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EXTRACTION AND CHARACTERIZATION OF FUCOIDAN FROM SIX BROWN MACROALGAE

Chun-Ying Wang[1] and Yean-Chang Chen[1, 2]

Key words: brown macroalgae, fucoidan, HPLC, *Sargassum*.

ABSTRACT

The content and composition of fucoidan in six brown macroalgae were investigated. The influence of different washing steps and two types of extraction methods on the yield and composition of fucoidan were assessed. The composition of the extract was determined by using a colorimetric method to measure the total sugar, sulfate, and protein contents of the fucoidan. The monosaccharide composition was assessed using high-performance liquid chromatography. The structures of the algal polysaccharide were studied using Fourier transform infrared-attenuated total reflectance spectroscopy. The fucoidan content extracted using method I was 13.13% (of dry weight), 24.00%, and 22.67% from *Sargassum glaucescens*, *Sargassum horneri*, and *Laminaria japonica*, respectively. By contrast, the fucoidan content extracted using method II was 4.20%, 4.80%, and 4.64% from *S. glaucescens*, *S. horneri*, and *L. japonica*, respectively. The samples extracted using method II were lower in total sugar content but higher in sulfated content than those extracted using method I. Using method II yielded the highest fucose content in *S. glaucescens* (20.89%) and *Hizikia fusiformis* (15.89%). The proportion of *H. fusiformis* was lower than that of *S. glaucescens* because the biomass of the *H. fusiformis* was much larger in the wild. Therefore, we consider *H. fusiformis* to be a rich source of fucoidan for human consumption.

I. INTRODUCTION

Fucoidan, or fucose-containing sulfated polysaccharides (FCSPs), is present in algae and has numerous functions relating to physiological activity (Ale et al., 2011; Fitton, 2011). In mammals, fucose-containing glycans have major roles in blood transfusion reactions, selectin-mediated leukocyte-endothelial adhesion, and other functions (Becker and Lowe, 2003). The value of brown algal fucoidan is preferred to fucose-containing glycans because of its different branches and molecular weights, which increase its applications in functional foods and pharmaceuticals (Ale et al., 2011). L-fucose is the major sugar constituent of fucoidan, as reported in many reviews (Li et al., 2008; Liu et al., 2012). Fucoidan has many health benefits, such as applying to antitumor, immunomodulatory, anti-inflammatory, antiviral, antithrombotic, anticoagulant, and antioxidant effects (Ale et al., 2011; Kraan 2012; Wijesinghe and Jeon, 2012). In addition, fucoidan can delay the symptoms of liver disease, osteoarthritis, and kidney disease, reduce the risk of radiation damage, and can even inhibit some snake venom (Fitton, 2011).

Marine macroalgae contain various polysaccharides such as frame polysaccharides, mucopolysaccharides, and storage polysaccharides (Murata and Nakazoe 2001). Fucoidan is classified as a mucopolysaccharide of brown macroalgae. It is a natural, heterogeneous sulfated polysaccharide unique to marine brown macroalgae. The carbon backbone of fucoidan comprises the fucose repeating chain (i.e., FCSPs). Fucoidan has been extracted from marine invertebrates such as the sea cucumber (Ribeiro et al., 1994; Fitton, 2005); however, brown macroalgae is the largest source (Hahn et al., 2012). Brown macroalgae that have been used for fucoidan extraction include *Sargassum filipendula* (Costa et al., 2011), *Undaria pinnatifida* (Yang et al., 2008), *Fucus vesiculosus*, *Ascophyllum nodosum* (Rioux et al., 2007), *Laminaria saccharina*, and *Cladosiphon okamuranus* (Cumashi et al., 2007). In *Laminaria japonica*, fucoidan is located in the inner boundary of cells or in the cell wall region to protect algae from drying (Mizuno et al., 2009). Recently, brown macroalgal fucoidan was found to possibly play a role in supporting unstable membranes, similar to the role of callose in plant cells (Nagasato et al., 2010).

Fucoidan is a heteropolysaccharide that mainly contains sulfated L-fucose residues such as fucose and sulfate; but it may also contain other monosaccharides (glucose, mannose, galactose, xylose, etc.), uronic acids, and even protein (Bilan et al., 2002; Holtkamp et al., 2009). The structures of fucoidan can be classified into two groups (Cumashi et al., 2007): repeating (1→3)-linked α-L-fucopyranose residues and alternating (1→3)- and (1→4)-linked α-L-fucopyranose residues.

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Fucoidan may also feature a (1→2)-α-L-fucopyranose structure (Li et al., 2008; Sinurat and Marraskuranto, 2013). The sulfate esters of fucoidan are located at positions 2 and 4, and rarely at 3; the position depends on the brown algae species (Ale et al., 2011; Hahn et al., 2012). The chemical composition of most fucoidans is varied and complex; therefore, the exact structure of fucoidan has not been determined.

The composition of fucoidan varies in brown algae species, environment, and collection season (Cumashi et al., 2007; Skriptsova et al., 2009). The molecular weight of fucoidan can range from 13 to 627 kDa (Hahn et al., 2012; Hickey, 2012), and even reach 3080 kDa (Kawamoto et al., 2006). The total sugar content of fucoidan is 8.9% in *Turbinaria turbinate* (García-Ríos et al., 2012) and 65.4% in *Ascophyllum nodosum* (Foley et al., 2011). The organic sulfate content of fucoidan is 0.41% in *Undaria pinnatifida* (Hemmingson et al., 2006) and 36.3% in *Fucus evanescens* (Cumashi et al., 2007). Finally, the uronic acid content of fucoidan is 3.5% in *Sargassum stenophyllum* (Duarte et al., 2001) and 23.4% in *C. okamuranus* (Cumashi et al., 2007). Hayakawa and Nagamime (2009) also reported that purified fucoidan contains less than 0.1% protein contamination.

