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Structural Study of Fucoidan from the Brown Seaweed *Hizikia fusiformis*

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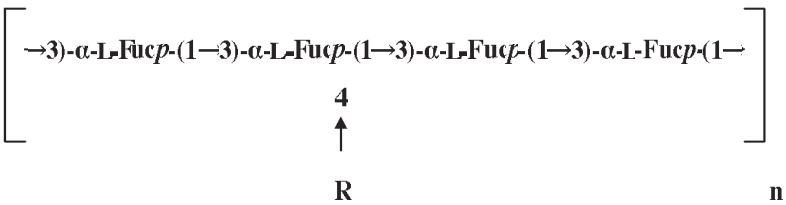
We investigated the structure of fucoidan from *Hizikia fusiformis*. Using DEAE-Sepharose column chromatography, fucoidan was fractionated into four groups. F4, main fraction of fucoidan, contained 38 % (w/w) of ester sulfate and had the simplest sugar component (Fuc : Gal=7:3). The structure of F4 was discussed using de-sulfation, 2D-NMR and methylation analysis and proposed as



R=α-L-Fucp-(1→4)-α-L-Fucp-(1→(50 %) or α-D-Galp-(1→4)-α-D-Galp-(1→(50 %).
58 % in molar ratio of the hydroxyl group is sulfated.

Keywords: fucoidan, *Hizikia fusiformis*, brown seaweed, de-sulfation, 2D-NMR, methylation

Introduction

Fucoidan, a cell-wall matrix polysaccharide in brown seaweed and some echinoderms, is composed of L-fucose and ester sulfate, including minor amounts of D-Gal, D-Man, D-Xyl, D-Glc, D-GlcA and acetic acid. Fucoidans have been extensively investigated because of their various biological activities as an anticoagulant (Chevolot *et al*, 1999; Doane and Whistler, 1963; Nagumo and Nishino, 1996), antitumor (Itoh *et al*, 1993), antiviral agent (Baba *et al*, 1988; Ponce *et al*, 2003) and inhibitor of *Helicobacter pylori* infection (Shibata *et al*, 2003), and these activities differ according to linkage pattern, content of ester sulfate, sugar component and molecular weight (Berteau and Mulloy, 2003). It is also important to elucidate the structure of fucoidans to understand the interactions between their structure and biological activities. Recently, the structure of fucoidan from the brown seaweed has been identified using 2D-NMR or electrospray ionization mass spectrometry (ESI-MS) techniques (Bilan *et al*, 2002; Chizhov *et al*, 1999; Daniel *et al*, 2007), although there is structural diversity.

In this study, *Hizikia fusiformis*, which is utilized for food material mainly in Japan and South Korea, was used for structural elucidation. Various reports have already addressed the preliminary characterization (Nishide *et al*, 1987; Nishino and Nagumo, 1987; Shiroma *et al*, 2003) and anticoagulant activity (Dobashi *et al*, 1989) of fucoidans from *H. fusiformis*, as well as the structure of *H. fusiformis* fucoidan containing a fucose-free core (Li *et al*, 2006). However, the structure of fucoidan containing high-fucose and ester sulfate has not yet been reported. Here, we discuss the structure of fucoidan containing high-fucose and ester sulfate from *H. fusiformis* in an acidic extract.

Materials and Methods

Materials. *H. fusiformis* used in this study was harvested in June 2002 from Yonabaru Town, Okinawa, Japan. The collected seaweed was washed with tap water and air-dried in an oven at 40°C for 24 h. The dried seaweed was then powdered using a mixer (MX-620G, Matsushita Electric In —

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dustrial Co., Ltd., Japan).

Isolation and fractionation of fucoidan from H. fusiformis The isolation of crude fucoidan was carried out as previously reported (Shirom*at al*,2003). Briefly, dried seaweed (20 g) was suspended in 0.1 M HCl (200 ml) and stirred at room temperature for 6 h. The suspension was filtered with a suction filter, and the filtrate was then neutralized with 0.5 M NaOH. The neutralized solution was concentrated and precipitated by the addition of 2 volumes of ethanol. The precipitate, crude fucoidan, was dried *in vacuo* and purified by cetylpyridinium chloride (CPC) precipitation. The CPC-purified fucoidan (100 mg) was applied to the DEAE-Sepharose column (Cl- form, i. d.; 26(h; 260 mm) and eluted with water followed by a liner gradient of θ→3.5 M NaCl at a flow rate of 0.5 ml/min. Fractions of 7 ml were collected, and total carbohydrate and uronic acid contents were estimated using phenol (Dubois*et al*,1956) and carbazole (Galambos, 1967)-H_2SO_4 reactions, respectively.

