collected from the east coast of Tasmania, Australia, during September 2000 to January 2001. Collections were dried within a day of harvest and combined throughout the period.

Extraction of galactofucan sulfates

Air-dried blades and sporophylls (70:30 w/w) were ground and extracted twice at ambient temperature for 6 h with 1% (w/w) H_2SO_4 (20 g alga L^{-1}). The filtered extracts were combined and neutralised with aqueous NaOH (10%). Salts and small molecules were removed by dialysis (MWCO ca. 14 kDa) or ultrafiltration and the galactofucan sulfate extract (GFS) was isolated by lyophilisation in 4.5% yield.

Fractionation of galactofucan sulfate extract (GFS)

Extract (185 mg) was dissolved in distilled water (8 mL) and applied to a DEAE Sepharose CL-6B column (3.7 cm i.d. × 14 cm) equilibrated with distilled water. The column was eluted, first with distilled water (430 mL) and then stepwise with 1 M (390 mL), 2 M (365 mL) and 4 M (285 mL) NaCl at a flow rate of 0.35 mL min^{-1}. Fractions (7.5 mL) were collected and polysaccharide was detected by the phenol-H_2SO_4 method (Dubois et al., 1956). Each salt concentration eluted a polysaccharide fraction which was isolated by dialysis and lyophilisation (F1M 33.7 mg, F2M 116.6 mg and F4M 6.7 mg). The total yield of recovered polysaccharide was 157.0 mg (85%).

Cellulose acetate electrophoresis (CAE)

The method was adapted from that described by Stanley et al., (1983). Samples (10 mg mL^{-1}, 8 μL) were applied to cellulose acetate strips (Sepraphor III) and electrophoresis was conducted in a Gelman semi-micro bath containing $ZnSO_4$ buffer (0.2 M, pH 5.1) for 60 min at 6 mA, 100 V. Strips were stained with 1% Alcian Blue and destained with 5% aq. HOAc containing 10% EtOH. Hyaluronic acid and chondroitin sulfate were used as standard charged polysaccharides.

Sulfate and nitrogen content

Elemental analyses (CHNS) were conducted by the Campbell Microanalytical Laboratory, University of Otago, Dunedin, New Zealand. The number of sulfate groups per sugar residue (DS) was estimated assuming all sulfur to be sulfate and all carbon to be in 6-carbon sugar residues. The protein content was estimated by multiplying %N by 6.25.

Uronic acid content

The uronic acid content was determined by the colorimetric method using 3,5-dimethylphenol described by Usov et al. (1995) with glucuronic acid as the standard.

Constituent sugar analysis

Neutral sugar analysis was conducted by the method of Stevenson and Furneaux (1991), involving reductive hydrolysis of the polysaccharide, followed by acetylation of the hydrolysate and analysis of the resulting alditol acetate derivatives by gas chromatography.

Methylation analysis

The four samples undergoing glycosyl linkage analysis (F1M, F2M and desulfated F1M and F2M, see below) were converted to the triethylammonium salt form. None of these samples had good solubility in Me_2SO, so they were twice subjected to methylation ($MeI/Me_2SO^-K^+$) using the method of Stevenson and Furneaux (1991). Each time they were isolated by dialysis (MWCO 1000) 1× versus distilled water, 2× versus triethylammonium hydrochloride (ca. 0.1 M, pH 7), 2× versus distilled water and then lyophilisation. Reductive hydrolysis and acetylation was followed by GCMS analysis of the resulting partially methylated alditol acetate derivatives. GCMS was conducted using an Agilent 6890N gas chromatograph with a 5973 mass selective detector operated in EI mode, helium carrier gas at 12 psi and splitless injection. After a 1 minute hold at 120 °C and a rapid ramp to 130 °C, the HP5MS (30 m × 0.25 mm i.d., 0.25 μm film) column was ramped from 130 °C to 230 °C at 3 °C min^{-1}.

Solvolytic desulfation

Desulfation prior to methylation analysis was conducted by converting samples of F1M and F2M (10 mg) to the pyridinium salt form and desulfating with Me_2SO-MeOH-pyridine (89:10:1 v/v) (Falshaw and Furneaux, 1994). The desulfation of both samples was repeated but F1M did not give a clear solution during either desulfation procedure. The desulfated samples were recovered in the triethylammonium salt form (9.5 mg and 6.5 mg, respectively) for methylation analysis as described above.

