J. Appl. Glycosci., **63**, 31−37 (2016)
doi:10.5458/jag.jag. JAG-2015_024

Regular Paper

Structural Characteristics and Antioxidant Activities of Fucoidans from Five Brown Seaweeds

(Received November 12, 2015; Accepted January 29, 2016)
(J-STAGE Advance Published Date: March 17, 2016)

Katsumi Ajisaka,[1, †] Tatsuya Yokoyama,[1] and Keita Matsuo[1]

[1]*Department of Applied Life Science, Niigata University of Pharmacy and Applied Life Sciences*
(*265−1 Higashijima, Akiha-ku, Niigata 956−8603, Japan*)

Abstract: Five kinds of fucoidans from the brown seaweeds *Cladosiphon okamuranus*, *Sargassum hornery*, *Kjellmaniella crassifolia* (*Saccharine sculpera*), *Nemacystus decipiens*, and *Fucus vesiculosus*, were isolated according to a previously reported procedure with slight modification. The scavenging activities of DPPH radical, superoxide radical, and hydroxyl radical, as well as the ORAC value were measured for the isolated fucoidans. Fucoidans from *S. hornery*, *F. vesiculosus,* and *K. crassifolia* showed higher antioxidant activity than that from *S. hornery* and *C. okamuranus*, except for the hydroxyl radical scavenging activity. The relationship between the antioxidant activity and the structure was examined for each fucoidan. Fucoidans with high amount of sulfate groups did not necessarily result in increased antioxidant activity, although the sulfate group itself was essential for the antioxidant activity. Furthermore, the fucoidan linked to a side chain monosaccharide, such as GlcA, demonstrated similar antioxidant activity. The antioxidant activity of the fucoidans was possibly due to a combination of the factors involved, such as the amount of sulfate groups, the position of the sulfate groups, the kind of side chain sugar, the linkage of a side chain sugar, and the molecular weight.

Key words: fucoidan, sulfated fucan, antioxidant activity, ORAC assay

INTRODUCTION

Fucoidans are known as water-soluble polysaccharides that can be easily isolated from brown seaweeds, such as mozuku or kombu, and are mainly composed of L-fucose and/or sulfated L-fucose, linked via an α-1,3- or α-1,4-linkage. Some fucoidans contain L-fucose, sulfated L-fucose, or D-glucuronic acid (GlcA) as branching sugars. Mozuku and kombu, which contain structurally different fucoidans, are consumed as a part of healthy diet over a wide region in Japan.

The biological activity of fucoidans is also important from the practical point of view, since they can be used as food supplements or medical applications. For example, fucoidans isolated from *L. saccharina*, *F. serratus, F. distichus,* and *F. vesiculosus* have demonstrated approximately 80 % reduction in MDA-MB 231 tumor cell adhesion to human platelets *in vitro*.[1)] Furtheremore, several fucoidans have been approved for the prevention of stomach ulcers due to its role in suppressing *Helicobactor pylori* in the stomach.[2)] These results prove the potential use of fucoidans for the development of new drugs against tumor progression.

Moreover, the antioxidant activity of fucoidans was suggested to show a relation to anti-aging.[3)] Therefore, it would be important to find the edible brown seaweeds with higher antioxidant activity. Regarding this subject, the antioxidant activity of fucoidans has been extensively studied and many findings on the antioxidant activity of fucoidans from various brown seaweed have been reported in recent years.[4−8)] However, there is substantial literatures that describes the antioxidant activity of only one kind of fucoidan or assesse it through one assay method. Therefore, it was necessary to review the whole profiles of antioxidant activity of the fucoidans from different origins.

The purpose of the present study is to investigate the factors influencing the antioxidant activity of the fucoidans. For this purpose, we compared the antioxidant activities of five kinds of representative fucoidans from five brown seaweeds: *Fucus vesiculosus* (kelp),[9−11)] *Cladosiphon okamuranus* (Okinawa-mozuku),[12)] *Sargassum horneri* (Nagamo or Akamoku),[1,13)] *Kjellmaniella crassifolia* or *Saccharine sculpera* (Gagome kombu),[14−16)] and *Nemacystus decipiens* (Hana-mozuku or Ito-mozuku).[17,18)] The structures of these fucoidans had already been reported in the literatures and ^{1}H and/or ^{13}C NMR spectra has already been disclosed except for the fucoidan from *K. crassifolia*. We firstly confirmed the structure of the fucoidans that were isolated from the above-mentioned brown seaweeds. Namely, we compared the NMR spectra of these fucoidans with those reported in the respective studies in the literatures. Then, we measured the antioxidant activity using four assay methods: DPPH radical, superoxide radical, hydroxyl radical scavenging activity, and ORAC assay, using Trolox as a standard. The relation between the observed antioxidant activities and structure of each fucoidan was examined.

† Corresponding author (Tel. +81−250−25-5000, Fax. +81−250−25−5021, E-mail: ajisaka@nupals.ac.jp).
Abbreviations: DPPH, 1,1-diphenyl-2-picrylhydrazyl; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; SOD, Superoxide dismutase; ORAC, Oxygen Radical Absorbance Capacity; GlcA, D-glucuronic acid.

