Isolation and Characterization of Fucoidan from *Hizikia fusiformis* (Hijiki)*

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Abstract: The polysaccharide was isolated from *Hizikia fusiformis* (Hijiki), which was harvested in Okinawa Prefecture, Japan. The yield of the polysaccharide was 0.4% (w/w) based on wet seaweed. The total carbohydrate, uronic acid, ash and sulfuric acid of the polysaccharide were 66.5, 13.7, 30.8 and 26.3%, respectively. After the acid hydrolysis of the polysaccharide, L-fucose, D-galactose, D-glucose, D-mannose, D-xylose and D-glucuronic acid were identified by liquid chromatography. The molar ratio of these sugar components and sulfuric acid was calculated to be L-Fuc : D-Gal : D-Glc : D-Man : D-Xyl : D-GlcA : H₂SO₄ = 5 : 1 : 1 : 0.5 : 0.5 : 2 : 7. The molecular mass of the polysaccharide was estimated by gel chromatography to be about 4.3×10^4 . The IR spectrum of the polysaccharide was in agreement with that of standard fucoidan. The L-fucose, D-glucuronic acid and acetyl group were identified by ¹H- and ¹³C-NMR spectroscopy. The ¹H-NMR spectral data suggests that fucoidan is mainly composed of 3-linked unsulfated, 4-linked unsulfated, 3-linked 2-mono-O-sulfated, 4-linked 2-mono-O-sulfated and 3-linked 2,4-di-O-sulfated L-fucopyranose.

Key words: fucoidan, *Hizikia fusiformis*, NMR spectroscopy

Fucoidan is a complex sulfated polysaccharide found in the cell-wall matrix of brown algae and some echinoderms. Fucoidan has been extensively investigated because of its various biological activities as an anticoagulant,¹⁻⁵⁾ antitumor⁶⁾ and antiviral^{7,8)} agents.

In previous paper, we isolated a fucoidan, which consisted of L-fucose, D-xylose, D-glucuronic acid, sulfuric acid and acetic acid in a molar ratio of 4.0 : 0.03 : 1.0 : 2.0 : 2.0, from *Cladosiphon okamuranus* (Okinawamozuku)⁹⁾ which had been commercially cultured on artificially seeded nets (1.5 × 20 m). The yield of the fucoidan was estimated to be 2.3% (w/w) based on wet algae. This suggested that fucoidan could be commercially produced on a simple industrial scale from artificially cultured Okinawamozuku.¹⁰⁾ The annual production of the algae in Okinawa is reported to have been approximately 13,200 t in 2002.

We also isolated a fucoidan, which consisted of L-fucose, D-galactose and sulfuric acid in a molar ratio of 1.0 : 0.05 : 1.0, from *Nemacystus decipiens* (Itomozuku) which had also been commercially cultured on artificially seeded nets.¹¹⁾ The yield of the fucoidan was estimated to be 0.5% (w/w) based on wet algae. The annual production of the algae in Okinawa is reported to have been approximately 2,000 t in 2002. These seaweeds (Okinawamozuku and Itomozuku) are used in salad (Sunomono), so their utilization by the food industry has recently been increasing.

Hijiki (*Hizikia fusiformis*), one of the brown algae, is widespread in nature between Hokkaido and Okinawa

Prefecture in Japan. The annual production of Hijiki, in Okinawa, is reported to have been approximately 120 t in 2002. Fucoidan from Hijiki has been reported by Nishino *et al.*,¹²⁾ Nishide *et al.*¹³⁾ and Dobashi *et al.*,¹⁴⁾ but the polysaccharide from the algae harvested in Okinawa has not yet been studied. We report now the isolation and chemical characterization of fucoidan from the Hijiki.

MATERIALS AND METHODS

Materials. Hijiki (*Hizikia fusiformis*) used in this study was harvested in June 2001 from Yonabaru Town, Okinawa. The collected seaweed was washed with tap water and air-dried in oven at 40°C for 24 h. The dried seaweed was powdered in a blender.

Fucoidan used as the standard was extracted from *Fucus vesiculosus* (Sigma Co., Ltd., USA).

Extraction and purification of the polysaccharide from Hijiki. The dried seaweed was suspended in 0.2 M HCl and stirred at room temperature for 24 h for extraction of polysaccharide. The extract was then filtered through Celite 545 and neutralized with 0.5 M NaOH. The neutralized solution was concentrated and precipitated by the addition of 2 volume of ethanol. The precipitate was dried *in vacuo*.

The crude polysaccharide was dissolved in 0.1 M CaCl₂ solution and passed through Celite 545. The filtrate was dialyzed against distilled water overnight at room temperature and freeze-dried.