Molecular weight

Weight average molecular weight values (Mw) were determined by high performance size-exclusion chromatography coupled with multi-angle laser light scattering (SEC-MALLS). The SEC-MALLS system consisted of a Waters 2690 Alliance separation module, a Waters 490E programmable multi-wavelength detector set at 280 nm, a DAWN-EOS multi-angle laser light scattering detector with a laser at 690 nm (Wyatt Technology Corp., Santa Barbara, CA) and a Waters 2410 refractive index monitor. Samples (2 mg mL^{-1} in 0.1 M $LiNO_3$) were hydrated for 24 h at ambient temperature and centrifuged (16100 × g, 10 min) before injection (100 μL). They were eluted with 0.1 M $LiNO_3$ containing 0.02% NaN_3 (0.7 mL min^{-1}) from two columns (TSK-Gel G5000PWXL and G4000PWXL, 300 × 7.8 mm, Tosoh Corp., Tokyo, Japan) connected in series and operating at 60°C. Data for molecular weight determination were analysed using ASTRA software (Version 4.73.04, Wyatt Technology Corp.) and a *dn/dc* of 0.145 mL g^{-1}.

Antiviral activity

HSV-1 and HSV-2

Human fibroblast (HF) cells were grown in MEM supplemented with glutamine, antibiotics and 10% foetal bovine serum (FBS). Maintenance medium (MEM below) contained 1% FBS. Laboratory strains F of HSV-1 and G of HSV-2 (American Type Culture Collection, Rockville, U.S.A.) were used. These strains are susceptible to acyclovir (ACV). The test materials were weighed, dissolved in distilled water and filtered (0.45 μm).

HF cells were inoculated into 96-well microtiter trays (7×10^3 cells per well) and the plates were incubated at 35 °C in 5% CO_2 until the cells were confluent. Four uninfected cell control wells, four virus-infected control wells and eight serial two-fold dilutions of each test material in MEM in quadruplicate rows were used for each virus. After removal of the growth medium, MEM was added to the cell control wells (100 μL) and the virus-infected control wells (50 μL). The test solutions (50 μL) were added to the remaining 32 wells. Dilutions of the virus were prepared in MEM for the inoculum [multiplicity of infection (MOI) = 0.05] and aliquots (50 μL) were added to all except the cell control wells. All plates were incubated at 35°C in 5% CO_2 for 48 h. The plates were then examined microscopically, the cytopathic effect (CPE) was scored and the cells were fixed. Following fixation, each well was washed four times with 300 μL of wash solution (PBS containing 0.2% BSA and 0.05%

Table 1 Properties of galactofucan sulfate extract and its fractions

	Yield (wt%)	Uronic acid (wt%)	Sulfate (DS)[1]	Mw[2] (kDa)	Nitrogen (as% protein)
GFS	-	3.4	0.75	710	1.2
F1M	18.2	10.1	0.41	150	0.9
F2M	63.0	1.0	0.94	290	<0.6
F4M	3.6	1.0	1.32	nd[3]	9.0
Total	84.8				

[1]Sulfate ester groups per sugar residue
[2]Weight average molecular weight
[3]Not determined

Tween 20). The antibodies used in the *in situ* enzyme-linked immunosorbent (ELISA) assay were obtained from Dako Corporation, Carpinteria, CA. They were prepared by immunising rabbits with an antigen prepared by sonication and extraction of HSV-1 or HSV-2 infected rabbit cornea cells and then conjugated to horseradish peroxidase. To determine the inhibitory concentration (IC_{50}), the enzyme-linked rabbit polyclonal antibody to HSV-1 or HSV-2 was diluted, added to each well (100 μL) and incubated at 35 °C for 2 h. After removal of the antibody solution, the wells were washed four times as described above. The enzyme substrate 3,3′,5,5′-tetramethylbenzidine (TMB, Sigma, St. Louis, MO) was then added to each well and the plates were incubated at room temperature for 3–4 min. The O.D. of each well was read in a dual wavelength ELISA plate reader at 630–450 nm and averaged for each quadruplicate set. The IC_{50} value corresponds to a 50% reduction in the O.D of the coloured product.

HCMV

HF cells were inoculated into 24-well culture plates (1×10^5 cells per well) and the plates were incubated at 35 °C in 5% CO_2 until the cells were confluent. Each test material was prepared in five dilutions (4.0 → 0.25 μgmL^{-1}) in MEM. The growth medium was removed from the HF cells and MEM was added to the control wells and dilutions of each test material to the remaining wells in duplicate. A dilution of HCMV strain AD 169 was added to each well at a concentration that would result in ca. 50 plaques per well. The plates were incubated at 35°C for 2 h. The inoculum was removed and an agarose overlay was added to the wells. The control overlay contained MEM in agarose. The test wells contained test material in MEM and agarose. The plaques were counted 14 days post inoculation. The IC_{50} value corresponds to the lowest concentration reducing the plaques by 50% or more.