The bioactivity of fucoidan is related to its molecular weight (Yang et al., 2008), the monosaccharide composition, the sulfate content, and the position of the sulfate ester group (Li et al., 2008). However, the activity may also depend on the extraction method, composition, and fucoidan structure. To date, a standard extraction protocol has not been established for fucoidan. Common extraction methods include various steps of pretreatment, using solvents for extraction, precipitation, and gel filtration chromatography. Pretreatments are necessary to remove chlorophylls, mannitol, salts, and other small compounds (Foley et al., 2011; Pettolino et al., 2012). Methanol-chloroform-water (MeOH-$CHCl_3$-H_2O, 4:2:1) (Ale et al., 2012) or 80 to 85% ethanol are usually used as pretreatment solvents (Yang et al., 2008). Acid (Zvyagintseva et al., 1999; Ale et al., 2012) or water (60-100°C) (Zvyagintseva et al., 1999; Yang et al., 2008) can also be used as extraction solvents, and $CaCl_2$ is sometimes used to precipitate alginate during the extraction process (Bilan et al., 2002). Extraction with acidic solutions, such as HCl, improved fucoidan yields (Kawamoto et al., 2006), but also partially decomposed the fucoidan (Ale et al., 2012). The addition of $CaCl_2$ to precipitate alginate can increase the purity of fucoidan but may also reduce its yield (Rioux et al., 2007). By contrast, using water (60-100°C) as a solvent is a method of extracting fucoidan that retains its natural bioactivity without decomposing its structure.

As mentioned, the uncertain composition of fucoidan and its health benefits result from the bioactivity of the fucoidan. Therefore, the structure of fucoidan in brown algae warrants further study. In this study, the content and composition of 6 species of brown macroalgal fucoidan were assessed using 2 extraction methods, followed by high-performance liquid chromatography (HPLC) and Fourier transform infrared-attenuated total reflectance (FTIR-ATR) spectroscopy.

II. MATERIALS AND METHODS

1. Algae Collection and Sample Preparation

All specimens were collected from coastal waters around Taiwan. *Sargassum glaucescens* and *S. hemiphyllum* were collected at Haikou Port, Pingtung; *S. horneri* was collected from Keelung; and *Laminaria japonica* was obtained from the Kinmen Fisheries Research Institute. *Endarachne binghamiae* was collected from Gongliao, and *Hizikia fusiformis* was collected from Yehliu. These algae were washed using tap water, dried in the sun, and then dried in a 60°C oven. Finally, they were milled (KIMAH Grinding Machine D3V-10, Taiwan) into a fine powder and stored in a 4°C refrigerator until further analysis.

1. Extraction Procedures

1) Method I

Following our lab protocol (Lee et al., 2012), 5 grams of algal powder from 6 species was individually added to 500-mL flasks containing 200 mL of deionized water (algal-water ratio of 1:40) and then boiled using a hot plate (PC-420D, Corning, USA) while stirring for 1 h. After cooling to room temperature, the extracts were centrifuged at 3273 × *g* (Allegra X-12R Centrifuge, Beckman Coulter, Inc., USA) for 10 min, and the supernatants were collected. A fraction of each algal supernatant was lyophilized (ScanVac MaxiVac Beta, Labogene ApS, Lynge, Denmark) and referred to as product A. This supernatant was treated with trichloroacetic acid (TCA) (Panreac, Spain) to precipitate the protein and then centrifuged at 3273 × *g* for 10 min. The sediment was lyophilized to yield product B. The supernatant was collected, and a portion of this supernatant was lyophilized to yield product C. The remaining supernatant was added to 4 volumes of 95% ethanol to precipitate the polysaccharides, which were recovered by centrifugation at 3273 × *g* for 10 min nd lyophilized to yield product D. These procedures are detailed in Fig. 1(A).

2) Method II

The methods of fucoidan extraction from brown macroalgae described by Yang et al. (2008), Rodriguez-Jasso et al. (2011), and Foley et al. (2011) were followed with some modification. Eight grams of algal powder from the 6 species were individually mixed with 100 mL of 85% ethanol and stirred for at least 12 h at room temperature to remove lipids and pigments. The solutions were then centrifuged at 3273 × *g* for 10 min to remove the supernatant. The remaining sediment of each algal species was then washed with acetone and left to dry at room temperature overnight. Five grams of the sediment of each algal species was extracted and stirred on a 65°C hot plate with 200 mL of deionized water for 1 h. After centrifugation at 3273 × *g* for 10 min, 1% $CaCl_2$ was added to the supernatant to precipitate the alginate, and the resultant solutions were stored in a 4°C refrigerator overnight. The solutions were then centrifuged at 3273 × *g* for 10 min, and the