Purified polysaccharide was redissolved in distilled water and the solution deionized by passage through a column of Amberlite 120 (H⁺), and then neutralized with 50 mM NaOH. The solution was filtered through Celite 545 again. The filtrate was dialyzed against distilled water

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over-night at room temperature and freeze-dried.

Deacetylation of polysaccharide. An aqueous 1.5% solution of the polysaccharide was treated under an atmosphere of nitrogen with 0.05 M NaOH at room temperature over-night to accomplish deacetylation. The solution was neutralized with 0.05 M HCl, and then the product was dialyzed against distilled water and freeze-dried.

Chemical procedure. Total carbohydrate content was determined by the phenol-sulfuric acid method,¹⁵ using L-fucose as a standard. Uronic acid content was determined by the carbazole-sulfuric acid method,¹⁶ using D-glucuronic acid as a standard.

Ester sulfate in the polysaccharide hydrolyzate, which had been treated by heating a 100°C for 3 h in a 2 M HCl solution, was determined by ion chromatograph DX500 (Dionex Co., Ltd., USA) on a column (AS4A 4×250 mm) at a flow rate of 1 mL/min at 35°C. The column was developed with a 1.7 mM NaHCO₃ and 1.8 mM Na₂CO₃ solution.

Acid hydrolysis. Acid hydrolysis was carried out by dissolving the polysaccharide from Hijiki in distilled water and adding sulfuric acid to a final concentration 2.0 M. The mixture was heated at 100°C for 3 h. The hydrolyzate was neutralized with BaCO₃.

Liquid chromatography. The polysaccharide hydrolyzate was applied to a liquid chromatograph DX500 (Dionex Co., Ltd., USA) on a column (Carbopac PAL4×250 mm) equilibrated with 3 mM NaOH for neutral sugar and 100 mM NaOH+150 mM CH₃COONa for D-glucuronic acid. The chromatography was carried out at a flow rate of 1 mL/min at 35°C.

Molecular mass. The molecular mass of the polysaccharide from Hijiki was determined by high-performance liquid chromatography (HPLC) (LC-6A; Shimadzu Co., Japan) with a column of TSKgel PW_{XL} (7.8×300 mm, Tosoh Co., Japan) and detected with a refractive index detector RID-6A (Shimadzu Co., Japan) at room temperature. The column was eluted by 150 mM NaCl in a 50 mM phosphate buffer (pH 7.2) at a flow rate of 0.3 mL/min. Pullulan P-5 ($M_w=0.59 \times 10^4$), P-20 (2.28×10^4), P-100 (11.2×10^4) and P-400 (40.4×10^4) (Showa Denko Co., Japan) were used as the molecular weight standards.

Optical rotation. Optical rotation of the polysaccharide from Hijiki was measured at 589 nm on a polarimeter DIP-180 (Japan Spectroscopic Co., Ltd., Japan) for a 0.2% (w/v) solution in distilled water.

Infrared spectra. Infrared spectra were recorded with an Infrared spectrometer FTS-3000 (Bio-Rad Lab., Inc., USA) for a sample dispersed in KBr discs.

¹H- and ¹³C-Nuclear magnetic resonance (NMR) spectroscopy. The ¹H- and ¹³C-NMR spectra were recorded on a FT-NMR spectrometer JNM-A500 (Nihondenshi Co., Ltd., Japan) at 500.00 and 125.65 MHz, respectively. The polysaccharide (5%) was dissolved in D₂O, and the data recorded at 60°C. The ¹H- and ¹³C-NMR chemical shifts are expressed in parts per million (ppm) relative internal sodium 3-(trimethylsilyl)propionic-2,2,3,3-d₄ (TSP, 0.00 ppm).

RESULTS AND DISCUSSION

The harvested seaweed (2000 g) was washed with tap water and then air-dried in an oven at 40°C for 24 h. The weight of the air-dried seaweed decreased to 280 g. The polysaccharide from *Hizikia fusiformis* (Hijiki) was extracted in 0.2 M HCl and purified as described in MATERIALS AND METHODS. The yield of the purified polysaccharide was 0.4% based on wet seaweed.

The total carbohydrate, uronic acid and ash of the polysaccharide were 66.5, 13.7 and 30.8%, respectively. The sulfuric acid content of the polysaccharide was estimated to be 26.3% (Table 1).

