Structural Characteristics and Antioxidant Activities of Fucoidans from Five Brown Seaweeds

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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 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.3) Furthermore, several fucoidans have been approved for the prevention of stomach ulcers due to its role in suppressing Helicobacter pylori in the stomach.5) 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.9-11) However, there is substantial literatures that describes the antioxidant activity of only one kind of fucoidan or assess 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 hornery (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 1H and/or 13C 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.
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. ¹H and ¹³C NMR spectra were measured in D₂O 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). ¹H and ¹³C NMR spectra were also published in the literature, except for the spectra of isolated fucoidans in the present study, and 1H and/or 13C spectra, and compared the spectra with those already been reported in the literature17) with a 75 mM phosphate buffer. Each carbohydrate solution). The oxygen radical absorbance activity (ORAC) assay. 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: (sample/blank) × 100, where Sample is the absorbance of the sample and Blank is the absorbance of the blank.

Measurement of hydroxyl radical scavenging activities. The hydroxyl radical scavenging activity was measured using the deoxyribose method.²⁰ 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 FeCl₃ (0.01 mL), 1mM EDTA (0.1 mL), and 10 mM H₂O₂ (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 – Sample/Blank) × 100, where Blank is the absorbance of the blank (10 mM potassium phosphate buffer instead of carbohydrate solution).

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

Fucoidans were isolated according to the literature17) 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).

RESULTS AND DISCUSSION

Purification of fucoidans from freeze-dried brown seaweed. Fucoidans were isolated according to the literature17) 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 ¹H and/or ¹³C NMR spectra were also published in the literature, except for those of the fucoidan from *K. crassifolia*. We measured ¹H and ¹³C 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 1H (600 MHz, 60 °C) and 13C 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-galactose (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 1H 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 13C 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

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

* The yield of this fucoidan was calculated from commercial one.

Fig. 1. 1H NMR spectra of the five fucoidans used in the present study. Fucoidans from a, Sargassum horneri; b, Cladosiphon okamuranus; 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 D2O, and spectra of c and e were measured at 400 MHz and 25 °C in D2O. The mark * is a methyl signal of acetonitrile referenced to 2.01 ppm.
The structural information of the fucoidans cited from the literature.

Table 2.

| 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                   | none                       | 13)  |
|                    | ShF2 59.3 %   | α-(1,3)-α-(1,4)-      | Rha        | C-2 0.17                   | none                       |      |
|                    | ShF3 3.7 %    | α-(1,4)-              |            | C-2 0.00                   | none                       |      |
|                    | 80 %          | α-(1,3)-              |            | C-2 0.17                   | none                       |      |
| F. vesiculosus     | 13 %          | α-(1,3)-              |            | C-2 0.36                   | none                       | 11)  |
|                    | 7 %           | α-(1,3)-              |            | C-2 0.15                   | none                       |      |
| C. okamura-nus     | α-(1,3)-      | GlcA-(1-2), Fucose     |            | C-2 0.36                   | none                       |      |
| N decipiens        | F-fucoidan    | α-(1,3)-              |            | C-2 0.07                   | none                       |      |
| K. crassifolia     | U-fucoidan    | GlcA-(1-2), Fucose     |            | C-2 0.33                   | none                       |      |

The spectrum was measured at 100 MHz and 25 °C in D2O. Assignments were made by referencing the report of Ermacova et al.13)

The 1H NMR spectra (data not shown) of fucoidan from K. crassifolia, there was no literature that we could find reporting the structural information of this fucoidan. This fucoidan was reported to be a mixture of polysaccharides of F-fucoidan and U-fucoidan.14,15) The fucoidan was reported to have a structure of GlcA-(1-2)-[Fuc-(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, and a sulfated L-fucose was linked at 2-position randomly.14)
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). 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 structures of isolated fucoidans in the present study were roughly analogous to those in the literatures (Table 2). The antioxidant activity and the structure assumed that the fucoidan used in the present study was supposed to contain a larger amount of sulfated hydroxyl groups. Therefore, from the $^1$H NMR spectrum in Fig. 1e, the present fucoidan showed remarkable oxidant scavenging activity compared with those from *N. decipiens*, *C. okamuranus*, and *S. horneri*, which showed about four-fold higher values than those from *C. okamuranus* and *N. decipiens*.

### 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* had four-fold higher values than those from *C. okamuranus* and *N. decipiens*.

### 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. vesiculosus*  | 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 Trolox/g fucoidan.
showed higher antioxidant activity than those from \textit{C. okamuranus} and \textit{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.

\textbf{Relation between the antioxidant activity and the amount of sulfate groups.}

Tsuji \textit{et al.} reported that the amount of sulfated group varied in the range of 17–21\% with the harvested months from January to May.\textsuperscript{29}\textsuperscript{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 \textit{S. horneri}, \textit{F. vesiculosus}, and \textit{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.\textsuperscript{30}\textsuperscript{30} Although the positive relation was suggested by three research groups, these groups performed similar experiments using two or three fractions of fucoidan from \textit{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 \textit{K. crassifolia} and from \textit{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 \textit{K. crassifolia} and \textit{S. horneri} showed higher antioxidant activity in the DPPH method, SOD assay, and ORAC value than the fucoidans from \textit{F. vesiculosus}, \textit{C. okamuranus}, and \textit{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 \textit{F. vesiculosus} showed higher activity than the fucoidans from \textit{C. okamuranus} and \textit{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 \textit{et al.} suggested\textsuperscript{31} 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 \textit{C. okamuranus}, \textit{F. vesiculosus}, and \textit{K. crassifolia} contained GlcA residue, even though the amount was not clear. Although fucoidans from \textit{F. vesiculosus} and \textit{K. crassifolia} showed higher activities, the fucoidan from \textit{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.

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