Novel Antiviral Fucoidan from Sporophyll of *Undaria pinnatifida* (Mekabu)

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Structural characterization and antiviral activities of fucoidan from sporophyll of *Undaria pinnatifida* (Mekabu) was examined. The fucoidan was composed of fucose and galactose with an approximately ratio of 1.0 : 1.1. Degree of substitution of sulfate was 0.72 and its apparent molecular weight was 9000. Methylation analyses showed that fucoidan had various sugar linkages, and revealed that the fucoidan might have complicated structure. This fucoidan showed potent antiviral activities against herpes simplex virus type 1 (HSV-1), HSV-2, and human cytomegalovirus.

Key words fucoidan; *Undaria pinnatifida*; sporophyll; antiviral activity; Mekabu

Blown algae like wakame (*Undaria pinnatifida*) and kombu (*Laminaria* sp.) are popular foods in East Asia. These edible algae have been used as rich sources of minerals, vitamins and dietary fibers. They have been attracted recently as multifunctional foods for maintaining our health.

Fucoidan (fucan sulfate) is a fucose-containing sulfated polysaccharide from brown algae such as *Fucus vesiculosus*,[1] *Ecklonia kurome*,[2] and *Cladosiphon okamuranus*.[3] Fucoidans showed various biological activities like anticoagulant[4] and antivirus.[5] In addition, fucoidan prevents microvascular thrombus formation induced by endothelial damage in arterioles and venules *in vivo*.[6] It is suggested that this activity is not attributable to inhibition of P- and L-selectin but instead to the anticoagulative capacity of fucoidan. Thus, fucoidan might be useful for anticoagulant, antiviral, and anti-thrombus formation agent.

Mekabu is a sporophyll of *U. pinnatifida* and it is also used as a safe, inexpensive and savory food in Japan. It has been reported that Mekabu has potential for chemoprevention of human breast cancer since Mekabu extract shows strong suppressive effect on rat mammary carcinogenesis.[7] In addition, Mekabu fucoidan showed antitumor effect *in vivo* and its mechanism was mediated by IFN-γ activated NK cells.[8] Although Mekabu fucoidan has been subjected to different kinds of biological evaluation, its structural feature is still unknown. This paper describes the structural characterization of Mekabu fucoidan as well as evaluation for its antiviral effects.

Results and Discussion

After washing and crushing the dried alga, it was treated with EtOH to remove lipophyllic compounds. The defatted alga was then extracted with 0.15 N HCl to prevent extraction of alginates. After neutralization and precipitation with 4 vol. of EtOH, crude fucoidan (CF) was obtained. CF was successively fractionated by ion exchange column chromatographies on DEAE Toyopearl 650M and Q-Sepharose FF and gel filtration on Sephacryl S-300 HR. As indicated in Fig. 1, CF was separated to four fractions. The most abundant fraction (CF-4) was further fractionated by Q-Sepharose (Fig. 2), and the second eluate (CF-4B) was collected. Then CF-4B was applied to a gel filtration on Sephacryl S-300 HR (Fig. 3) and the most abundant fraction was collected as purified fucoidan.

The fucoidan was eluted as a single peak, and its apparent molecular weight was estimated to be 9000 by HPLC on TSK gel $GMPW_{XL}$. In addition, the electrophoretic pattern indicated that the electric charge of fucoidan was homogeneous (Fig. 4). These results revealed that the fucoidan might be a homogeneous polysaccharide on the basis of molecular

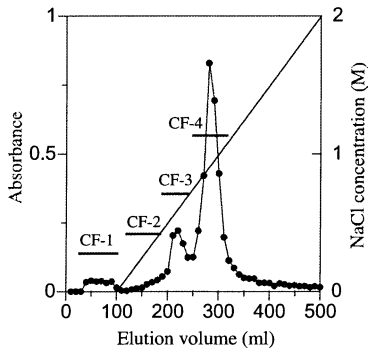

Fig. 1. Elution Profile of Crude Fucoidan on DEAE Toyopearl 650M

The column was eluted with a linear gradient of NaCl (0—2 M) in 50 mM sodium acetate (pH 5). Each fraction was monitored by phenol–H_2SO_4 method at 480 nm.