The polysaccharide hydrolyzate was applied to a liquid chromatograph and L-fucose, D-galactose, D-glucose, D-mannose and D-xylose were detected (Fig. 1(A)). The uronic acid of polysaccharide component was confirmed to D-glucuronic acid by a liquid chromatograph (Fig. 1(B)). According to each percentage of total carbohydrate, uronic acid and sulfuric acid content, the molar ratio of these sugar components and sulfuric acid was calculated to be L-Fuc : D-Gal : D-Glc : D-Man : D-Xyl : D-GlcA : H₂SO₄ = 5 : 1 : 1 : 0.5 : 0.5 : 2 : 7 (Table 2). The results differed from fucoidans isolated from Hijiki which were harvested on the main islands of Japan (Fuc : Xyl : Man : Gal : GlcA : H₂SO₄ = 6 : 1 : 1 : 3 : 4.5 : 9 by Nishino *et al.*¹²); Fuc : Uronic acid : H₂SO₄ = 1 : 0.56 : 3.03 by Nishide *et al.*¹³); Fuc : Xyl : Man : Gal : GlcA : H₂SO₄ = 100 : 1 : 6 : 6 : 3 : 186 by Dobashi *et al.*¹⁴).

The molecular mass of purified polysaccharide was es-

Table 1. Chemical components of polysaccharide from Hijiki (% w/w)

	Carbohydrate	Uronic acid	Ash	H ₂ SO ₄
Polysaccharide	66.5	13.7	30.8	26.3

Table 2. Molar ratio of the sugar components and sulfuric acid of polysaccharide from Hijiki.

L-Fuc	D-Gal	D-Glc	D-Man	D-Xyl	D-GlcA	H ₂ SO ₄
5	1	1	0.5	0.5	2	7

Fig. 1. Liquid chromatogram of the polysaccharide from Hijiki.

(A): (1) L-fucose, (2) D-xylose, (3) D-galactose, (4) D-glucose, (5) D-mannose. Column, Carbopac PAL (4×250 mm); eluent, 3 mM sodium hydroxide; eluent flow, 1 mL/min. (B): (6) D-glucuronic acid. Column, Carbopac PAL (4×250 mm); eluent, 100 mM sodium hydroxide+150 mM sodium acetate; eluent flow, 1 mL/min.

timated with pullulan as standards to be about 4.3×10^4 by gel permeation chromatography with a column of TSKgel PWXL (Fig. 2).

The optical rotation of the polysaccharide (0.2%, w/v) showed a value of -0.032° at 60°C and decreased a little with decreasing temperature to a value of -0.035° at 10°C (Table 3).

The infrared spectrum of the polysaccharide is shown in Fig. 3(A). The spectrum indicated a hydroxyl group at 3400 cm^{-1} and a sulfate group at 1255 cm^{-1} .¹⁷⁾ Besides chemical analysis, it also became clear that a sulfate group is included in the polysaccharide from Hijiki with IR spectrum. A small shoulder of absorption at 1720 cm^{-1}

Fig. 2. Gel permeation chromatogram of polysaccharide from Hijiki.

Column, TSKgel PWXL ($7.8 \times 300\text{ mm}$); eluent, 150 mM sodium chloride in a phosphate buffer (pH 7.2); eluent flow, 0.3 mL/min. The calibration curve was made with pullulan with molecular masses of 40.4 , 11.2 , 2.28 and 0.59×10^4 as a standard.

Fig. 3. Infrared spectra of the purified polysaccharide from Hijiki at $4000\text{--}400\text{ cm}^{-1}$.

(A), native polysaccharide; (B), alkaline-treated polysaccharide. Arrow shows disappearance of absorption at 1720 cm^{-1} .

Table 3. Optical rotation of the polysaccharide from Hijiki at 589 nm.

	Temperature ($^\circ\text{C}$)						
	10	20	25	30	40	50	60
α degree	-0.035	-0.034	-0.033	-0.033	-0.033	-0.032	-0.032

was observed in the polysaccharide. The spectrum was in agreement with that of standard fucoidan (Figure not shown). The spectrum of alkaline treated polysaccharide is shown in Fig. 3(B). After the alkaline treatment of native polysaccharide, a small shoulder of absorption at 1720 cm^{-1} disappeared. This may be due to de-esterification of the polysaccharide.¹⁸⁾

The $^1\text{H-NMR}$ spectra of native (A) and alkaline treated (B) polysaccharide are shown in Fig 4. The chemical shift of the envelope of anomeric signals of the native polysaccharide (A) at $5.0\text{--}5.5\text{ ppm}$ is consistent with the presence of $\alpha\text{-L-fucopyranosyl}$ units.¹⁹⁾ The signals at 5.41 , 5.33 , 5.22 , 5.11 and 5.04 ppm can be assigned to 4-linked 2-mono-*O*-sulfated, 3-linked 2,4-di-*O*-sulfated, 3-linked 2-mono-*O*-sulfated, 4-linked unsulfated and 3-linked unsulfated L-fucopyranose.^{11,18-20)} The signals at $2.2\text{--}2.0\text{ ppm}$ were assigned to the methyl protons of the acetyl group.¹⁰⁾ The signals at $1.5\text{--}1.1\text{ ppm}$ were assigned to the methyl proton group of the L-fucopyranose¹⁰⁾ where 1.41 , 1.24 and 1.18 ppm can be assigned to 4-linked 2-mono-*O*-sulfated, 3-linked 2-mono-*O*-sulfated and 3-linked unsulfated L-fucopyranose,¹⁸⁾ respectively. The signals at $2.2\text{--}2.0\text{ ppm}$ of the alkaline treated polysaccharide (B) disappeared. The result indicates that the acetyl groups were removed by alkaline treatment.¹⁰⁾