MATERIALS AND METHODS

Materials. Four brown seaweeds were obtained from several local markets: *S. horneri* from Kouda Co., Ltd. (Sado, Japan), *C. okamuranus* from Kaiso-tonya (Okinawa, Japan), *N. decipiens* from Kawashimaya (Niigata, Japan), and *K. crassifolia* from Hakodate-Kaisoya (Hakodate, Japan). These brown seaweeds were harvested at the sea near the district of the market. Crude fucoidan from *F. vesiculosus* was purchased from Sigma-Aldrich Co., (St. Louis, USA) and was purified from the second step of the purification procedure described below.

NMR analyses. ^{1}H and ^{13}C NMR spectra were measured in D_2O using the Avance III 400 NMR spectrometer at 25 ˚C (Bruker Corporation, Billerica, USA), or the JEOL JNM-EC600 spectrometer at 60 ˚C (Tokyo, Japan).

Fucoidan purification. As the first step of the purification, fucoidan was extracted form freeze-dried brown seaweed using 0.1 M hydrochloric acid by stirring slowly for 2 h at room temperature. After neutralization with a 0.1 M sodium hydroxide solution and filtration, the fucoidan was precipitated with three volumes of ethanol. Then, the precipitate was filtered, dissolved in water, and freeze-dried. As the second step of purification, the freeze-dried crude fucoidan was solubilized in 3 % barium chloride solution. After filtration, the filtrate was dialyzed against distilled water using Spectrum dialysis tube (molecular weight cut off; 12−14 kDa), and subsequently, the chelating barium ion was exchanged with protons by passing the dialyzed solution through an ion exchange column of Amberlite IR-120 [H^+]. The flow-through solution was concentrated to a small volume, neutralized with 0.1 M NaOH, and freeze-dried to obtain a white powder of purified fucoidan. The yields of the purified fucoidans are summarized in Table 1.

General treatments in the antioxidant activity measurement. The antioxidant activities of the purified fucoidans were measured in triplicate. After confirming linearity in the graph of inhibition percentage *versus* concentration (1−20 mg/mL), the antioxidant activity was calculated as a Trolox equivalent value (mg Trolox/g fucoidan) from the inhibition percentage at 10 or 20 mg/mL of fucoidan, and the calibration curve of Trolox.

Measurement of DPPH radical scavenging activity. The DPPH radical scavenging activity of each fucoidan was measured according to a general DPPH procedure.[19,20] Briefly, 100 μM DPPH in ethanol (1 mL) and each fucoidan solution (1, 2.5, 5, 10, 15, and 20 mg/mL in 100 mM Tris-HCl buffer (pH 7.4), 1 mL) were mixed, and the reaction mixture was then incubated for 20 min, in the dark, at room temperature. The absorbance at 515 nm was measured against a blank control (100 mM Tris-HCl buffer instead of fucoidan solution). The DPPH radical scavenging activity (inhibition percentage) was calculated as: Inhibition (%) = $(1 - A_{sample}/A_{blank}) \times 100$, where A_{blank} is the absorbance of the blank.

Measurement of superoxide radical scavenging activity. Superoxide radical scavenging activity was measured through the SOD assay kit-WST™ (Dojin Chemicals Co., Kumamoto, Japan) according to the manufacturer's instructions. The method is based on the reports of Ukeda *et al.*[21,22] Briefly, the mixed solution (20 μL) of fucoidan at various concentrations (1, 2, 5, 10, and 20 mg/mL in water) were placed in a 96-well microplate. The WST working solution™ (200 μL) containing 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2-H-tetrazolium in 50 mM carbonate buffer (pH 10.2), and the Enzyme working solution™ (20 μL) containing xanthine oxidase in the same buffer were added. Then, the microplate was incubated at 37 ˚C for 20 minutes, and the absorbance at 450 nm was measured. The SOD activity (inhibition percentage) was calculated as: Inhibition (%) = $(1 - A_{sample}/A_{blank}) \times 100$, where A_{blank} is the absorbance of the blank (dilution buffer instead of carbohydrate solution).

Measurement of hydroxyl radical scavenging activities. The hydroxyl radical scavenging activity was measured using the deoxyribose method.[23] The fucoidan solution (0, 0.1, 0.25, and 0.5 mg/mL, 1 mL), 50 mM potassium phosphate buffer (pH 7.4, 0.33 mL), and 1 mM ascorbic acid (0.1 mL) were added to a mixture containing 10 mM 2-deoxyribose (0.36 mL), 10 mM $FeCl_3$ (0.01 mL), 1mM EDTA (0.1 mL), and 10 mM H_2O_2 (0.1 mL). This mixture was then incubated for 1 h at 37 ˚C. A solution of 1 % (w/v) thiobarbituric acid (TBA) in 50 mM NaOH (1 mL) and 2.8 % (w/v) trichloroacetic acid (TCA) in distilled water (1 mL) was added next, and the mixture was heated in a boiling water bath for 20 min. The amount of chromogen produced was measured at 535 nm. The hydroxyl radical scavenging activity (inhibition percentage) was calculated as: scavenging activity (%) = $(1 - A_{sample}/A_{blank}) \times 100$, where A_{blank} is the absorbance of the blank (10 mM potassium phosphate buffer instead of carbohydrate solution).

The oxygen radical absorbance activity (ORAC) assay. The ORAC assay was performed by use of 96-well black opaque plate.[24] Trolox and sample solutions (12.5−100 μM) were prepared using a 75 mM phosphate buffer. Each solution (25 μL) and 40 nM fluorescein (150 μL) were put into each well of the microplate. Then 150 mM 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH, 25 μL) was added, and the microplate was inserted into the fluorometer. The fluorescence intensity (excitation 485 nm, emission 527 nm) was recorded at every 30 s for 1.5 h through a programmed reader. The ORAC values were calculated as μmole Trolox/g fucoidan.