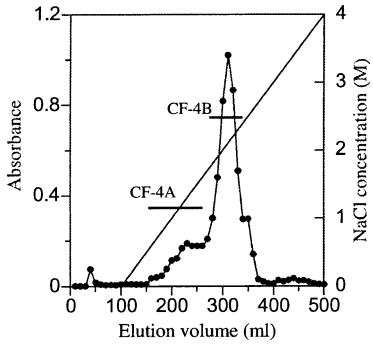

Fig. 2. Elution Profile of CF-4 on Q Sepharose FF Column Chromatography

The column was eluted with a linear gradient of NaCl (0—4 M) in 25 mM Tris–HCl (pH 7.2). Each fraction was monitored by phenol–H_2SO_4 method at 480 nm.

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weight and charge distribution.

Total sugar and uronic acid contents of the fucoidan were 37.9% by phenol–H_2SO_4 method and 1.9% by *m*-hydroxydiphenyl method, respectively. Bradford assay revealed that the fucoidan does not contain protein portion. Its sulfur content was estimated to be 10.4% and degree of sulfate substitution was calculated as 0.72. This value indicated that about 7 sulfated esters were substituted per 10 sugar residues.

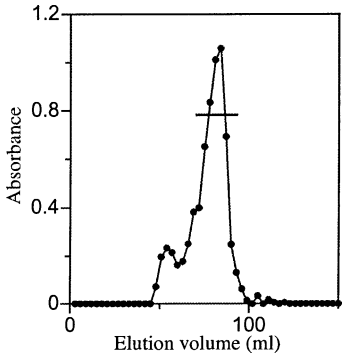

Fig. 3. Elution Profile of CF-4B on Sephacryl S-300 HR Column Chromatography

The column was eluted with 50 mM sodium acetate (pH 5) containing 0.1 M NaCl. Each fraction was monitored by phenol–H_2SO_4 method at 480 nm.

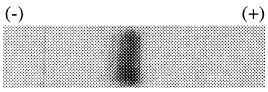

Fig. 4. Cellulose Acetate Membrane Electrophoresis of Fucoidan

Fucoidan was applied to a cellulose acetate membrane and run for 2 h at 0.5 mA/cm in a 0.2 M calcium chloride. Fucoidan was stained with 0.25% toluidine blue.

Sugar compositional analysis revealed that the fucoidan was composed of fucose and galactose with approximate ratio of 1.0 : 1.1. Then, fucoidan and desulfated fucoidan was submitted to methylation in order to elucidate the sugar linkage and substitution position of sulfate esters. Although methylation analysis of sulfated polysaccharide is not quantitative, it may offer valuable information concerning the position of the glycosidic linkage and the site of sulfation. The results of fucoidan might be loss of degradation products under methylation during dialysis of the derivatized sample. As shown in Table 1, desulfated fucoidan contained various sugar linkages such as non-reducing end fucose and galactose, 1,3-linked fucose, and 1,3-, 1,4-, and 1,6-linked galactose. From the comparison of the analytical results of the fucoidan with those of the desulfated fucoidan, sulfate ester was suggested to be substituted at 2-position of fucosyl residue and 3- or 6-position of galactosyl residue. These results revealed that Mekabu fucoidan might possess complicated structure containing various sugar linkages with various substitution patterns of sulfate.

To assess antiviral potency of the fucoidan, its effects on the growth of three host cell lines and six viruses were examined (Table 2). The 50% inhibitory concentrations of the fucoidan for cell growth (CC_{50}) of fucoidan were higher than $>2000\,\mu$g/ml against all tested cell lines. The 50% inhibitory concentration of the fucoidan for virus replication (IC_{50}) of HSV-1, HSV-2, HCMV, and influenza A virus were 2.5, 2.6,

Table 1. Results of Methylation Analysis of Fucoidan and Desulfated Fucoidan from Mekabu

Identification	Deduced linkage	Primary mass fragments (*m/z*)	Fucoidan (mol%)	Desulfated fucoidan (mol%)
2,3,4-Me_3-Fuc[a)]	t-Fuc	117, 131, 161, 175	7.8	11.6
2,4-Me_2-Fuc	3-Fuc	117, 131, 233, 247	9.1	24.7
2,3-Me_2-Fuc	4-Fuc	117, 161, 203, 247	4.2	7.8
4-Me-Fuc	2,3-Fuc	131, 261	11.3	5.5
3-Me-Fuc	2,4-Fuc	189. 203	4.9	2.4
2,3,4,6-Me_4-Gal	t-Gal	45, 117, 161, 205	6.0	12.8
2,4,6-Me_3-Gal	3-Gal	45, 117, 161, 233, 277	7.3	6.8
2,3,6-Me_3-Gal	4-Gal	45, 117, 161, 233, 277	6.3	10.1
2,3,4-Me_3-Gal	6-Gal	117, 161, 189, 233	14.3	15.0
2,6-Me_2-Gal	3,4-Gal	45, 117, 305	4.6	
2,4-Me_2-Gal	3,6-Gal	117, 189, 233, 305	15.4	1.0
2,3-Me_2-Gal	4,6-Gal	117, 161, 261, 305	8.8	2.4

a) 2,3,4-Me_3-Fuc means 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylfucitol.