Results

Structural properties of GFS and its fractions

GFS from *Undaria pinnatifida* was fractionated by anion exchange chromatography on Sepharose CL-6B eluting with 1 M, 2 M and 4 M NaCl to give three fractions (F1M, F2M and F4M) in 18.2%, 63.0% and 3.6% yield, respectively (Table 1). The two major fractions differed markedly in their uronic acid and sulfate contents. The fraction eluted with 1 M NaCl (F1M) had a high uronic acid/low sulfate content, while the larger fraction eluted with 2 M NaCl (F2M) had a low uronic acid/high sulfate content. The minor fraction eluted with 4 M NaCl (F4M) had a low uronic acid content, a higher sulfate content than the 2 M fraction and also a much higher nitrogen content (9.0% cf < 0.6%). The nitrogen content of F4M was also much higher than those of F1M and GFS (both ca. 1%).

The weight average molecular weights of fractions F1M and F2M (150 and 290 kDa, respectively, Table 1), were less than half that of GFS (710 kDa), presumably due to depolymerisation during the fractionation and recovery process. GFS, after purification by dissolution at 33 mg mL^{-1} and centrifugation of the solution to remove insoluble material (3%), had half the Mw of the original material (340 cf 710 kDa), indicating both removal of high Mw material and slight depolymerisation.

Cellulose acetate electrophoresis of GFS (Fig. 1) showed three mobile bands, the one having the highest mobility (highest charge /mass ratio) being the most intense. Two diffuse bands of weak intensity followed.

Fig. 1 Cellulose acetate electrophoresis of F1M (top), F2M (centre) and GFS (bottom)

A significant amount of non-mobile material was also present. Fraction F2M gave one intense band running slightly ahead of the intense band of GFS, while fraction F1M showed two intense bands running in the same positions as the diffuse second and third bands of GFS (Figure 1). The fastest band in GFS ran slightly ahead of the chondroitin sulfate standard and twice as fast as the hyaluronic acid standard (not shown). Only traces of the non-mobile material in GFS were present in the fractions. These results are consistent with F2M being more highly sulfated than F1M, which appears to contain at least two components differing in their charge/mass ratios.

Constituent sugar analysis, which does not detect uronic acid components, showed that fucopyranosyl (Fuc) and galactopyranosyl (Gal) were the predominant units in GFS (Fuc/Gal 1.5, Table 2). Small amounts of rhamnopyranosyl, xylopyranosyl, mannopyranosyl and glucopyranosyl units were also detected. F2M had fewer fucopyranosyl units and more galactopyranosyl units than GFS (Fuc/Gal 1.2), while F1M had a markedly higher Fuc/Gal ratio (5.3). Of the minor sugar units, F2M contained only rhamnopyranosyl, while F1M was enriched in all of them.

Table 2 Constituent sugar composition (normalised mol%) of galactofucan sulfate extract and fractions F1M and F2M

	Fuc	Rha	Xyl	Man	Gal	Glc	Fuc/Gal
GFS[1]	57	1	1	2	38	1	1.5
F1M	74	2	3	5	14	2	5.3
F2M	54	1	0	0	45	0	1.2

[1]F4M not determined

Methylation analysis of fractions F1M and F2M (Table 3) showed that both the fucopyranosyl and galactopyranosyl sugar units had complex linkage/substitution patterns. No fucofuranosyl units were detected, hence the pyranosyl terminology is used throughout this paper. [Small amounts have been found in fucans from some species, but none in fucans from others (Ponce et al., 2003)]. The analysis does not differentiate between glycosyl linkages and sulfate ester substitution, so the linkage/substitution patterns are referred to simply as "substitution patterns".

In the high uronic acid/low sulfate fraction (F1M), the most abundant fucopyranosyl units were substituted at the 3-; 2,3-; or 2,3,4-positions and smaller amounts were substituted at the 3,4- or 4-positions (Table 3). The substitution patterns of the galactopyranosyl units were also complex, with substitution at the 3- or 3,4-positions predominant. Unexpectedly, methylation analysis of desulfated F1M gave very similar results to those given by the original material, indicating only partial desulfation, despite two desulfation and two methylation treatments. This fraction will be re-examined after further fractionation, desulfation and carboxyl reduction, and reported elsewhere.