RESULTS AND DISCUSSION

Purification of fucoidans from freeze-dried brown seaweed.

Fucoidans were isolated according to the literature[17] with a minor modification. Each seaweed was freeze-dried before the purification and weighed. The yields of fucoidans isolated from four freeze-dried brown seaweeds were in the range of 1.2−5.1 % based on the dry seaweed weights (Table 1).

Comparison of NMR spectra of isolated fucoidans with the literature.

The structures of the fucoidans in the present study have already been reported in the literature. Their ^{1}H and/or ^{13}C NMR spectra were also published in the literature, except for those of the fucoidan from *K. crassifolia*. We measured ^{1}H and ^{13}C NMR spectra, and compared the spectra with those in the literature, to confirm that the isolated polysaccharides were surely fucoidans with no impurity.

Fucoidans are a mixture of polysaccharide, which causes

every proton signal to overlap and makes it difficult to assign them individually. However, several empirical rules are known, for example, when a hydroxyl group in the sugar is changed to a sulfate ester, the ring proton signal shifts 0.5−0.7 ppm to lower field.[25] Therefore, if several hydroxyl groups were sulfated in the fucan chain, the profile of NMR spectrum would change remarkably. Therefore conversely, if the profile of NMR spectrum of the isolated fucoidan is analogous to that of the reported one, it may be said that, not only the structure of main chain of fucoidan, but also the amount of the sulfate groups are not largely different.

The ^{1}H (600 MHz, 60 °C) and ^{13}C NMR (100 MHz, 25 °C) spectra of the fucoidan from *S. horneri* isolated in the present study are shown in Figs. 1a and 2, respectively. In the report of Ermakova *et al.*,[13] the fucoidan from *S. horneri* is composed of three different polysaccharide fractions, ShF1, ShF2, and ShF3, with the ratio of approximately 1.0:1.6:0.1, respectively. In the present study, we did not separate these fractions and measured the NMR spectra as a mixture of these fractions. According to the report of Ermakova *et al.*,[13] ShF1 was a linear α-1,3-linked homo-fucan with sulfate group at 2-position (14.9 % sulfate) and D-galctose (8 %) and L-rhamnose (7 %) as the side chain, ShF2 was composed of an alternating α-1,3- and α-1,4-linked homo-fucan without sulfate group, and ShF3 was a sulfated rhamno-fucan (16.9 % sulfate, Fuc:Rha = 1:0.4) though the content was very low. In ^{1}H NMR spectrum (Fig. 1a), H-1 signals due to linear unsubstituted L-fucose chain resonated at 4.96 and 5.10 ppm, and the signals at 5.44 and 5.58 ppm are supposed to be H-1 signals of substituted L-fucose and side chain D-galactose residue. Signals of 3.7−4.1 ppm are assumed as ring protons of D-galactose residue. The ^{13}C NMR spectrum (Fig. 2) showed approximately a similar profile with the overlapped spectra of ShF1 and ShF2 in Ermakova *et al*.[13] Although two sets of C-1 and C-6 signals from D-galactose residues were observed in Fig. 2, the two peaks were assigned to the substituted and non-substituted D-galactose residues, as suggested by Ermakova *et al*. The peak intensities due to D-galactose residues in Fig. 2 are a

Table 1. Yield of isolated fucoidans from various brown seaweeds.

Origin of fucoidan	Starting mass (g)	Purified fucoidan (mg)	Yield of fucoidan (%)
S. horneri	5	225	4.5
F. vesiculosus	1	501	50.0[a]
C. okamuranus	11	443	4.0
N. decipiens	7	83	1.2
K. crassifolia	5	254	5.1

[a] The yield of this fucoidan was calculated from commercial one.