Chemical procedure and Liquid chromatography Acid hydrolysis was carried out by dissolving fucoidan in distilled water and adding sulfuric acid to a final concentration 2.0 M. The mixture was heated at 100℃ for 3 h and then neutralized with $BaCO_3$. Neutral monosaccharide analysis of the fucoidan hydrolyzate was carried out by liquid chromatography on DX500 (Dionex Co., Ltd., USA) using a column of Carbopac PA 1 (i. d.; 4×h; 250 mm, Dionex Co., Ltd.) equilibrated with 3 mM NaOH. Chromatography was carried out at a flow rate of 1 ml/min at 35˚C. Ester sulfate in fucoidan hydrolyzate, which had been treated by heating at 100℃ for 3 h in 2 M HCl solution, was determined by the $BaCl_2$-gelatin method (Dodgson, 1961). The molecular weight of fucoidan was determined by a high-performance liquid chromatography (HPLC) (LC-6A; Shimadzu Co., Japan) on a column of TSKgel PW$_{XL}$ (i. d.; 7.8×h; 300 mm, Tosoh Co., Japan) and detected with a refractive index detector RID-6A (Shimadzu Co., Japan) at room temperature. The column was eluted by 150 mM NaCl in 50 mM phosphate buffer (pH 7.2) at a flow rate of 0.3 ml/min. Pullulan P-5 (MW=0.59×10^5), P-20 (2.28 ×10^5), P-100 (11.2×10^5) and P-400 (40.4×10^5) (Showa Denko Co., Japan) were used as the molecular weight standards.

De-sulfation Chemical desulfation of sample was carried out according to the method of Nagasawa*et al*(1977). Sample (100 mg) was dissolved in distilled water (10 ml) and mixed with DOWEX 5W×8 (H^+, 200-400 mesh). After neutralization with pyridine, the solution was lyophilized. The lyophilized pyridinium salt was dissolved in DMSO: MeOH (9: 1; v/v, 20 ml). The mixture was heated at 80 °C for 4 h, and the desulfated product was dialyzed against distilled water and lyophilized.

NMR spectroscopy The spectra were recorded using a JNM-A500 (Nihondenshi Co., Ltd., Japan) at room temperature or 333 K. The samples (10 mg/ml) were dissolved in D_2O, and chemical shifts are expressed in ppm relative to internal standard [3-(Trimethylsilyl) propionic-2, 2, 3, 3-d_4 acid sodium salt, as 0.00 ppm]. Two-dimensional spectra (COSY, HOHAHA, NOESY and HMQC) were recorded using the pulse programs supplied with the instrument.

Methylation analysis The methylation analysis was carried out by the modified method of Needs and Selvendran (1993). The NaOH-DMSO reagent was prepared by the addition of MeOH (0.2 ml) and DMSO (6 ml) to a 50 % NaOH solution (0.1 ml). The DMSO-layer was collected and back-washed with DMSO, then, DMSO (2 ml) was added. A solution of sample (3 mg) in DMSO (2 ml) was mixed with NaOH-DMSO reagent (1 ml) and stirred at room temperature for 90 min. To this mixture, CH_3I (1 ml) was added and stirred at room temperature for 60 min in the dark. Next, distilled water (4 ml) was added, and the mixture was dialyzed against distilled water and then evaporated in a vacuum to dryness. The above procedure was repeated two times. The methylated sample was extracted by the addition of $CHCl_3$ (2 ml), and the $CHCl_3$ layer was evaporated in a vacuum to dryness. The extract was added to 2 M TFA (2 ml) and hydrolyzed at 120℃ for 2 h. The hydrolyzate was evaporated in a vacuum to dryness, and then 1M NH_4OH (100 µl) and $NaBH_4$ (10 mg) in DMSO (0.5 ml) was added. The reaction mixture was reduced at 40℃ for 90 min and then neutralized with AcOH (100 µl). Next, 1-methylimidazol (100 µl) and (CH_3CO)$_2$O (0.5 ml) was added, and the mixture was then acetylated at room temperature for 10 min. The reaction was stopped by the addition of distilled water (1.5 ml). Aldital acetates were extracted with $CHCl_3$ and analyzed by GC-MS on a QP-5000 (Shimadzu Co., Japan) using a DB-1 (i. d.; 0.25 mm×l; 30 m, Agilent Technologies, USA) capillary column. The temperature program was 150 °C for 5 min and then raised to 250 °C at 5 °C/min and held for 5 min. The carrier gas was He at flow rate of 1.2 ml/min.