In the low uronic acid/high sulfate fraction (F2M), the most abundant fucopyranosyl units were substituted at the 2,3,4- or 3,4-positions and smaller amounts were substituted at the 2,3-; 3- or 4-positions. The substitution patterns of the galactopyranosyl units were again complex, with substitution at the 3-; 3,6- or 3,4-positions predominant. As expected, methylation analysis of desulfated F2M gave markedly different

Table 3 Methylation analysis data for fractions F1M and F2M (normalised mol%)

Position of methylation of residue	Deduced substitution of residue	F1M	DeS[1] F1M	F2M	DeS[1] F2M
Fucosyl	Fucosyl				
2, 3, 4	T	0	0	0	0
2, 3	4	1	2	5	3
2, 4	3	24	29	7	37
2	3, 4	11	16	21	23
4	2, 3	22	17	10	3
3	2, 4	0	0	0	0
–	2, 3, 4	20	14	24	Tr
Total Fuc		78	78	67	66
Galactosyl	Galactosyl				
2, 3, 4, 6	T	0	0	0	1
2, 4, 6	3	5	6	13	14
2, 3, 6	4	0	0	0	10
2, 3, 4	6	0	0	0	3
2, 6	3, 4	5	4	7	1
2, 3	4, 6	0	0	1	Tr
2, 4	3, 6	1	1	8	1
2	3, 4, 6	0	0	1	0
–	2, 3, 4, 6	0	1	0	0
Total Gal		11	12	30	30
Misc.[2]		11	10	3	4

[1]Desulfated
[2]Miscellaneous residues, primarily xylosyl and mannosyl

substitution patterns for both the fucopyranosyl and galactopyranosyl sugar units (Table 3). Compared to the original sample, there was a complete absence of 2,3,4-trisubstituted fucopyranosyl units, as well as a significant reduction in 2,3-disubstituted fucopyranosyl units. This coincided with a similar increase in 3-substituted fucopyranosyl units, which is consistent with the most abundant units in the original sample (F2M) being 3-linked fucopyranosyl 2,4-disulfate units and with a smaller number being 3-linked fucopyranosyl 2-sulfate units. In contrast, desulfation had little effect on the number of 3,4-disubstituted fucopyranosyl units, which were the second most abundant in F2M. These units could be branch points, but the absence of terminal units does not support this assignment.

The proportion of the predominant 3-substituted galactopyranosyl units was almost the same in the desulfated material as in the original sample, indicating the presence of unsulfated 3-linked galactopyranosyl units in the original material. However, a significant amount of 4-substituted galactopyranosyl units was observed only in the desulfated material. This was accompanied by a reduction in the amount of 3,4-disubstituted galactopyranosyl units, indicating the presence of 4-linked galactopyranosyl 3-sulfate units in the original material. A reduction in the amount of 3,6-substituted galactopyranosyl units on desulfation was not associated with a corresponding increase in any one particular unit, although some 6-substituted galactopyranosyl units were observed only in the desulfated material (Table 3). This indicated the presence of some 6-linked galactopyranosyl 3-sulfate units in the original material.

The abundance of 3-linked fucopyranosyl and galactopyranosyl units indicates a (1→3)-linked backbone predominates in both sugar components.

Antiviral activities of GFS and its fractions

The antiviral activities of GFS and the low uronic acid/high sulfate fraction (F2M) were the same, within experimental error, against all three herpes viruses (Table 4). The activity of the high uronic acid/low sulfate fraction (F1M) was markedly lower against all three viruses, while the activity of the minor, low uronic acid/high sulfate fraction (F4M) was similar to that of F1M not F2M.

Table 4 Antiviral activity (IC_{50}) of galactofucan sulfate extract and its fractions

	HSV-1 μg/mL	HSV-2 μg/mL	HCMV μg/mL
GFS	1.1	0.2	0.5
F1M	4.6	1.0	4.0
F2M	1.1	0.1	0.5
F4M	3.1	1.0	2.0

Discussion

Structural properties

The three fractions obtained by a single stepwise fractionation of the extract (GFS) derived from the blade and sporophyll of *Undaria pinnatifida* differed markedly in their composition and antiviral activity. The fraction (F2M) obtained in highest yield (63.0%) gave only one band on CAE analysis. It most closely resembled the low MW, highly purified fraction, reported by Lee et al. (2004), in uronic acid content (1.0% cf 1.9%), DS (0.94 cf 0.72), protein (<0.6% cf 0%) and fucose/galactose ratio (1.2 cf 0.9). However, the very large difference in Mw between F2M and the three times fractionated material of Lee et al. (320 kDa cf 9 kDa, both values based on pullulan standards) suggests that repetitive fractionation severely depolymerised the galactofucan. A single fractionation of GFS gave major fractions each with less than half the molecular weight of the original material. The lower sulfate content (DS value) of the 9 kDa material suggests some loss of sulfate groups also occurred on repetitive fractionation.