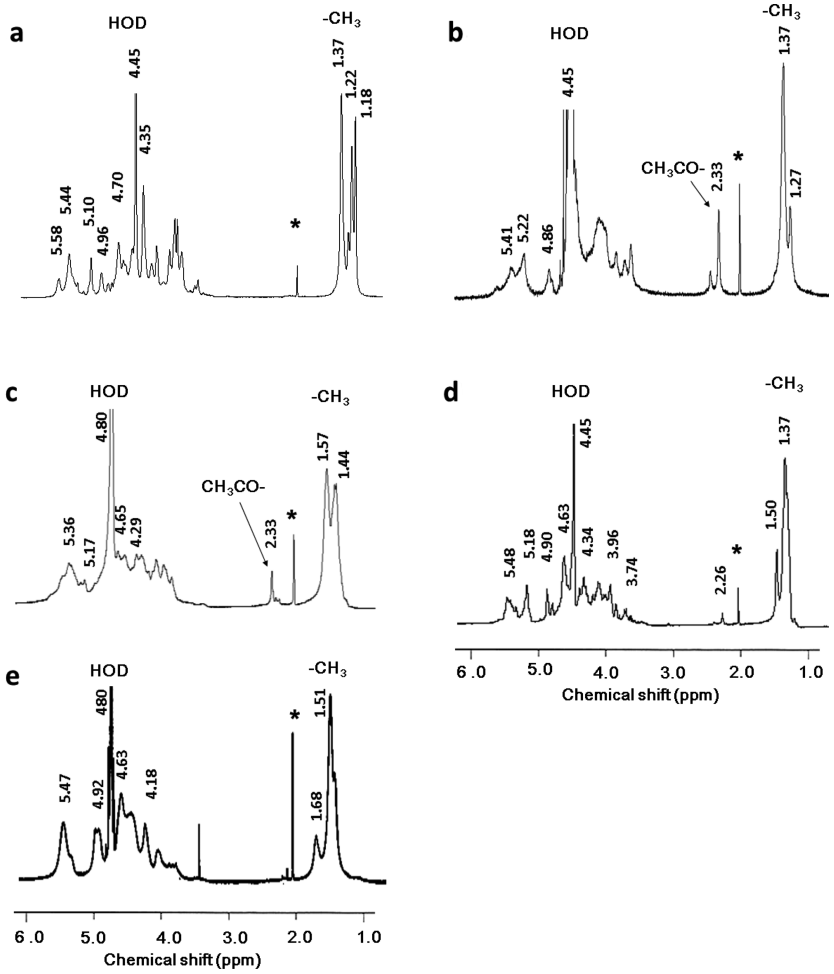

Fig. 1. ^{1}H NMR spectra of the five fucoidans used in the present study.

Fucoidans from a, *Sargassum horneri*; b, *Cladosiphon okimuranus*; c, *Fucus vesiculosus*; d, *Nemacystus decipiens*; and e, *Kjellmaniella crassifolia*. The spectra of a, b, and d were measured at 600 MHz and at 60 °C in D_2O, and spectra of c and e were measured at 400 MHz and 25 °C in D_2O. The mark * is a methyl signal of acetonitrile referenced to 2.01 ppm.

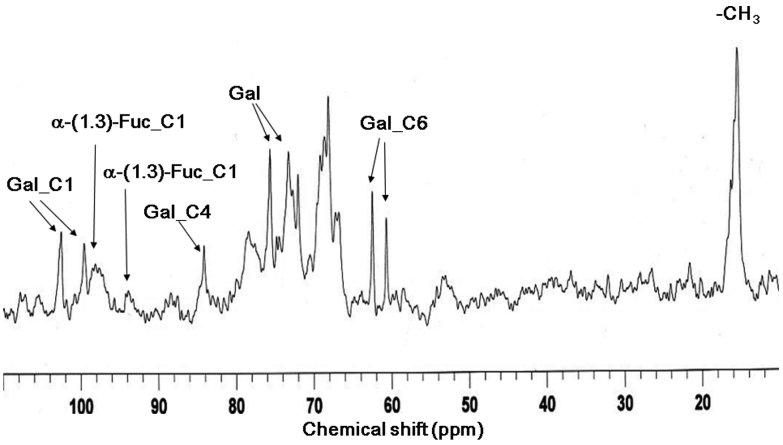

Fig. 2. ^{13}C NMR spectrum of the fucoidan from *S. hornery*.

The spectrum was measured at 100 MHz and 25 °C in D_2O. Assignments were made by referencing the report of Ermacova *et al*.[13]

little higher than in the spectrum of Ermakova *et al.*,[13] which means that the ratio of ShF1 or the ratio of D-galactose side chain in ShF1 may be more than that reported by them. From these spectra, the present fucoidan from *S. horneri* were recognized to be a mixture of fuco-galactans as reported by Ermakova *et al*.[13]

The ^{1}H NMR spectrum of the fucoidan from *C. okamuranus* is shown in Fig. 1b, which is roughly analogous to the one reported by Tako *et al*.[26] Namely in Fig. 1b, the signals at 5.22 and 5.41 ppm may be assigned to the H-1 of non-branched fucan chain and to the H-1 of L-fucose residue linked to GlcA, respectively. Furthermore, the peak at 2.33 ppm can be assigned to the CH_3 signal of acetyl group.[27] In the ^{13}C NMR spectrum (data not shown), the chemical shifts of every carbon signals coincided with those of the spectrum reported by Nagaoka *et al*.[12] From these results, the fucoidan from *C. okamuranus* that we isolated was assumed to be almost the same structure that reported in the literature (Table 2). Namely, this fucoidan was assumed to have a linear chain of α-1,3-linked fucan with a half sulfate group substitution at 4-position and a few acetyl group at 4-position randomly. Moreover, it was characteristic that GlcA residue was attached randomly at 2-position of fucan chain through an α-linkage. It was reported that the ratio of L-fucose:GlcA:sulfate group was about 6.1:1.0:2.8.

The ^{1}H NMR spectrum of fucoidan from *F. vesiculosus* (Fig. 1c) showed an analogous feature with the one reported by Ruperez *et al*.[9] The structure suggested by Patankar *et al.*[10] was basically composed of α-1,3-linked fucans with an L-fucose side chain binding through α-1,2- or α-1,4-linkage to every two or three L-fucose residues, and with a sulfate group at 4 position on some of the L-fucose residues. The H-1 signals at 5.17 and 5.36 ppm support the presence of two kinds of linkages of linear and substituted fucan chains, respectively. In addition, it is noticeable that a CH_3 signal from an acetyl group was observed at 2.33 ppm, and it may be the first time that a CH_3 signal of an acetyl group was observed in the ^{1}H NMR spectrum of this fucoidan.

The ^{1}H NMR spectrum of fucoidan from *N. decipiens* (Fig 1d) also showed roughly resembled profile with that reported in the literature of Tako *et al*.[17] The structure of this fucoidan was reported as the α-1,3-linked linear fucan without side chain. The peaks at 4.63, 4.90, 5.18, and 5.48 ppm were observed in their spectrum too, although the chemical shift values were slightly different. The assignments were already performed and described in their report.