Results and Discussion

The CPC-purified fucoidan was obtained in yield of 1.3 % (w/w, dry material) and applied to the DEAE-Sepharose column using a linear gradient of θ→3.5 M NaCl aqueous solution. Although 20 mM Tris-HCl buffer (pH 7.3) was also used as the eluate, the elution profile was almost same as that of the former solvent system, and thus the elution with θ→3.5 M NaCl aqueous solution was used.

The elution profile of the CPC-purified fucoidan was divided into four fractions based on the result of total carbohydrate analysis (Fig. 1). Each fraction was pooled, dialyzed against distilled water and lyophilized. The yields and chem

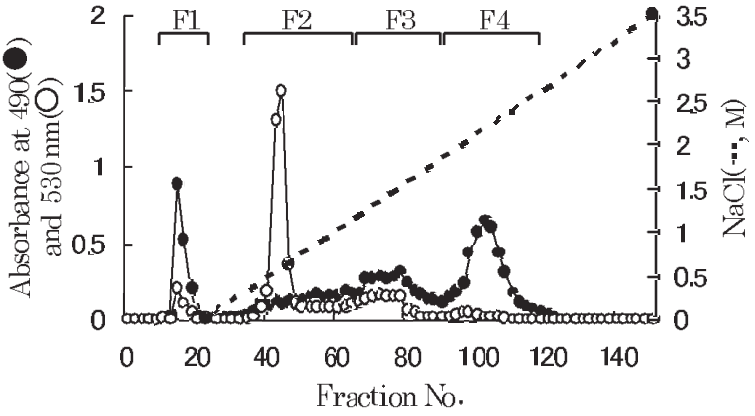

Fig. 1. Elution profile of the CPC-purified fucoidan on the DEAE- Sepharose column (φ26×260 mm). The flow rate of the column was 0.5 ml/min. Fractions of 7 ml were collected and checked by phenol-H_2SO_4 (490 nm) and carbazole-H_2SO_4 (530 nm). The dotted line shows NaCl concentration.

Table 1. Yields and Chemical components of F1-F4 obtained by the DEAE-Sepharose column chromatography of the CPC-purified fucoidan.

Fraction	Yield (%, w/v)	Neutral monosaccharides (%)					SO_3Na (%)	MW ($\times 10^4$)
		Fuc	Gal	Man	Xyl	Glc		
F1	13.3	44.8	36.1	7.0	9.8	2.3	19.3	35.2
F2	16.0	48.8	31.4	16.1	3.7	—	16.5	0.74
F3	10.8	56.7	31.4	5.9	4.7	1.3	33.0	29.0
F4	31.9	80.0	20.0	—	—	—	38.0	1.8

ical compositions of the four fractions are shown in Table 1. F1, a through fraction of DEAE-Sepharose contained 19.3 % of ester sulfate and had a high molecular weight. Thus, it appeared to be a fucoidan fraction tightly-bonded with CPC. The ^{1}H-NMR spectrum of F2, the highest uronic acid fraction, is shown in Fig. 2(A). As it is similar to that of alginate (Shiroma *et al.*, 2007), it appears to be a low molecular weight soluble alginate fraction. In the ^{1}H-NMR spectrum of F3, shown in Fig. 2(B), signals (2.0-2.2 ppm) arising from acetyl group were observed (Taki *et al.*, 2000). Thus, F3 appears to be a fucoidan fraction containing acetylated sugars. We obtained the highest yield (31.9 %) from F4, which had the simplest sugar composition (Fuc:Gal=8: 2, Table 1), and was thus used for structural elucidation.