The next most abundant fraction (F1M) obtained in 18.2% yield differed markedly from F2M in composition. It contained at least two components separable by CAE as opposed to one. It also had a much higher uronic acid content (10.1% cf 1.0%), a much lower sulfate content (DS 0.41 cf 0.94), a higher nitrogen content, a much higher fucose/galactose ratio (5.3 cf 1.2) and all of the minor sugar units (xylopyranosyl, mannopyranosyl and glucopyranosyl) absent from F2M (Table 2). This is consistent with the more heterogeneous nature of 'uronofucoidan' fractions of brown seaweed polysaccharides cf 'galactofucan' fractions (Ponce et al., 2003).

The minor fraction (F4M) obtained in 3.6% yield most resembled F2M in its uronic acid and sulfate content, but differed markedly in having a high nitrogen content. This is assigned to protein, but could also be due to the presence of an amino sugar component as found in *Fucus vesiculosis* (Nishino et al., 1994). Further work on this very minor fraction is required to elucidate its composition.

Although the fucan extracts from other species of brown algae can contain a significant proportion of galactopyranosyl units (eg *Sargassum stenophyllum*, Fuc/Gal 1.8, Majczak et al., 2003), the extract from *Undaria pinnatifida* has a particularly high content (Fuc/Gal 1.5). The content of galactopyranosyl units is slightly higher in the major, low uronic acid/high sulfate fraction F2M (Fuc/Gal 1.2), but greatly reduced in the high uronic acid/low sulfate fraction F1M (Fuc/Gal 5.3).

Methylation analysis conducted on these two fractions showed that the most abundant fucopyranosyl substitution patterns in F2M were 3-linked fucopyranosyl-2,4-disulfate, 3,4-disubstituted fucopyranosyl and 3-linked fucopyranosyl-2-sulfate. Smaller amounts of unsubstituted, 3- and 4-linked fucopyranosyl units were also present. No terminal fucopyranosyl units (or fucofuranosyl units, Ponce et al., 2003) were present in this component and only 1 mol% of terminal galactopyranosyl units was present in the desulfated polymer. This indicates that the 3,4-disubstituted fucopyranosyl units are not branched. A sulfate group resistant to desulfation at the 4-position of a 3-linked unit is another possibility. Bilan et al. (2002) reported residual sulfate at *O*-4 of some 3-linked residues and attributed it to axial sulfate groups being more resistant to solvolysis than equatorial ones. However, such high stability does not seem to be supported by data for fucans from other species (Duarte et al., 2001; Ponce et al., 2003). The galactopyranosyl component of F2M is predominantly a mix of 3-linked galactopyranosyl, 4-linked galactopyranosyl-3-sulfate and 6-linked galactopyranosyl-3-sulfate units.

These results are consistent with predominantly (1→3)-linked backbones for both of the fucopyranosyl and galactopyranosyl components, but the relationship between the two components in the overall structure is unclear. Backbones of fucopyranosyl and galactopyranosyl units either as blocks or interspersed in one polymer are two possibilities. Another possibility is the presence of two separate polymers. In a recent paper, Ponce et al. (2003) reviewed a number of backbone structures proposed since 1990. The absence of terminal units does not support the presence of galactopyranosyl units in branch chains. However,

in methylation analyses, the mol% of branched units often exceeds the mol% of terminal units, possibly due to relative total ion current values not being an absolutely accurate measure of relative mol% (Chizhov et al., 1999). Consequently, a small number of branched units could be present, but these would only be a small proportion of the 3,4-disubstituted fucopyranosyl units.