Although we measured the ^{1}H NMR (Fig. 1e) and ^{13}C NMR spectra (data not shown) of fucoidan from *K. crassifolia*, there was no literature that we could find showing the NMR spectra of this fucoidan. This fucoidan was reported to be a mixture of polysaccharides of F-fucoidan and U-fucoidan,[14,15] F-fucoidan was reported to be composed of an α-1,3-linked, but partly α-1,2-linked poly-sulfated fucan, and a sulfated L-fucose was linked at 2-position randomly.[14] U-fucoidan was reported to have a structure of GlcA-(1-2)-[Fucα-(1-3)-]Man repeating tri-saccharide unit, whose hydroxyl group was sulfated randomly. The ratio of U-fucoidan to F-fucoidan was about 1:2. In Fig. 1e, and the signal at 4.92 ppm can be H-1 signals due to L-fucose residues in F-fucoidan, and the signal at 5.47 ppm can be those of L-fucose that had side chain, D-mannose, and GlcA residues of U-fucoidan. The peak area at 4.5−5.0 ppm was

Table 2. Structural information of the fucoidans cited from the literature.

Origin of fucoidan	Name of chain	Linkage of main chain	Side chain	Position of sulfate group	Sulfate group (mol/mol Fuc)	Ref.
S. horneri	ShF1 37.0 %	α-(1,3)-	Gal, Rha, Xyl	C-2	0.15	13)
	ShF2 59.3 %	α-(1,3)-α-(1,4)-	Rha	none	0.00	
	ShF3 3.7 %	α-(1,4)-	Rha	C-2	0.17	
F. versiculosus	80 %	α-(1,3)-	Fuc(C-2, C-4), Uronic acid, -OAc	C-4	0.36	11)
	13 %	α-(1,3)-	Fuc(C-2, C-4), Uronic acid, -OAc	C-4	0.18	
	7 %	α-(1,3)-	Fuc(C-2, C-4), Uronic acid, -OAc	C-4	0.12	
C. okamuranus		α-(1,3)-	GlcA(C-2), Xyl, Man, -OAc(C-4)	C-4	0.35	12)
N decipiens		α-(1,3)-	none	C-4	0.31	17)
K. crassifolia	F-fucoidan 67 %	α-(1,3)-	Fuc(C-2), GlcA(C-2)	C-4	0.67	14)
	U-fucoidan 33 %	GlcA-(1-2)-[Fuca-(1-3)-]Man	none	C-2, C-2,3, C-6(Man)	0.33	

clearly larger than that of the other fucoidans, probably due to the lower field shifts of the ring protons, whose hydroxyl group was sulfated, since this polysaccharide was supposed to contain a larger amount of sulfated hydroxyl groups. Therefore, from the ^{1}H NMR spectrum in Fig. 1e, the present fucoidan may be a mixture of F-fucoidan and U-fucoidan. In contrast, Zhang *et al.* reported a study of the structure and anti-complement activity of the fucoidan from *K. crassifolia* using polysaccharides extracted through water (KCW) or by dilute HCl (KCA).[28] It is not clear the relation between the U- and F- fucoidan in Table 2 and KCA or KCW.

The structure of each fucoidan isolated in the present study was confirmed to be a structure analogous to those in the literature, though the structure of fucoidan isolated from *K. crassifolia* was not identified, since the spectrum for comparison was not disclosed in the literature. It is remarkable that the structures of isolated fucoidans in the present study were roughly analogous to those in the literatures despite the harvested place, the harvested season, the isolation procedure, and so on being probably different. According to the reason mentioned above, not only the whole profile of the structure, but also the amount of sulfate group of the fucoidan used in the present study was supposed to be nearly analogous to those reported in the literature. The structures reported in literatures are summarized in Table 2.

The following discussion about the relation between the antioxidant activity and the structure assumed that the structure of the fucoidans isolated in the present study were almost same as those reported in the literatures (Table 2).

Antioxidant activities of fucoidans by four assay methods.

In DPPH radical scavenging activity, the plots of the inhibition percentage *versus* concentration showed high linearity as demonstrated in Fig. 3 as an example. The DPPH radical scavenging activities of five fucoidans calculated as Trolox equivalent value are summarized in Table 3. The fucoidan from *K. crassifolia* showed a relatively higher antioxidant activity as a whole, and those from *C. okamuranus* and *N. decipiens* showed relatively lower antioxidant activities. The fucoidan from *F. vesiculosus* and *S. horneri* showed the medium antioxidant activity. The antioxidant activity of the fucoidan from *K. crassifolia* showed about four-fold higher values than those from *C. okamuranus* and *N decipiens.*

Also in the superoxide radical scavenging activity assay, the percent inhibition of each fucoidan showed a good linearity with the concentration of the fucoidan (data not shown). However, the order of the Trolox equivalents of every fucoidan was quite different from those in the DPPH radical scavenging activity assay (Table 3). The fucoidan from *F. vesiculosus* showed the highest activity, and one from *C. okamuranus* had the lowest activity, and the other three fucoidans showed medium activities. However, the ratio of the highest activity over the lowest activity was only 1.9.

The hydroxyl radical scavenging activity also did not show the evident difference as summarized in Table 3. Although the fucoidan from *K. crassifolia* showed the highest activity, and the one from *F. vesiculosus* showed the lowest activity, the ratio of the highest activity over the lowest activity was only 1.8.