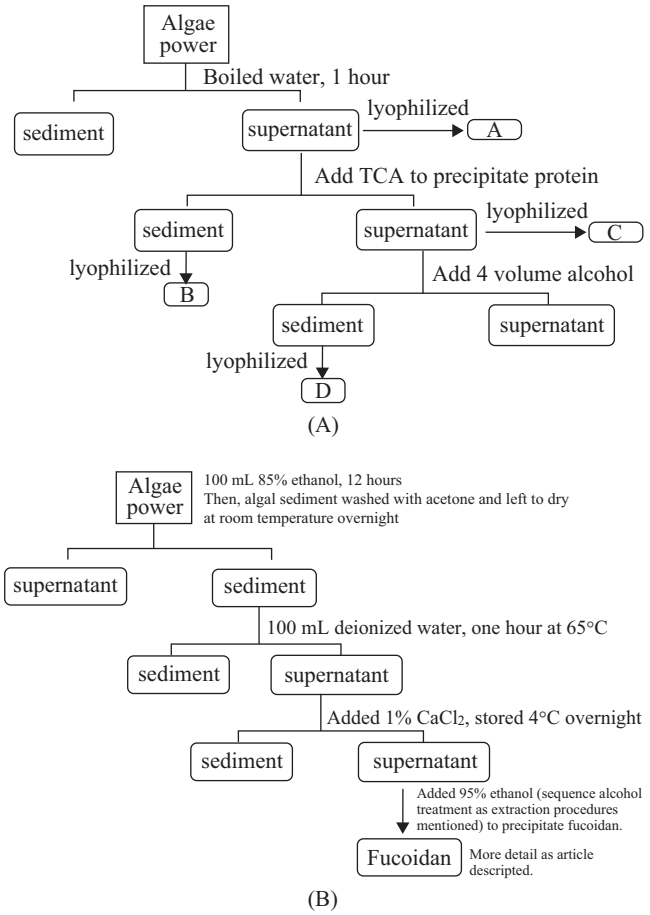

Fig. 1. Procedures for the extraction of water-soluble polysaccharides from brown macroalgae by (A) method I and (B) method II.

supernatants were harvested and subsequently added to 95% ethanol to obtain a final ethanol concentration of 30% (v/v). The solutions were then centrifuged at 3273 × *g* for 10 min to remove unwanted impurities. The supernatants were collected and individually added to 95% ethanol to reach a total ethanol concentration of 70% (v/v), and the resultant mixtures were stored at 4°C overnight. Finally, the fucoidan was recovered by centrifuging at 3273 × *g* for 10 min and stored at 4°C after being lyophilized. These procedures are outlined in Fig. 1(B). Fucoidan from *Fucus vesiculosus* (Sigma, USA) were used as a reference. We also extracted fucoidan from different collection times to gain different batches for analyzing the composition of polysaccharide.

3. Total Sugar Content

The total sugar content was analyzed using the phenol-sulfuric acid method according to the DuBois study (DuBois et al. 1956). L-fucose (Sigma, USA) was used as the standard (Duarte et al. 2001; Yang et al., 2008). Serially diluted standards were prepared at 20, 40, 80, 160, 320, and 640 μg/mL. Two hundred microliters of eluted samples or eluted standards were transferred into 10-mL test tubes containing 0.5 mL of 5% phenol, followed by the addition of 2.5 mL of 18 M H_2SO_4. Each tube was mixed thoroughly and incubated for 15 min. The absorbance was measured at 480 nm on a spectrophotometer (Model SP-830 plus, Taiwan). Serially diluted standards were calculated to obtain the standard curve.

4. Sulfate Groups

The sodium rhodizonate method (Terho and Hartiala, 1971) was followed to determine the sulfate content of the polysaccharide. First, 10 mL of 2 M acetic acid, 2 mL of 5 mM $BaCl_2$, and 8 mL of 0.02 M $NaHCO_3$ were diluted to 100 mL with 95% ethanol to serve as the $BaCl_2$ buffer. Subsequently, 5 mg of sodium rhodizonate (Acros, USA) was dissolved in 20 mL of distilled water, 100 mg of L-ascorbic acid was added, and the solution was brought to 100 mL with ethanol to serve as the sodium rhodizonate reagent. This reagent was light-sensitive; it had to be used within 2 days. Serially diluted Na_2SO_4 samples were used as a standard (0, 20, 40, 60, 80, 100, 120, and 140 μg/mL). HCl (1 N, 0.5 mL) was added to the lyophilized sample, and the resultant mixture was boiled for 2 h in a water bath. After HCl hydrolysis, the solvent was removed and concentrated using a vacuum concentrator (ScanSpeed MaxiVac Beta, Labogene) at 60-65°C, and the sample was then eluted using distilled water. One hundred microliters of the sample or standard solution was then added to 400 μL of ethanol (95%), 1 mL of $BaCl_2$ buffer, and 1.5 mL of sodium rhodizonate, and then the entire solution was performed in the dark for 10 min and measured at 520 nm on a spectrophotometer (Model SP-830 plus, Taiwan).

5. Determination of Protein Content

A protein assay kit (Bio-Rad Laboratories, USA) was utilized to measure the protein content of the extracted samples according to the Bio-Rad operation manual. Bovine serum albumin (New England BioLabs, USA) was used as a standard.

6. Monosaccharide Analysis of Polysaccharides

The monosaccharide content was assessed using an HPLC system comprising a pump (JASCO PU-2089, Japan), injection valve with a 20-μL sample loop, PL Hi-Plex H column (300.0 × 7.7 mm, Polymer Laboratories, England), and refractive index detector (JASCO RI-2031, Japan). Five to 10 mg of the sample was treated with 1 to 2 mL of 2 M trifluoroacetic acid at 121°C for 1 h. After trifluoroacetic acid hydrolysis, the reaction medium was dried with a vacuum concentrator, and distilled water was added to redissolve the sample. The resultant mixture was neutralized to approximately pH 7 by using 1 N NaOH. One milligram per milliliter of polysaccharide sample was injected into the HPLC system. The column was kept in a 65°C column oven (COLBOX, Taiwan), and distilled water was used as the mobile phase at a flow rate of 0.6 mL/min. The monosaccharide standard was L-fucose. The data were analyzed using the software ChromManager 6.93 (Analytical Based Development Center, Taiwan).