The ^{1}H- and ^{13}C-NMR spectra of F4 are shown in Fig. 3. Since these spectra were complex by their ester sulfates, and because it was difficult to elucidate the structure, F4 was de-sulfated as described in the Materials and Methods. De-sulfated F4 (DeS-F4) was obtained in yield of 10.7 % (w/w) from native F4. As this was very small value compared with the theoretical value (about 60 %), it was considered that the main chain of F4 degraded during the solvolytic desulfation. Almost no ester sulfate was detected in DeS-F4 by the $BaCl_2$

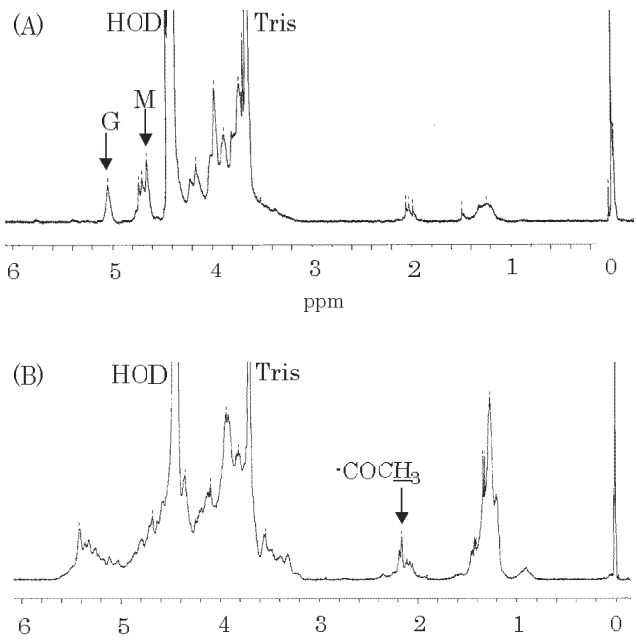

Fig. 2. ^{1}H-NMR spectra of F2 (A) and F3 (B) in D_2O at 60°C. Chemical shifts are referenced to internal TSP (0.00 ppm). M; anomeric proton of mannuronic acid, G; anomeric proton of glucuronic acid.

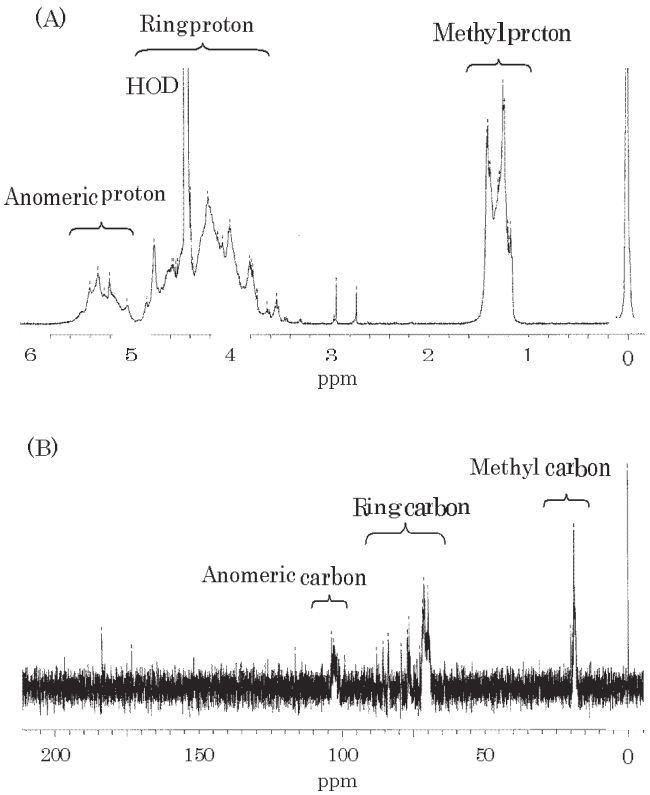

Fig. 3. ^{1}H (A)- and ^{13}C (B)-NMR spectra of F4 in D_2O at 60°C. Chemical shifts are referenced to internal TSP (0.00 ppm).