The ORAC values for five fucoidans are shown in Fig. 4 and Table 3. The fucoidans from *F. vesiculosus*, *S. horneri*, and *K. crassifolia* showed remarkably higher activity in comparison with those from *C. okamuranus* and *N. decipiens.* In this case, the ratio of the highest activity over the lowest activity was 4.5.

In summary, it should be noticed in Table 3 that DPPH radical scavenging activity, superoxide radical scavenging activity, and ORAC value showed similar tendency, i.e. that fucoidans from *F. vesiculosus*, *S. horneri*, and *K. crassifolia*

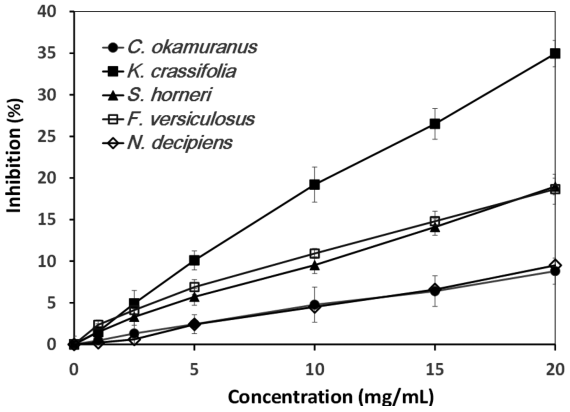

Fig. 3. DPPH radical scavenging activity by fucoidans from five kinds of brown seaweeds.

Concentration of fucoidans (*x*-axis) is final concentration of the incubated solution in the measurement of the activity.

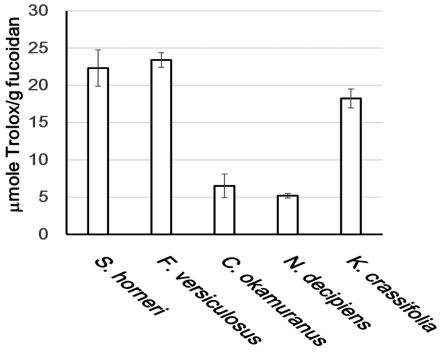

Fig. 4. Results of ORAC assay of fucoidans from five kinds of brown seaweeds.

ORAC value (μmol Trolox/g fucoidan) was calculated from the average of the results using 1.25−10 mg/mL fucoidan solution.

Table 3. DPPH radical, superoxide radical, hydroxyl radical scavenging activity, and ORAC values of the fucoidans from various origins.

Origin of fucoidan	DPPH radical[a]	Superoxide radical[a]	Hydroxyl radical[a]	ORAC value[b]
S. horneri	2.48 ± 0.42	14.3 ± 2.4	1.20 ± 0.25	22.3 ± 2.4
F. versiculosus	2.45 ± 0.24	21.2 ± 3.1	0.89 ± 0.12	23.4 ± 1.0
C. okamuranus	1.20 ± 0.48	11.1 ± 2.3	1.27 ± 0.23	6.5 ± 0.6
N. decipiens	1.15 ± 0.21	13.8 ± 2.4	1.43 ± 0.14	5.2 ± 0.3
K. crassifolia	4.50 ± 0.13	15.7 ± 4.2	1.58 ± 0.16	18.2 ± 1.3

[a] Values are expressed as mg Trolox/g fucoidan. [b] Values are expressed as mmol Trolx/g fucoidan.

showed higher antioxidant activity than those from *C. okamuranus* and *N. decipiens*. On the other hand, a distinct tendency could not be observed in hydroxyl radical scavenging activities since the ratio of the highest activity over the lowest activity was less than two.

Relation between the antioxidant activity and the amount of sulfate groups.

Tsuji *et al*. reported that the amount of sulfated group varied in the range of 17−21 % with the harvested months from January to May.[29] However, the difference of 4 % was not significant for the purpose of the present study, since the purpose of the present study was not to estimate the quantitative relation between the antioxidant activity and the amount of sulfate group, but to elucidate the factors controlling the antioxidant activity of fucoidans qualitatively.

From the general view of Table 3, fucoidans from *S. horneri, F. vesiculosus,* and *K. crassifolia* showed higher antioxidant activities except for the hydroxyl radical scavenging activity, than the other fucoidans. Therefore, the structural difference between the former three fucoidans and the latter two fucoidans was considered. Previous reports suggest the relation between the antioxidant activity and the amount of sulfate group or molecular weight of the fucoidan.[30−32] Although the positive relation was suggested by three research groups, these groups performed similar experiments using two or three fractions of fucoidan from *Laminaria japonica,* and measured only the superoxide radical scavenging activity. Therefore it may be significant to confirm whether a similar conclusion could be obtained or not.

At first, the relation between the antioxidant activity and the amount of sulfate group was examined. The fucoidans from *K. crassifolia* and from *S. horneri* were supposed to contain the highest and the lowest amount of sulfate group, respectively, among the five fucoidans in the present study (Table 2). In Table 3, both fucoidans from *K. crassifolia* and *S. horneri* showed higher antioxidant activity in the DPPH method, SOD assay, and ORAC value than the fucoidans from *F. vesiculosus, C. okamuranus,* and *N. decipiens*, which were supposed to contain medium amounts of sulfate group. Among the fucoidans containing a medium amount of sulfate group, the fucoidan from *F. vesiculosus* showed higher activity than the fucoidans from *C. okamuranus* and *N. decipiens*. These results indicate that the amount of sulfate group is not directly related to the antioxidant activity.