Table 1. The extraction rate and chemical composition of fucoidan resulted from different pre-treatments of washing-cleaned in *Sargassum horneri*.

Method I						
	Sample	Extraction rate (%)[a]	Total sugar (%)[b]	Sulfate content (%)[b]	Protein (%)[b]	Fucose (%)[b]
Sargassum horneri WS	A	42	8.80 ± 2.86	2.23 ± 1.24	2.48 ± 0.28	2.09 ± 0.28
	B	-	16.64 ± 7.42	3.69 ± 0.50	2.99 ± 0.50	1.48 ± 0.20
	C	-	6.24 ± 2.34	2.36 ± 1.04	2.62 ± 0.36	1.73 ± 0.08
	D	6	43.88 ± 12.11	7.85 ± 0.47	2.42 ± 0.19	8.02 ± 0.99
S. horneri WL	A	38.5	11.48 ± 2.94	2.63 ± 1.37	2.02 ± 0.33	1.61 ± 0.04
	B	-	16.18 ± 3.62	0.38 ± 0.55	1.62 ± 0.50	1.32 ± 0.03
	C	-	7.82 ± 2.35	2.75 ± 0.48	2.45 ± 0.04	1.39 ± 0.15
	D	9.5	63.37 ± 4.79	12.30 ± 1.86	2.9 ± 0.04	8.11 ± 1.31
Method II						
Sargassum horneri WS		7.8	46.00 ± 1.34	11.94 ± 0.20	1.6 ± 0.09	10.15 ± 0.91
S. horneric WL		6.4	62.86 ± 1.19	13.64 ± 0.06	2.18 ± 0.13	8.12 ± 1.65

-: not recorded.

a: % means dry weight of algal biomass.

b: % means dry weight of fucoidan.

S. horneri WS: using tap-water washed for a short period of time (fifteen seconds).

S. horneri WL: using tap-water washed for a long time (ten minutes).

7. Infrared Spectra of Brown Algae

The infrared spectra of isolated polysaccharides (freeze-dried) were recorded using an FTIR-ATR spectrometer (model DA8.3, Bomem, Canada) with a universal ATR sampling device. The spectra were recorded at an absorbance mode of 4000 to 600 cm^{-1}.

8. Data Analysis

Statistically significant differences between samples were analyzed using a one-way analysis of variance (ANOVA) test with the GraphPad Prism 6 program. P values less than 0.05 were considered statistically significant.

III. RESULTS AND DISCUSSION

1. Chemical Composition of Fucoidan Resulting from Different Washing Pretreatments

Collected algae were first cleaned with filtered seawater to remove contamination and then dipped in tap water to remove salt. *S. horneri* was used to assess the effect of the duration of the tap-water wash. A short wash (15 s) was labeled *S. horneri* WS, and a long wash (10 min) was labeled *S. horneri* WL. The 2 aforementioned extraction methods were used to obtain the fucoidan, and the chemical composition was examined (Table 1). With method I, the total sugar content of sample D was 43.88% and 63.37% for *S. horneri* WS and *S. horneri* WL, respectively (n = 3). With method II, the total sugar content was 46.00% and 62.86% in *S. horneri* WS and WL, respectively (n = 3). The washing duration was positively correlated with the sugar content, irrespective of the extraction method. We hypothesize that the salt and ion content was higher in the WS algae than in the WL algae, and these remnants affect the extraction efficiency of the algae. By contrast, the fucose content of sample D was 8.02% and 8.11% in *S. horneri* WS and WL, respectively, with method I. With method II, these figures were 10.15% and 8.12% for *S. horneri* WS and WL, respectively. This result demonstrates that the fucose content was independent of the wash duration.

2. Fucoidan Composition of Algae Extracted Using Methods I and II

In this study, 3 algae species, *S. glaucescens*, *S. horneri*, and *L. japonica*, were extracted by using methods I and II to determine a preferred method for fucoidan extraction. As shown in Table 2, method I yielded a total sugar content of 38.66% for sample A and 67.12% for sample D from *S. glaucescens*, 25.60% for sample A and 44.58% for sample D from *S. horneri*, and 31.82% for sample A and 52.89% for sample D from *L. japonica*. The sulfate content was 10.02% in sample A and 12.37% in sample D from *S. glaucescens*, 5.26% in sample A and 12.09% in sample D from *S. horneri*, and 5.78% in sample A and 9.78% in sample D from *L. japonica*. The current findings illustrate that, except for *S. glaucescens*, the total sugar and sulfate content increased after treatment with TCA and ethanol to remove protein and precipitate polysaccharides to obtain sample D. Table 2 presents the increases in the total sugar and sulfate content. The total sugar content increased from 8.80% to 43.88% and 11.48% to 63.37% in *S. horneri* WS and WL, respectively; the sulfate content increased from 2.23% to 7.85% and 2.63% to 12.30% in *S. horneri* WS and WL, respectively. Sample D of the 3 algae species yielded polysaccharides with more sulfated groups irrespective of the pretreatment method.

Table 2. The extraction rate and chemical composition of fucoidan of the algae extracted by methods I and II.