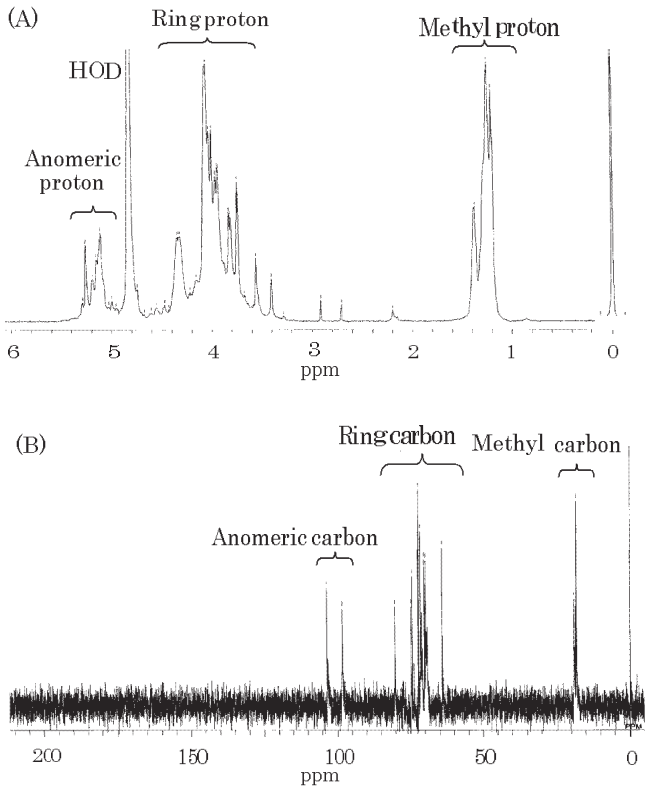

Fig. 4. ^{1}H (A)- and ^{13}C (B)-NMR spectra of DeS-F4 in D_2O at room temperature. Chemical shifts are referenced to internal TSP (0.00 ppm).

Table 2. ^{1}H and ^{13}C-NMR data of DeS-F4.

Residues	^{1}H Chemical shifts (ppm)					
	H1	H2	H3	H4	H5	H6
→3)-α-L-Fuc*p*-(1→	5.10	3.99	4.09	3.84	4.31	1.24
→3,4)-α-L-Fuc*p*-(1→	5.18	3.93	—	4.09	4.38	1.37
→4)-α-L-Fuc*p*-(1→	5.14	3.94	4.09	4.15	4.36	1.20
→4)-α-D-Gal*p*-(1→	5.26	3.81	3.94	4.05	3.99	3.74
	^{13}C Chemical shifts (ppm)					
	C1	C2	C3	C4	C5	C6
→3)-α-L-Fuc*p*-(1→	98.42	—	—	—	—	18.17
→3,4)-α-L-Fuc*p*-(1→	98.42	—	—	—	—	18.48
→4)-α-L-Fuc*p*-(1→	98.42	—	—	—	—	18.17
→4)-α-D-Gal*p*-(1→	103.49	71.63	69.79	80.37	72.19	64.19

gelatin method (Dodgson, 1961). The ^{1}H- and ^{13}C-NMR spectra of DeS-F4 are shown in Fig. 4. As these spectra were simple enough, DeS-F4 was then used for 2D-NMR and methylation analyses.

The four signals, 5.10, 5.14, 5.18 and 5.24 ppm, were mainly observed in anomeric region of the ^{1}H-NMR spectrum of DeS-F4. Using the COSY and HOHAHA experiments, the chemical shifts of ring protons of the four sugar residues were determined from these anomeric signals (Table 2). In the NOE spectrum of DeS-F4, the correlation peaks of (1→3)- or (1→4)-linked sugar residues were not identified because the H3 and H4 region were still complex. However, it was reported by Bilan *et al.* (2006) that the H1' (the other sugar residue)/H3 and H1'/H4 correlation peaks appear for (1→3)-linked fucobioside fragments as well as H1'/H4 and H1'/H6 peaks for (1→4)-linked fucobioside fragments. In