Next, we focused on the amount of GlcA residues. In the study of antioxidant activity of chondroitin sulfate, Campo *et al.* suggested[33] the importance of the carboxyl group of GlcA residue together with the sulfate group of *N*-acetyl-D-galactosamine residue. In the present study, fucoidans from *C. okamuranus, F. vesiculosus,* and *K. crassifolia* contained GlcA residue, even though the amount was not clear. Although fucoidans from *F. vesiculosus* and *K. crassifolia* showed higher activities, the fucoidan from *C. okamuranus* showed lower activity. Therefore, the evident contribution of GlcA residue was not demonstrated.

In conclusion, the antioxidant activities of five kinds of fucoidans from different origins were compared in relation to the amount of sulfate group or the linkage of GlcA. However, the fucoidans containing higher and lower amount of sulfate group did not necessarily give the higher and lower antioxidant activity, respectively, even though the sulfate group itself was essential for the antioxidant activity. In addition, some monosaccharides, GlcA for example, did not enhance the antioxidant activity. Therefore, the antioxidant activity should be a consequence of the combination of factors, such as the amount of sulfate groups, the position of the sulfate groups, the kind of side chain sugar, including GlcA, the linkage of side chain sugar, the complexity of sugar chain, molecular weight, *etc*.

ACKNOWLEDGEMENTS

The authors acknowledge to Dr. Ichiro Matsuo and Ms. Nozomi Ishii of Gunma University for the measurement and the discussion on the 600 MHz NMR spectra of fucoidans. The authors would also like to thank Dr. Toru Shigematsu and Dr. Shinnosuke Kumakura of Niigata University of Pharmacy and Applied Life Sciences for the assistance in the measurement of ORAC values. This work was supported by the MEXT of Japan under a Grant of Strategic Research Foundation Grant-Aided Project for Private Universities (S1001030).

REFERENCES

1) A. Philchenkov, M. Zavelevich, T. Imbs, T. Zvyagintseva, and T. Zaporozhets: Sensitization of human malignant lymphoid cells to etoposide by fucoidan, a brown seaweed polysaccharide. *Exp. Oncol.*, **29**, 181−185 (2007).

2) H. Shibata, I. Kimura-Takagi, M. Nagaoka, S.M. Hashimoto, H. Sawada, S. Ueyama, and T. Yokokura: Inhibitory effect of *Cladosiphon* fucoidan on the adhesion of *Helicobacter pylori* to human gastric cells. *J. Nutr. Sci. Vitaminol*. (Tokyo), **45**, 325−336 (1999).

3) D. Fusco, G. Colloca, M.R.L. Monaco, and M. Cesari: Effects of antioxidant supplementation on the aging process. *Clin. Interv. Aging*, **2**, 377−387 (2007).

4) T. Barahona, N.P. Chandia, M.V. Encinas, B. Matsuhiro, and E.A. Zuniga: Antioxidant activity of sulfated polysaccharides from seaweeds, a kinetic approach. *Food Hydrocoll.*, **25**, 529−535 (2011).

5) K. Bernhard, R. Stohr, L. Piker, and C. Plieth: The antioxidative properties of fucoidan are possibly related to their superoxide scavenging activities. *Marine Drugs*, **9**, 1−10 (2011).

6) M.C. Rocha de Souza, C.T. Marques, C.M.G. Dore, R.F.S. Fernando, H.A.O. Rocha, and E.L. Leite: Antioxidant activities of sulfated polysaccharides from brown and red seaweeds. *J. Appl. Phycol.,* **19**, 153−160 (2007).

7) K. Ajisaka, S. Agawa, S. Nagumo, K. Kurato, and T. Miyazaki: Evaluation and comparison of the antioxidative activity of various carbohydrates using different methods. *J. Agric. Food Chem*., **57**, 3102−3107 (2009).

8) G.P. Fidelis, R.B. Gamara, M. Gomes, M.F. Queiroz, M.S.S.P. Costa, O.C. Santos, H.A.O. Rocha, and L.S. Costa: Proteolysis, NaOH and ultrasound-enhanced extraction of anticoagulant and antioxidant sulfated polysaccharides from the edible seaweed, *Gracilaria birdiae*. *Molecules*, **19**, 18511−18526 (2014).

9) P. Ruperez, O. Ahrazem, and A. Leal: Potential antioxidant activity of sulfated polysaccharides from the edible marine brown seaweed, *Fucus versiculosus*. *J. Agric. Food Chem*., **50**, 840−845 (2002).

10) M.S. Patankar, S. Oehninger, T. Barnett, R.L.Williams, and G.F. Clark: A revised structure for fucoidan may explain some of its biological activities. *J. Biol. Chem*., **29**, 21770−21776 (1993).

11) M.C. Zaragoza, D. Lopez, M.P. Saiz, M. Poquet, J. Perez, P. Puig-Parellada, F. Marmol, P. Simonetti, C. Gardana, Y. Lerat, P. Burtin, C. Inisan, L. Rousseau, M. Besnard, and T. Mitjavila: Toxicity and antioxidant activity *in vitro* and *in vivo* of two *Fucus vesiculosus* extracts. *J. Agric. Food Chem*., **56**, 7773−7780 (2009).