The $^{13}\text{C-NMR}$ spectra of native (A) and alkaline treated (B) polysaccharide from Hijiki are shown in Fig. 5. The signals of the spectrum of native polysaccharide (A) in the range of $20.0\text{--}18.5\text{ ppm}$ assigned to the methyl carbon group of the L-fucopyranose,¹⁷⁾ in the range of $87.4\text{--}63.6\text{ ppm}$ assigned to the ring carbons and in the range of

Fig. 4. $^1\text{H-NMR}$ spectra of the polysaccharide from Hijiki in D_2O , at 60°C .

(A), native polysaccharide; (B), alkaline-treated polysaccharide. 1,4-linked 2-mono-*O*-sulfated L-fucopyranose; 2,3-linked 2,4-di-*O*-sulfated L-fucopyranose; 3,3-linked 2-mono-*O*-sulfated L-fucopyranose; 4,4-linked unsulfated L-fucopyranose; 5,3-linked unsulfated L-fucopyranose.

Fig. 5. ^{13}C -NMR spectra of the polysaccharide from Hijiki in D_2O , at 60°C .

(A), native polysaccharide; (B), alkaline-treated polysaccharide.

105.8–102.6 ppm assigned to the anomeric carbons. The signals were complicated and these analysis are difficult. However, the disappearance of four signals were observed in the spectrum of alkaline treated polysaccharide (B). The disappearing signals of 25.3 and 23.4 ppm were methyl carbons of acetyl group^(10,20) and that of 177.2 and 175.9 ppm were considered to be carbonyl carbons of acetyl group.⁽¹⁰⁾ Therefore, the remaining signal of 187.4 ppm in low-field was assigned to the carbonyl carbon of D-glucuronic acid.

These results indicated that the polysaccharide from Hijiki is a fucoidan. Sulfate and acetyl groups were included in Hijiki fucoidan which were confirmed by IR and NMR spectroscopy, particularly, the sulfate groups attached to the C-2 or C-2 and C-4 of α -L-fucopyranosyl residues which was confirmed by ^1H -NMR spectroscopy. It is reported that fucoidan's molar ratio of neutral sugars and ester sulfate affect its biological activities.^(21,22) In particular, fucoidan from Hijiki collected in the main islands had anticoagulant activity,⁽¹⁴⁾ so fucoidan from Hijiki collected in Okinawa also has to have this activity. Other biological activities of fucoidan from Hijiki collected in Okinawa, including more detailed structural analysis, are now under investigation.

In conclusion, it was elucidated here that Hijiki (*Hizikia fusiformis*) from Okinawa can be used as a source of fucoidan, the same as Okinawamozuku (*Cladosiphon okamuranus*)⁽¹⁰⁾ and Itomozuku (*Nemacystus decipiens*).⁽¹¹⁾

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沖縄産ヒジキからのフコイダンの分離・同定

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沖縄産ヒジキから塩酸溶液により多糖を抽出した。常法により精製した多糖の全糖、ウロン酸、灰分および硫酸含量はそれぞれ 66.5, 13.7, 30.8 および 26.3% であった。構成糖は L-Fuc, D-Gal, D-Glc, D-Man, D-Xyl および D-GlcA で、その比率は L-Fuc : D-Gal : D-Glc : D-Man : D-Xyl : D-GlcA : H₂SO₄ = 5 : 1 : 1 : 0.5 : 0.5 : 2 : 7 と算出された。プルランを標準物質としたゲル濾過法により、ヒジキ精製多糖の分子量は約 43,000 と見積もられた。精製多糖の IR スペクトルは標本フコイダンと広い波数領域でのよい一致を示し、¹H-および¹³C-NMR スペクトルから L-フコース残基, D-グルクロン酸残基およびアセチル基の存在が確認された。¹H-NMR スペクトルより 3-linked unsulfated, 4-linked unsulfated, 3-linked 2-mono-O-sulfated, 4-linked 2-mono-O-sulfated および 3-linked 2,4-di-O-sulfated L-fucopyranose が存在することが示唆された。