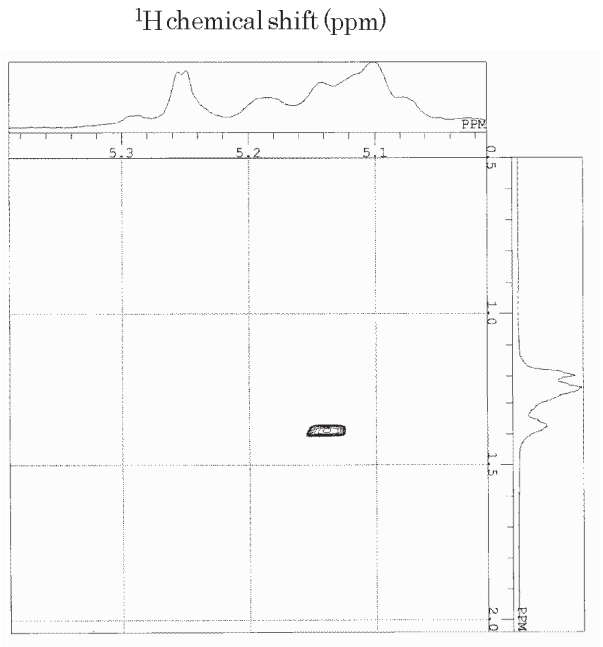

Fig. 5. A part of NOESY spectrum containing H1 and H6 regions of DeS-F4 in D_2O at room temperature. Chemical shifts are referenced to internal TSP (0.00 ppm).

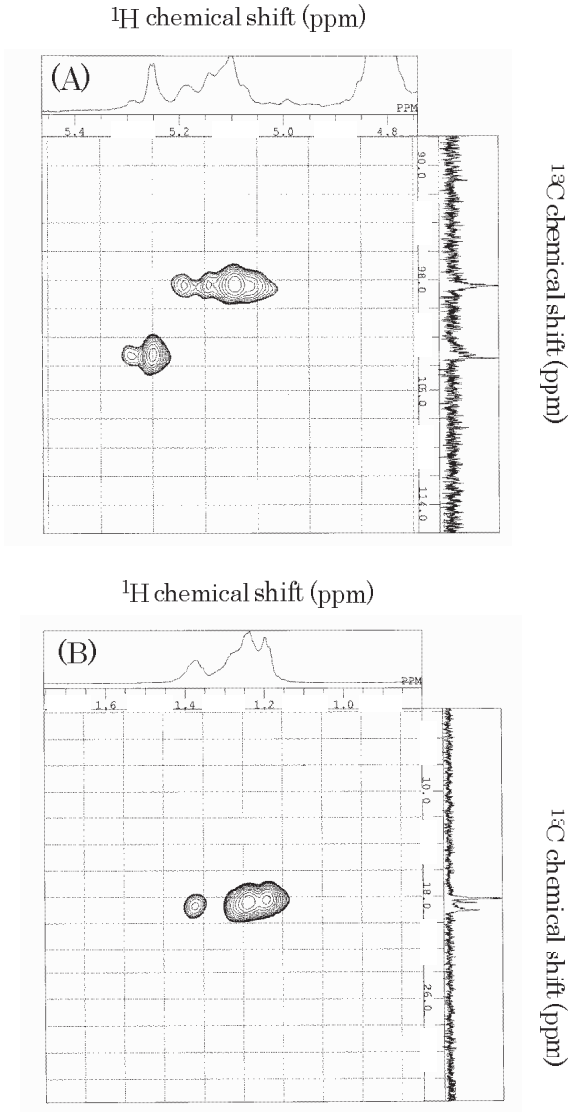

Fig. 6. Parts of HMQC spectra containing H1/C1 (A) and H6/C6 (B) regions of DeS-F4 in D_2O at room temperature. Chemical shifts are referenced to internal TSP (0.00 ppm).

the H1 and H6 region of the NOE spectrum of DeS-F4 (Fig. 5), the correlation peaks of 5.14/1.37 ppm corresponding to H1'/H6 was observed. Thus, the signal of 5.14 ppm was assigned as (1→4)-linked α-L-fucopyranosyl residue. The independent signal, 5.26 ppm, was assigned as (1→4)-linked α-D-galactopyranosyl residue, compared with the data of Faria *et al.* (2000). In the HMQC spectrum of DeS-F4 (Fig. 6), although the assignments of ring carbon signals were not completed because the ring proton and carbon region were also complex, the correlation peaks of H1/C1 and H6/C6 were observed and assigned respectively (Table 2).