12) M. Nagaoka, H. Shibata, I. Kimura-Takagi, S. Hashimoto, K. Kimura, T. Makino, R. Aiyama, S. Ueyama, and T. Yokokura:

Structural study of fucoidan from *Cladosiphon okamuranus* Tokida. *Glycoconj. J.*, **16**, 19−26 (1999).
13) S. Ermakova, R. Sokolova, S-M. Kim, B-H. Um, V. Isakov, and T. Zvyzgintseva: Fucoidan from brown seaweeds *Sargassum horneri*, *Eclonia cava*, *Costaria costata*: structural characteristics and anticancer activity. *Appl. Biochem. Biotechnol.,* **164**, 841−850 (2011).
14) Takara Bio Inc., S. Mizutani, S. Deguchi, E. Kobayashi, E. Nishiyama, H. Sagawa, and I. Kato: Fucoidan-containing cosmetics. *U.S. Patent*, US 7678368 B2 (2010).
15) T. Sakai, H. Kimura, K. Kojima, K. Shimanaka, K. Ikai, and I. Kato: Marinebacterial sulfated fucoglucurono mannan (SFGM) lyase digests brown algal SFGM into trisaccharides. *Marine Biotechnol*., **5**, 70−78 (2003).
16) M.K. Widjaja, A. Airanthi, M. Hosokawa, and K. Miyashita: Comparative antioxidant activity of edible Japanese brown seaweeds. *J. Food Sci*., **76**, C104−C110 (2011).
17) M. Tako, T. Nakada, and F. Hongou: Chemical characterization of fucoidan from commercially cultured *Nemacysus decipiens*. *Biosci. Biotechnol. Biochem.*, **63**, 1813−1815 (1999).
18) T. Teruya, Y. Tamaki, T. Konishi, and M. Tako: Rheological characteristics of alginate isolated from commercially cultured *Nemacystus decipiens* (Itomozuku). *J. Appl. Glycosci.,* **57**, 7−12 (2010).
19) M. Wang, J. Li, M. Rangarajan, Y. Shao, E.J. Lavoie, T-C. Huang, and C-T. Ho: Antioxidative phenolic compounds from sage (*Salvia offcinalis*). *J. Agric. Food Chem.,* **46**, 4869−4873 (1998).
20) Y. Lu and L.Y. Foo: Flavonoid and phenolic glycosides from *Salvia officinalis*. *Phytochemistry*, **55**, 263-267 (2000).
21) H. Ukeda, A.K. Sarker, D. Kawata, and M. Sawamura: Flow-injection assay of superoxide dismutase based on the reduction of highly water-soluble tetrazolium. *Anal. Sci*., **15**, 353−357 (1999).
22) H. Ukeda, D. Kawata, S. Maeda, and M. Sawamura: Spectrophotometric assay for superoxide dismutase based on the reduction of highly water-soluble tetrazolium salts by xanthine-xanthine oxidase. *Biosci. Biotechnol. Biochem*., **63**, 485−488 (1999).
23) J.B. Halliwell, M.C. Gutteridge, and O.I. Aruoma: The deoxyribose method: A simple "test-tube" assay for determination of rate constants for reactions of hydroxyl radicals. *Anal. Biochem*., **165**, 215−219 (1987).
24) D. Huang, B. Ou, M. Hamch-Woodill, J.A. Flanagan, and R.L. Prior: High-throughput assay of oxygen radical absorbance activity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. *J. Agric. Food Chem.,* **50**, 4437−4444 (2002).
25) N.E. Ustyuzhanina, M.I. Bilan, N.A. Ushakova, A.I. Usov, M.V Kiselevskiy, and N.E. Nifantiev: Fucoidans: pro- or antiangiogenic agents? *Glycobiology*, **24**, 1265−1274 (2014).
26) M. Tako: A novel acetylfucoidan and its preparing methods from commercially cultured *Cladosiphon okamuranus*. *Japanese Patent*, 3371124 (2002).
27) T. Teruya, H. Tatemoto, T. Konishi, and M. Tako: Structural characteristics and *in vitro* macrophage activation of acetyl fucoidan from *Cladosiphon okamuranus. Glycoconj. J.*, **26**, 1019−1028 (2009).
28) W. Zhang, W. Jin, D. Sun, L. Zhao, J. Wang, D. Duan, and Q. Zhang: Structural analysis and anti-complement activity of polysaccharides from *Kjellmaniella cracifolia*. *Marine Drugs*, **13**, 1360−1374 (2015).
29) M. Tsuji, Y. Sudou, M. Enoki, M. Tako, and T. Konishi: Variation in the contents and structure of fucoidan from cultivated *Cladosiphon okamuranus* Tokida. *Bull. Appl. Glycosci.*, **3**, 248−252 (2013). (in Japanese)
30) X. Zhao, C.H. Xue, Y.P. Cai, D.F. Wang, and Y. Fang: The study of antioxidant activities of fucoidan from *Laminaria japonica*. *High Tech. Lett*., **11**, 91−94 (2005).
31) L.H. Li, C.H. Xue, Y. Xue, Z.J. Li, and X.Y. Fu: The effects of fucoidans from *Laminaria japonica* on AAPH mediated oxidation of human low-density lipoprotein. *Acta Oceanol. Sin.,* **25**, 124−130 (2006).
32) J. Wang, Q. Zhang, Z. Zhang, and Z. Li: Antioxidant activity of sulfated polysaccharide fractions extracted from *Laminaria japonica*. *Int. J. Biol. Macromol.,* **42**, 127−132 (2008).
33) G.M. Campo, A. Avenoso, S. Campo, A.M. Ferlazzo, and A. Calatron: Antioxidant activity of chondroitin sulfate. *Adv. Pharmacol*., **53**, 417−431 (2005).