Method I						
	Sample	Extraction rate (%)[a]	Total sugar (%)[b]	Sulfate content (%)[b]	Protein (%)[b]	Fucose (%)[b]
Sargassum glaucescens	A	13.13	38.66 ± 3.79	10.02 ± 0.36	5.19 ± 0.33	5.74 ± 0.12
	B	-	23.49 ± 19.9	2.32 ± 0.01	3.07 ± 0.27	1.29 ± 0.20
	C	-	13.55 ± 0.64	3.28 ± 0.21	2.27 ± 0.14	3.65 ± 0.20
	D	2.00	67.12 ± 18.31	12.37 ± 0.16	1.75 ± 0.09	2.60 ± 0.20
S. horneri	A	24.00	25.60 ± 4.29	5.26 ± 0.30	1.49 ± 0.30	3.34 ± 0.04
	B	-	14.26 ± 0.79	1.75 ± 0.06	2.59 ± 0.14	1.48 ± 0.47
	C	-	9.97 ± 5.58	1.97 ± 0.10	0.19 ± 0.29	1.96 ± 0.02
	D	6.00	44.58 ± 0.52	12.09 ± 0.19	2.00 ± 0.25	3.37 ± 0.07
Laminaria japonica	A	22.67	31.82 ± 8.79	5.78 ± 0.21	1.98 ± 0.37	3.39 ± 0.09
	B	-	22.48 ± 1.39	1.13 ± 0.19	2.23 ± 0.23	1.92 ± 1.15
	C	-	7.95 ± 3.23	2.41 ± 0.06	0.04 ± 0.03	2.16 ± 0.18
	D	6.00	52.89 ± 1.69	9.78 ± 0.32	nd.	4.51 ± 0.07
Method II						
Sargassum glaucescens		4.20	39.72 ± 14.57	15.28 ± 2.15	nd.	13.42 ± 1.40
S. horneri		4.80	38.98 ± 3.18	14.08 ± 1.32	0.62 ± 0.21	7.30 ± 2.23
Laminaria japonica		4.64	59.08 ± 33.53	18.36 ± 0.78	3.33 ± 1.04	8.30 ± 1.35

-: not recorded.
nd: not detectable.
a: % means dry weight of algal biomass.
b: % means dry weight of fucoidan.
Each values represents the mean ± SD of triplicate measurements.

In method I, TCA was used to remove protein from the algal materials to yield sample B, and the total protein content was 3.07%, 2.59%, and 2.23% in *S. glaucescens*, *S. horneri*, and *L. japonica*, respectively. The total sugar content was 23.49%, 14.26%, and 22.48% in *S. glaucescens*, *S. horneri*, and *L. japonica*, respectively. These data demonstrate that using TCA to remove proteins may also reduce the sugar content because of the interaction of fucoidan with protein (Jones and Williams, 1990).

Method II yielded fucose content of 13.42%, 7.30%, and 8.30% for *S. glaucescens*, *S. horneri*, and *L. japonica*, respectively. The sulfate content was 15.28%, 14.08%, and 18.36% in *S. glaucescens*, *S. horneri*, and *L. japonica*, respectively. The fucose and sulfate fucoidan content were greater than those obtained by method I for *S. glaucescens*, *S. horneri*, and *L. japonica*. These differences were due to the use of 85% ethanol in method II to remove other compounds, such as pigments and small compounds, as well as the use of $CaCl_2$ to remove alginate. Therefore, method II extracted more purified fucoidan than did method I. Because fucoidan mainly comprises sulfated L-fucose (Berteau and Mulloy, 2003; Usov and Bilan, 2009; Patel, 2012), fucose, sulfate, and L-fucose can be used to represent the quality of the fucoidan. Cho et al. (2010) reported that the bioactivity of fucoidan was positively correlated with sulfate content. In this study, the fucose and sulfate content levels yielded using method II were higher than those recorded using method I. Thus, we believe that method II yielded higher quality fucoidan than did method I.

As shown in Fig. 2(A), the total sugar content in fucoidan extracted using method II depended on the batches. Katohda et al. (1976) reported that the total sugar content of cell walls varied from 56% to 72%; therefore, using the total sugar content to represent the amount of fucoidan is inappropriate because the samples contain many other sugar types. To date, the structure and composition of fucoidan have been difficult to determine because many factors, such as the extraction method, environment, and complexity of fucoidan, affect experimental results (Ale et al., 2011), and thus affect the color and composition of fucoidan by batch (Holtkamp et al., 2009). The content of fucoidan cannot be easily quantified. However, Fig. 2(B) shows the fucose content of fucoidan extracted from the same batch: the highest fucose content was extracted from *S. glaucescens*, followed by *S. horneri* and *L. japonica*, irrespective of the extraction batch. Hence, the fucose content can be used as a quantitative indicator to identify the algal species with the highest fucoidan content. Consequently, suitable algal species can be selected to improve their utility during commercialization.

3. Fucoidan Extracted Using Method II

According to the results in Section III-2, method II improved the purity of fucoidan more than method I did. Therefore, method II was used to conduct all subsequent research, which was aimed at determining the fucoidan compositions of the 6 algal species.