Table 2. Antiviral Activities of Fucoidan from Mekabu

Virus	Host cell	Cytotoxicity (CC_{50}, μg/ml)	Antivirus activity (IC_{50}, μg/ml)		Selectivity index (CC_{50}/IC_{50})	
			A[a)]	B[b)]	A	B
HSV-1	Vero	>2000	2.5	14	>800	>140
HSV-2	Vero	>2000	2.6	5.1	>770	>390
HCMV	HEL	>2000	1.5	16	>1300	>125
Influenza A virus	MDCK	>2000	15	55	>130	>36
Poliovirus	Vero	>2000	>100	>100	≧20	≧20
Coxsackie virus	Vero	>2000	>100	>100	≧20	≧20

a) Sample was added to the medium at the time of viral infection for 1 h and throughout the incubation thereafter. *b*) Sample was added to the medium immediately after viral infection.

1.5, and 15 μg/ml, respectively, under conditions in which the fucoidan was added at the same time as viral infection (treatment A). The resulting selectivity indices (SIs; CC_{50}/IC_{50}) indicated that fucoidan has potent antiviral activity against these enveloped viruses. However, when the fucoidan was added to the medium after viral infection (treatment B), the activity was less than that on treatment A. On the other hand, the fucoidan showed no antiviral effect against non-enveloped viruses including polio- and coxsackie viruses.

It is well known that key antiviral agents contained in brown algae are sulfated polysaccharides.[9] They are considered to act principally by inhibiting the entry of enveloped viruses into host cells. Our results also revealed that Mekabu fucoidan interfered with the virus-host cell binding step, since antiviral activity at treatment A was more potent than that at treatment B (Table 2).

Major sulfated polysaccharide present in *U. pinnatifida* is characterized to be a galactofucan sulfate.[10,11] These results agreed well with our present data, although slight differences in sugar composition and molecular weight were observed.

Water extract from *U. pinnatifida* has been reported to show various biological activities including antiviral and immuno-modulating ones on human lymphocytes.[12,13] The antiviral principle of *U. pinnatifida* was suggested to be a polyanionic compounds such as sulfated polysaccharide. Present study also revealed that antiviral principle in Mekabu may be a sulfated polysaccharide since Mekabu fucoidan possess potent antiviral efficiencies against various enveloped viruses.

Experimental

General Absorbance was measured with a JASCO V-530 UV/VIS spectrophotometer. GC was carried out on a GL-Science GC353 gas chromatograph equipped with a hydrogen frame-ionization detector (FID). GC-MS analysis was carried out on a Shimadzu GCMS-QP5000 GC-MS. Carbohydrates were determined by the phenol–H_2SO_4 method.[14]

Extraction of Crude Fucoidan The dried young thullus of *U. pinnatifida* (10 kg) was washed with tap water at 15 °C for 10 min, and then milled. After the addition of 25 l of EtOH, it was refluxed at 80 °C for 2 h and filtered through gauze to obtain defatted alga. The defatted alga (3 kg) was extracted with 0.15 N HCl (11.2 l). After centrifugation and filtration, the extract was neutralized with 3 N NaOH and then applied to ultrafiltration (Asahi-Kasei Co. Ltd., LX2201 with ACP-1050 module, MW 13000 UF). After concentration, 4 vol. of EtOH was added to the solution and then filtered through mesh (300 μm). After washing the precipitate with EtOH, it was dried to yield crude fucoidan (52 g).

Purification of Fucoidan from Crude Fucoidan Crude fucoidan (200 mg) was dissolved in 5 ml of 25 mM sodium acetate (pH 5), and then applied to a DEAE Toyopearl 650 M column (2 i.d.×8 cm) equilibrated in 25 mM sodium acetate (pH 5). The column was eluted with 150 ml of the same buffer, and then eluted by a linear gradient prepared by mixing 300 ml of 25 mM sodium acetate (pH 5) with 300 ml of 2 M NaCl in the same buffer. The flow rate was 1 ml/min. Fractions of 10 ml were collected and checked by the phenol–H_2SO_4 method. Each fraction was dialyzed and lyophilized. Yield: CF-1, 32.1 mg; CF-2, 17.7 mg; CF-3, 28.7 mg; CF-4, 146 mg.