The data reported here for F2M differs markedly from the methylation analysis data for the highly depolymerised, low uronic acid/high sulfate fraction prepared by Lee et al. (2004) from the sporophyll of *Undaria pinnatifida.* 3-Linked fucopyranosyl-2,4-disulfate and 3,4-disubstituted fucopyranosyl units were not observed by Lee et al., consistent with cleavage of linkages in a (1→3)-linked backbone, while terminal and 2,4-disubstituted fucopyranosyl units, not found in F2M, were present in substantial amounts. In contrast, similar amounts of 3-substituted, 4-substituted and 2,3-disubstituted units to those in F2M were present. The galactopyranosyl component of the highly depolymerised material was also more complex, as it contained substantial amounts of 4,6-disubstituted, 4-substituted, 6-substituted and terminal units, in addition to the 3-substituted, 3,6-disubstituted and 3,4-disubstituted units present in F2M. Unfortunately, the nature of the substitution in the 3,4-disubstituted fucopyranosyl units of F2M was not clarified by these data. The proportion of fucopyranosyl units was low cf F2M (37 mol%, cf 67 mol%) and it rose to 52 mol% (cf 66 mol%) with desulfation prior to methylation analysis. This makes accounting for the differences in substitution patterns between the two fractions difficult. The desulfated, low Mw polymer fraction obtained by Lee et al. (2004) appears to be primarily 3-linked with one terminal unit for every 3.3 of the remaining fucopyranosyl units. Consequently, it is clear from these data that the highly purified, low Mw, low uronic acid/high sulfate fraction obtained by Lee et al. (2004) from the sporophyll of *Undaria pinnatifida* is markedly different in structure from the less purified, high Mw, low uronic acid/high sulfate fraction we obtained from the blade plus sporophyll.

As discussed in the results section, only methylation analysis data without significant prior desulfation are presented in this paper for the more complex and heterogeneous F1M fraction. Substitution patterns are similar to those of F2M, but their abundances differ and assignation to units such as discussed above for F2M must await further work on this fraction and the role of the uronic acid component in its structure.

Antiviral activity

The antiherpetic activity of F1M is poor compared to that of F2M, as found with high uronic acid/low sulfate compared to low uronic acid/high sulfate fractions of fucan extracts from other species of brown seaweeds, such as *Adenocystis utricularis* and *Sargassum stenophyllum* (Ponce et al., 2003; Majczak et al., 2003). However, the poor activity of F4M shows that high sulfate alone is not a good indicator of activity. The IC_{50} values for F2M compare well with the ranges of values reported for the most active fractions of extracts from other species of brown seaweeds, such as those given above and *S. patens* (0.2–5.5 μgmL^{-1} for HSV-1 and 0.5–1.3 μgmL^{-1} for HSV-2, Zhu et al., 2003; Ponce et al., 2003; Majczak et al., 2003). Lee et al. (2004) reported IC_{50} values of 2.5, 2.6 and 1.5 μgmL^{-1}, respectively, for the anti-HSV-1, HSV-2 and HCMV activity of their highly purified, low Mw fraction from *U. pinnatifida* sporophyll. These values are consistently higher than ours, particularly for HSV-2. We also found lower activity (HSV-1, IC_{50} 3.1 μgmL^{-1}) in a fraction prepared by CTAB fractionation from GFS, which had a similar uronic acid and sulfate content to F2M, but Mw 19 kDa cf 290 kDa.

Within the constraints of comparing different sets of assays, these results suggest that isolation and purification procedures that markedly depolymerise the most active component of an extract may adversely affect its antiviral activity. The HSV-1 activity of dextran sulfate samples decreased at low MW (<5000 Da, Witvrouw & De Clercq, 1997). It is also relevant that the most active fraction F2M was not more active than GFS, despite GFS containing up to 37% less active material and having a ratio of F1M to F2M of 1:3.5. The potency of GFS against the HSV-1 virus was reproduced by 1:3 and 1:1 mixtures of F1M and F2M, but 3:1 mixtures were only half as active. The potency of GFS is, therefore, relatively insensitive to the presence of its less active components.

The potency of GFS against the HSV-2 virus is comparable to that found by Thompson and Drager (2004), (IC_{50} 0.2 μgmL^{-1} cf 0.5 μgmL^{-1}), but the potency against the HSV-1 virus is 15–29 times greater than

that found by these authors (IC_{50} 1.1 μgmL^{-1} cf 16, 32 μgmL^{-1}). The reason for the discrepancy between the two extracts is unclear.

The cytotoxicity of GFS and its fractions was not determined in this study, but other studies of *Undaria* extracts report relatively high concentrations, $CC_{50} >$ 4 $mg.mL^{-1}$ (Thompson & Drager, 2004), $CC_{50} > 2$ $mg.mL^{-1}$ (Lee et al., 2004). These authors have also shown that the antiviral effect is most likely due to prevention of viral entry by inhibition of virus-host cell binding.

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