The fucoidan content varied considerably from 0.31% to

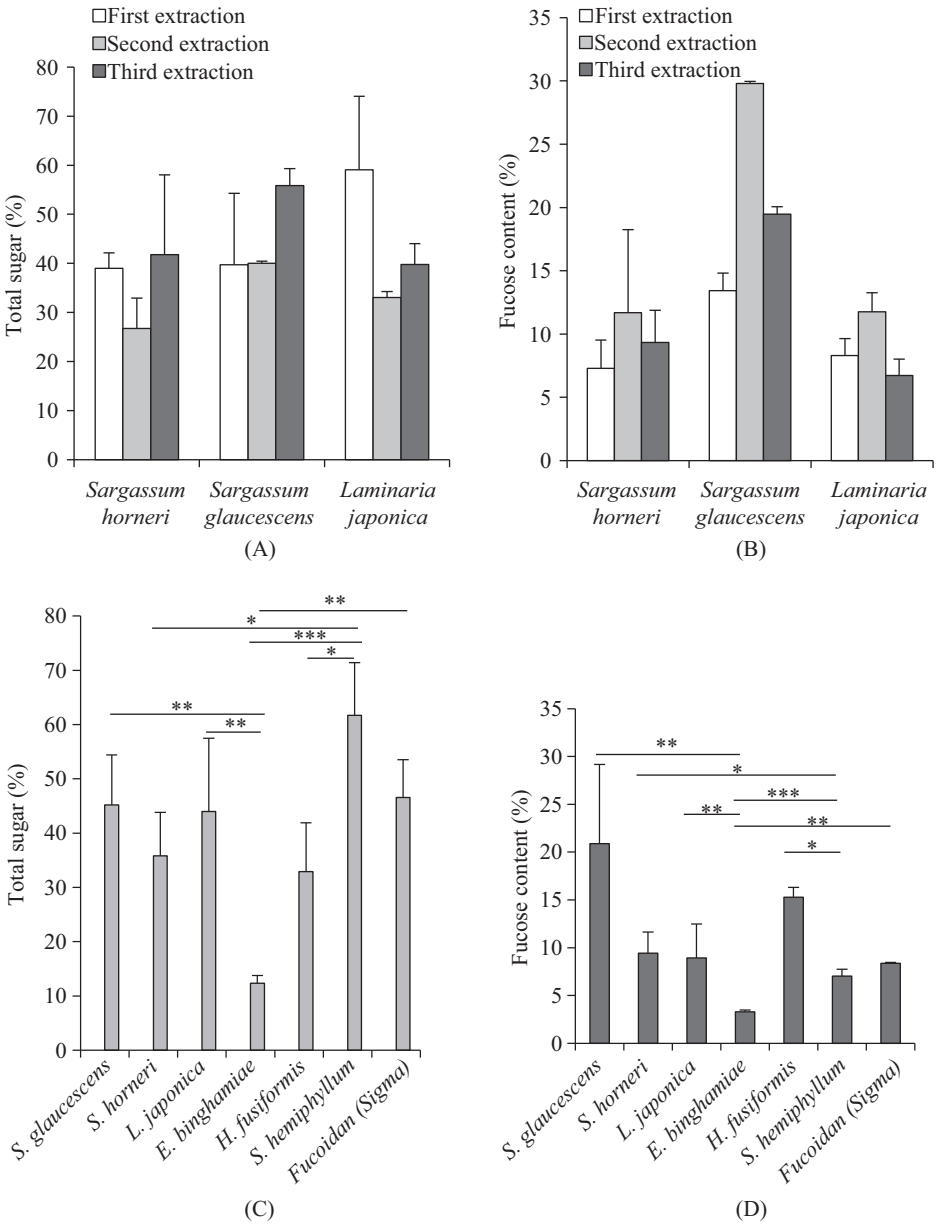

Fig. 2. Total sugar (A) and fucose content (B) were determined by method II. Each bar indicated different extraction batches. Total sugar content (C) and fucose content (D) of fucoidan from different species were all extracted by method II. Data were analyzed by one-way ANOVA. * means P < 0.05, **P < 0.01 and ***P < 0.005. Each values represents the mean ± SD of triplicate measurements.

7.00% dry weight in the 6 algal species, among which *S. horneri* was 6.90%, followed by *E. binghamiae* (6.34%) and *H. fusiformis* (6.00%). These proportions were higher than those reported by Foley et al. (2011) and Wang et al. (2008), both of which used multistep extraction in *S. horneri* and *L. japonica*, yielding 5.17% and 2.3% fucoidan, respectively. As for *S. glaucescens*, *S. hemiphyllum*, and *L. japonica*, the maximal fucoidan content was 4.00%, 3.40%, and 3.00%, respectively.

This study reports the yields of fucoidan for only *S. glaucescens* (4.00%) and *E. binghamiae* (6.34%). *E. binghamiae* is a common economic food (Huang and Lee, 2005) and is a potential material for extracting fucoidan; however, the fucose content of *E. binghamiae* was the lowest among the 6 species, at 3.29% (Table 3). Hence, the extraction rate and fucose content must be considered together to maximize the yield of fucoidan.

4. Composition of Fucoidan Extracted Using Method II

Fucoidan is a heteropolysaccharide that mainly comprises sulfated L-fucose residues such as fucose and sulfate; but it may also contain other monosaccharides (glucose, mannose, galactose, xylose, etc.), uronic acids, and even protein (Bilan et al., 2002; Holtkamp et al., 2009).

Figs. 2(C) and 2(D) present the total sugar and fucose

Table 3. The composition of fucoidan from six species of brown algae (% means dry weight of fucoidan).