The most abundant fraction (CF-4; 200 mg) was dissolved in 3 ml of 20 mM Tris–HCl (pH 7.2), and then applied to a Q-Sepharose FF column (2 i.d.×8 cm) equilibrated with same buffer. The column was eluted with 100 ml of the same buffer, then eluted by a linear gradient prepared by mixing 200 ml of 20 mM Tris–HCl (pH 7.2) with 200 ml of 4 M NaCl in the same buffer. The flow rate of the column was 1 ml/min. Fractions of 10 ml were collected and monitored by the phenol–H_2SO_4 method. Most abundant fraction was dialyzed and lyophilized to yield CF-4B (115.7 mg).

CF-4B (50 mg) was applied to a Sephacryl S300 HR gel filtration (1.1 i.d.×100 cm) and eluted with 25 mM sodium acetate (pH 5) containing 0.1 M NaCl. Fractions (each 6 ml) were collected and monitored by the phenol–H_2SO_4 method. Thus obtained fraction was dialyzed and lyophilized to give a colorless polysaccharide (28.4 mg).

Cellulose-Acetate Membrane Electro-phoresis Mekabu fucoidan (10 μg) were applied to a cellulose-acetate membrane (Separax) in 0.2 M calcium acetate and run at 0.5 mA/cm for 2 h. Membranes were stained with 0.5% toluidine blue.

Estimation of Apparent Molecular Weight The apparent molecular weight of the fucoidan was estimated by HPLC analysis. The sample was applied on a TSK $GMPW_{XL}$ gel filtration column (7.6×300 mm×2, Tosoh Co., Tokyo, Japan) and eluted with 0.2 M NaCl at 0.5 ml/min. Commercially available pullulans (Shodex P-52, Showa Denko K.K., Tokyo, Japan) were used as standard molecular markers.

Chemical Analyses Uronic acid contents were determined by *m*-hydroxydiphenyl method.[15] Sulfur contents were determined by the flask combustion method under O_2 gas in the presence of H_2O_2.[16] Briefly, after the sample in the combustion flask was ignited under the O_2 atmosphere with an aliquot of H_2O_2, the inner solution was titrated with a standard solution of 50 mM $BaCl_2$ using 0.1% dimethylsulfonazo III reagent as an indicator.

Desulfation of Fucoidan Desulfation of fucoidan was performed by solvolytic desulfation method.[17] Briefly, 30 mg of fucoidan was passed through Dowex 50 Wx 8 column (H^+ form, 2×15 cm) with H_2O. After neutralization with pyridine, the solution was lyophilized. The resulting pyridinium salt of fucoidan was dissolved in 10% MeOH/DMSO (15 ml) and then incubated at 80 °C for 5 h with continuous stirring. The solution was exhaustively dialyzed against distilled water and lyophilized to yield desulfated fucoidan (15.6 mg).

Analysis of Glycosyl Residues Fucoidan was methanolyzed with 1 M HCl/MeOH at 80 °C for 16 h. The methanolic HCl was removed by adding *t*-BuOH and then evaporating with a N_2 stream at room temperature. The methyl glycosides were silylated using hexamethyldisilazene–trimethylchlorosilane–pyridine (2 : 1 : 10) solution at 80 °C for 20 min. The derivatives were analyzed by GC using a 30 m SPB-1 fused silica capillary column (Supelco Inc., PA, U.S.A.) with following oven temperature: an initial temperature of 140 °C, then an immediate increase to 180 °C at a rate of 2 °C/min. Linkage analysis of the fucoidan and desulfated fucoidan was performed as follows. The fucoidan was converted to a triethylamine salt by Furneaux's method.[18] Polysaccharides were methylated by Hakomori method.[19] Methylated polysaccharides were hydrolyzed with 6 M TFA at 100 °C for 5 h, reduced with $NaBH_4$, and acetylated.

Cells and Viruses Vero, MDCK, and HEL cells were grown in Eagle's minimal essential medium (MEM) (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 5% fetal bovine serum (FBS). HSV-1 (HF strain), HSV-2 (UW-268 strain), poliovirus and coxsackie virus (Conn-5 strain) were grown on Vero cells. HCMV (Towne strain) and influenza A virus (NWS strain) were grown on HEL and MDCK, respectively.