The methylated sugars and their relative molar ratio derived from DeS-F4 by GC-MS analysis are shown in Table 3. 2,4-di-O-methyl-fucitol, corresponding to→3)-α-L-fucopyranosyl-(1→ residue, occupied about half of methylated sugars, and 2-O-methyl-fucitol, corresponding to (3,4)-(-L- fucopyranosyl-(1(residue, occupied about one third of the former residue. Compared these data with integral value of anomeric protons, the signals of 5.10 and 5.18 ppm were assigned as→3)-α-L-fucopyranosyl-(1→ and→3,4)-α-L-fucopyranosyl-(1→ residue, respectively.

From the above data, we proposed the structure of F4 from *H. fusiformis* in Fig. 7. The main chain of F4 is a (1→3)-linked α-L-fucopyranosyl residue with branches of α-L-fucopyranosyl-(1→4)-α-L-fucopyranosyl-(1→ (50 %) or α-D-galactopyranosyl-(1→4)-α-D-galactopyranosyl-(1→ (50 %), at C-4 every four residues, and this core structure is sulfated to 58 % of the hydroxyl group. The sulfated positions were not determined because of incomplete methylation of native F4. Clear evidence that galactosyl residue is attached to the main chain was also not obtained. Although our data did not agree with the findings of Nishino *et al.* (1994), Dobashi *et al.* (1989) reported that fucoidan from *H. fusiformis* had sulfated galactofucan fractions using electrophoresis, which supported that the exist of galactosyl residue in F4 is not due to the contamination of galactan. We thus consider that F4 from *H. fusiformis* is sulfated galactofucan, and this is the first report to elucidate the structure of fucoidans containing high-fucose and sulfate from *H. fusiformis* with an acidic extraction. The biological activities and interactions between the structure and activities of fucoidans containing high-fucose and ester sulfate from *H. fusiformis* are now under investigation.

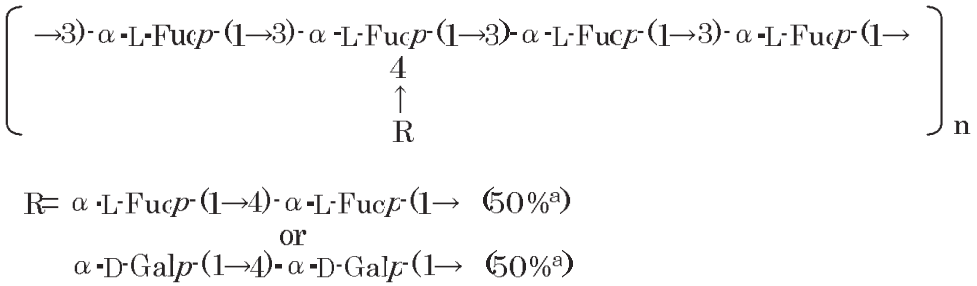

Fig. 7. Proposed structure of fucoidan fraction F4 from *H. fusiformis*.

Table 3. Methylated sugars derived from DeS-F4.

Peak No.	Alditol		Relative molar ratio
1	2, 3, 4-Me_3-Fuc	(Non-reducing Fuc*p*)	13
2	2, 3-Me_2-Fuc	[→4)-α-L-Fuc*p*-(1→]	10
3	2, 4-Me_2-Fuc	[→3)-α-L-Fuc*p*-(1→]	53
4	2, 3, 4, 6-Me_4-Gal	(Non-reducing Gal*p*)	5
5	2-Me-Fuc	[→3, 4)-α-L-Fuc*p*-(1→]	16
6	2, 3, 6-Me_3-Gal	[→4)-α-D-Gal*p*-(1→]	5

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