	Total sugar (%)	Sulfate content (%)	Protein (%)	Fucose (%)
Sargassum glaucescens	45.19 ± 9.22	13.51 ± 0.22	0.75 ± 0.83	20.89 ± 8.28
S. horneri	35.83 ± 8.00	13.63 ± 0.57	0.68 ± 0.32	9.44 ± 2.20
Laminaria japonica	43.97 ± 13.51	13.55 ± 0.16	0.22 ± 0.31	8.93 ± 3.55
Endarachne binghamiae	12.33 ± 1.45	13.19 ± 0.13	0.12 ± 0.05	3.29 ± 0.18
Hizikia fusiformis	32.89 ± 9.00	13.43 ± 0.14	1.00 ± 0.01	15.29 ± 1.00
S. hemiphyllum	61.71 ± 9.71	14.32 ± 1.47	1.13 ± 0.05	7.02 ± 0.72
Fucoidan (Sigma)	46.55 ± 7.00	14.29 ± 0.06	1.80 ± 0.01	7.69 ± 1.52

Each values represents the mean ± SD of triplicate measurements.

contents of fucoidan extracted using method II in 6 algal species. The total sugar content ranged from 12.33% (*E. binghamiae*) to 61.71% (*S. hemiphyllum*) among the 6 brown algae. In the Sargassaceae family, the total sugar content in *S. glaucescens*, *S. horneri*, *S. hemiphyllum*, and *H. fusiformis* was 45.19%, 35.83%, 61.71%, and 32.89%, respectively. Other studies have reported a total sugar content ranging from 41.4% to 66.0% in *S. filipendula* (Costa et al., 2011), 56.6% in *S. stenophyllum* (Duarte et al., 2001), 31.8% in *S. hemiphyllum* (Huynh et al., 2011), and 30.6% in *H. fusiformis* (Pereira, 2011). The total sugar content of the *Sargassum* species in this study ranged from 30.6% to 66.0%. In algae not from the Sargassaceae family, the total sugar content was 43.97% and 12.33% in *L. japonica* and *E. binghamiae*, respectively. In contrast to the study by Xue et al. (2004), the current study found that *L. japonica* contained 324.1-468.5 g/kg (32.41%-46.85%) of total sugar. Table 3 shows that the fucoidan of *Sargassum* and *Laminaria* consisted of approximately 40% total sugar, and that the total sugar content in *S. hemiphyllum* (61.71%) was the highest among the 6 species.

The sulfate content of fucoidan may influence its bioactivity (Ale et al., 2011). For example, the anticoagulant activity of fucose-containing sulfated polysaccharides from *Ecklonia kurome* depended on both the molecular weight and sulfate content (Nishino et al., 1991). In the present study, the sulfate content of fucoidan was approximately 13% in all 6 species. The sulfate content of *L. japonica* was lower than those reported by Cumashi et al. (2007) (27.5%-29.6%), but that of *S. hemiphyllum* (14.32%) was higher than that reported by Huynh et al. (2011) (3.6%). This difference may be due to the differing habitats and seasons (Skriptsova et al., 2009) examined in the studies.

In this study, the protein content of the 6 algal species was lower than that of the commercial fucoidan from Sigma, indicating that our extraction method removed more protein than did Sigma's method. The protein content ranged from 0.2% to 2.5% in *S. filipendula* (Costa et al., 2011; García-Ríos et al., 2012), and reached 12.4% in *S. longicruris* (Rioux et al., 2007). According to Hayakawa and Nagamime (2009), the protein content of fucoidan should be less than 0.1%. Method II of our study resulted in the lowest protein content of 0.12%, which is near 0.1%. Consequently, method II is simpler and more convenient for removing protein from fucoidan than advanced methods such as gel filtration, which may reduce the fucoidan yield.

As shown in Table 3, a monosaccharide analysis of fucoidan using HPLC determined that the maximum fucose content of fucoidan was 22.89% and 15.29% in *S. glaucescens* and *H. fusiformis*, respectively. This is also shown in Fig. 2(D). Eluvakkal et al. (2010) extracted 232.5 mg/g of fucoidan on a dry weight basis from *S. wightii* (equivalent to 23.25% fucoidan), which is similar to the value for *S. glaucescens* (20.89%) obtained in the present study. Wang et al. (2008) and Cumashi et al. (2007) have reported that the fucose content in the Laminariaceae family ranged from 29.12% to 36.7%. However, the present study found values that were 8.93% lower. This difference may be due to the different extraction methods, habitat, and time of acid hydrolysis (Emaga et al., 2012).

The composition of fucoidan differed among the 6 algal species (Tables 1 and 2). The results were similar to those of Ale et al. (2011, 2012), which confirm that the extraction method significantly influenced the composition and yield of fucoidan.

S. glaucescens is native to Taiwan; however, the biomass available for industrial fucoidan extraction is low. *H. fusiformis* is a commercially cultivated brown algae that grows quickly (approximately 3 cm d^{-1}) (Pang et al., 2005); therefore, it may be an effective material for extracting fucoidan.