Antiviral Activity and Cytotoxicity Plaque yield reduction assay for antiviral activity has been described previously.[20] Cell monolayers were infected with a virus at 0.1 plaque forming unit (PFU) per cell at room temperature. After 1 h of viral infection, the monolayers were incubated with fucoidan. Virus yields were determined by plaque assay at 3-d incubation point for HCMV or at 1-d incubation point for HSV-1, HSV-2, influenza A virus, poliovirus and coxsackie virus. The 50% inhibitory concentration (IC_{50}) was obtained from dose–response curves. For cell growth inhibition studies, cells were incubated in 48-well plates at an initial density of 1.2×10^4 cells/well). After cells had been incubated for 8 h at 37 °C, fucoidan was added and the incubation was continued for 3 d. Viable cell yield was determined by the trypan blue exclusion test. The 50% cytotoxic concentration (CC_{50}) was obtained from dose–response curves.

References

1) Patankar M. S., Oehringer S., Barnett T., Williams R. J., Clark G. F., *J. Biol. Chem.*, **268**, 21770—21776 (1993).
2) Nishino T., Yokoyama G., Dobashi K., Fujihara M., Nagumo T., *Carbohydr. Res.*, **186**, 119—129 (1989).
3) Nagaoka M., Shibata H., Kimura-Takagi I., Hashimoto S., Kimura K., Makino T., Aiyama R., Ueyama S., Yokokura T., *Glycoconjugate J.*, **16**, 19—26 (1999).
4) Nagumo T., Nishino T., "Fucan Sulfates and Their Anticoagulant Activities," ed. by Dumitriu S., Marcel Dekker, Inc., New York, 1996, pp. 545—574.
5) Baba M., Snoeck R., Pauwels R., De Clercq E., *Antimicrob. Agents Chemother.*, **32**, 1742—1745 (1988).
6) Thorlacius H., Vollmar B., Seyfert U. T., Vestweber D., Menger M. D.,

Eur. J. Clin. Invest., **30**, 804—810 (2000).
7) Funahashi H., Imai T., Mase T., Sekiya M., Yokoi K., Hayashi H., Shibata A., Hayashi T., Nishikawa M., Suda N., Hibi Y., Mizuno Y., Tsukamura K., Hayakawa A., Tanuma S., *Jpn. J. Cancer Res*., **92**, 483—487 (2001).
8) Maruyama H., Tamauchi H., Hashimoto M., Nakano T., *In Vivo*, **17**, 245—249 (2003).
9) Witvrouw M., De Clercq E., *Gen. Pharmacol*., **29**, 497—511 (1997).
10) Mori H., Kamei H., Nishide E., Nisizawa K., "Sugar Constituents of Some Sulphated Polysaccharides from the Sporophylls of Wakame and Their Biological Activities," ed. by Hoppa H. A., Levring T., Walter de Gruyter, New York & Berlin, 1982, pp. 109—121.
11) Koo J.-G., Jo K.-S., Do J.-R., Woo S.-J., *J. Korean Fish. Sci*., **28**, 227—236 (1995).
12) Hudson J. B., Kim J. H., Lee M. K., DeWreede R. E., Hong Y. K., *J. Appl. Phycol*., **10**, 427—434 (1999).
13) Shan B. E., Yoshida Y., Kuroda E., Yamashita U., *Int. J. Immunopharmacol*., **21**, 59—70 (1999).
14) Dubois M., Gilles K. A., Hamilton J. K., Robers P. A., Smith F., *Anal. Chem*., **28**, 350—356 (1956).
15) Blumenkrantz N., Asboe-Hansen G., *Anal. Biochem*., **54**, 484—489 (1973).
16) Ohta S., *Bunseki Kagaku*, **15**, 689—692 (1966) [*Chem. Abstr*., **65**, 14428*e* (1996)].
17) Nagasawa K., Inoue Y., Kamata T., *Carbohydr. Res*., **58**, 47—55 (1977).
18) Steavenson T. T., Furneaux R. H., *Carbohydr. Res*., **210**, 277—289 (1991).
19) Hakomori S., *J. Biochem*. (Tokyo), **55**, 205—208 (1964).
20) Hayashi K., Hayashi T., Morita N., *Antimicrob. Agents Chemother*., **36**, 1890—1893 (1992).