5. FTIR Analysis of Polysaccharides

The FTIR-ATR spectra from 4000 to 600 cm^{-1} of the polysaccharide are shown in Figs. 3(A) and (B). The infrared spectra in the 1200-800 cm^{-1} region provide information about the major polysaccharides present in complex polysaccharide mixtures (Kacurakova, et al., 2000). Freitas et al. (2011) indicated that the broad band near 3400 cm^{-1} represents the O-H stretching of hydroxyls common to all polysaccharides, and that the peak near 2930 cm^{-1} indicates the C-H stretching vibration of carbohydrates (Kim et al., 2010; Freitas et al., 2011). These peaks were evident in the FTIR-ATR spectra obtained in the present study. Two peaks at 1620 cm^{-1} and 1416 cm^{-1} indicated the asymmetric and symmetric stretching vibrations of carboxylate ($RCOO^{-}$), respectively, and were identified according to the results of Na et al. (2010) and

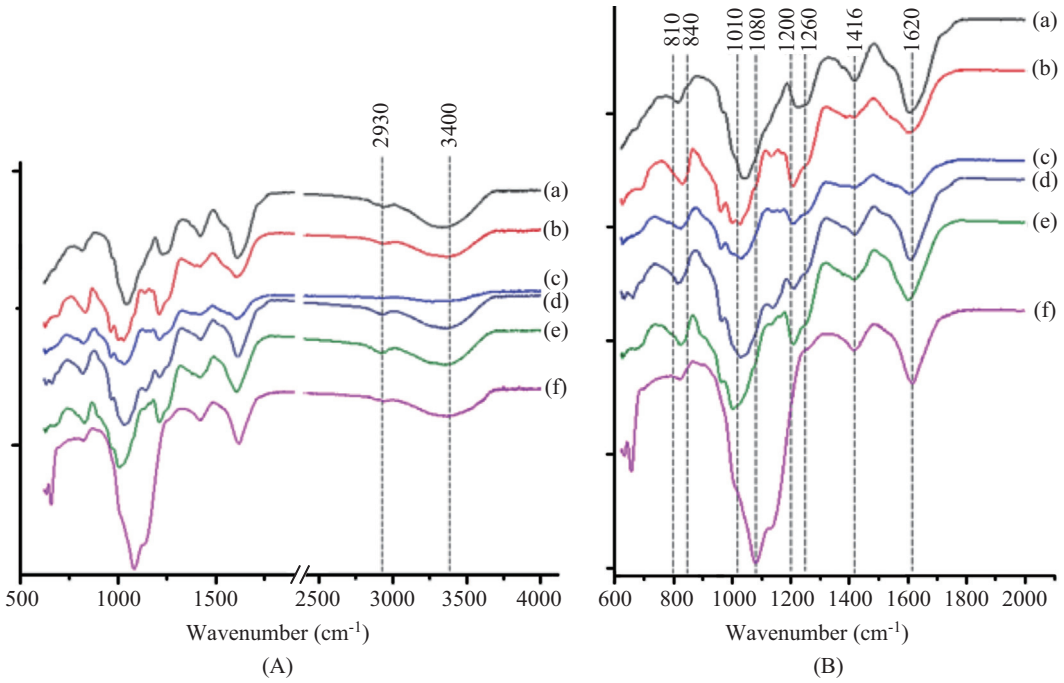

Fig. 3. Infrared spectra of the isolated polysaccharide. (A), The infrared spectra range from 4000-600 cm^{-1}: (a) *Laminaria japonica*; (b) *Sargassum glaucescens*; (c) *S. hemiphyllum*; (d) *S. horneri*; (e) *Hizikia fusiformis*; (f) *Endarachne binghamiae*. (B), Partial infrared spectra enlarge from Fig. 3 A (range from 2000-600 cm^{-1}).

Synytsya et al. (2003). The peak near 1260-1220 cm^{-1} was described by Cabassi et al. (1978) and Pereira et al. (2009) and was assigned to primary and secondary O-sulfate groups, which are a characteristic component of fucoidan and sulfated polysaccharides in marine algae (Pereira et al., 2013). These peaks were also identified in the present study. Duarte et al. (2001) reported that polysaccharide fractions from *S. stenophyllum* showed a band at 837 cm^{-1}, which indicated sulfate groups at the C-4 positions of the structural monosaccharides; moreover, Maciel et al. (2008) suggested that the band near 840-820 cm^{-1} (C-O-S) indicated a complex substitution of 4-sulfate and 6-sulfate monosaccharide units. The absorption band at 1080-1010 cm^{-1} was attributed to glycosidic links (Şen and Avcı, 2005). Accordingly, the fucoidan from all 6 algal species in the present study were found to be sulfated polysaccharides. The FTIR-ATR spectra of the 6 algal species identified the functional groups and structure of fucoidan. However, the spectra of fucoidan are determined by the backbone composition and influenced by side-chain constituents (Kacurakova et al., 2000). Additional equipment, such as nuclear magnetic resonance spectroscopy, electrospray ionization-tandem mass spectrometry, and quadrupole-time-of-flight mass spectrometry, is required to clearly identify the structure of fucoidan for future investigations and applications.

IV. CONCLUSIONS

The present study found that the duration of washing pretreatments affected the fucoidan composition of algal materials. The duration of washing was positively correlated with the total sugar content of the algae irrespective of fucose content. Moreover, the total sugar content depended on the batch. However, the fucose content can be used to estimate the fucoidan content.

The present study also found that treating algal materials with $CaCl_2$ (method II) can increase fucoidan yields. Although the composition of fucoidan differed among the 6 algal species examined, the extraction methods significantly influenced the fucoidan composition and yield. The present study found that *S. glaucescens* and *H. fusiformis* were the optimal sources for extracting fucoidan among the 6 algae species, and that *H. fusiformis* was easily obtained because of its rapid growth rate. Consequently, *H. fusiformis* was suggested to be the optimal algal material for the extraction of fucoidan. The overall FTIR spectra of the polysaccharide fucoidan indicated similar structures with slightly different side-chain constituents that differed by species. The fucoidan content varied among the brown macroalgae species; thus, species-specific extraction methods and further investigation of the bioactive mechanism of fucoidan are necessary.

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