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Structural study of fucoidan from *Cladosiphon okamuranus* TOKIDA

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A structural study was carried out on a fucoidan isolated from the brown seaweed *Cladosiphon okamuranus*. The polysaccharide contained fucose, glucuronic acid and sulfate in a molar ratio of about 6.1 : 1.0 : 2.9. The results of Smith degradation showed that this polysaccharide has a linear backbone of 1→3-linked α-fucopyranose with a half sulfate substitution at the 4-positions, and a portion of the fucose residues was *O*-acetylated. The data obtained from partial acid hydrolysis, a methylation analysis and NMR spectra indicated that the α-glucuronic acid residue is linked to the 2-positions of the fucose residues, which were not substituted by a sulfate group. These results indicated that the average structure of this fucoidan is as follows: $\text{-[(}\rightarrow\text{3Fuc-4(}\pm\text{OSO}_3^-\text{)}\alpha\text{1-)}_5\rightarrow\text{3[GlcA}\alpha\text{1}\rightarrow\text{2]Fuc}\alpha\text{1-]}_n\text{-}$. (Half of each fucose residue was sulfated. One *O*-acetyl ester was present in every 6 fucose residues.)

Keywords: **fucoidan, sulfated fucan, seaweed**

Introduction

Fucoidan is a complex sulfated polysaccharide, derived from marine brown algae [1–3], the jelly coat from sea urchin eggs [4–7], and the sea cucumber body wall [7–9]. Most investigations of the biological activity of fucoidans have been of fucoidans from brown algae such as *Fucus vesiculosus* and *Ecklonia kurome*. The fucoidans from brown seaweed mediate a variety of significant biological effects on mammalian cells. These fucoidans have anticoagulant activity [10–14] and are potent activators of both anti-thrombin III and heparin cofactor II [14]. *Fucus* fucoidan inhibits both the initial binding of sperm and subsequent recognition events necessary for penetration of the human zona pellucida [15]. This fucoidan also blocks the infection of human cell lines with several enveloped viruses, including human immunodeficiency virus (HIV), herpes virus, and cytomegalovirus [16]. This polysaccharide inhibits heterodimeric HIV reverse transcriptase activity in vitro [17]. Fucoidan also blocks cell-cell binding mediated by P- or L-selectin but not E-selectin [18]. Furthermore, fucoidan demonstrates differential binding to interleukin IL-1α and β, IL-2, and IL-6 [19] and hepatocyte growth factor (HGF) [20]. Since this polysaccharide causes no toxicity or irritation, fucoidan may useful for anticoagulant, antiviral, anti-inflammatory and contraceptive agents [21–23]. In our previous study, bacterial polysaccharides and fucoidan were found to be effective for the healing of gastric ulcers [24]. In particular, fucoidan from *Cladosiphon okamuranus* was more effective for ulcer healing than that from *F. vesiculosus*. The *Cladosiphon* fucoidan also had an inhibitory effect on the adhesion of *Helicobactor pylori* to sulfatide and Le^b antigen [25]. Despite the level of interest shown in the functional aspects of fucoidan, their structural properties have been relatively little studied. The structure originally proposed for the sulfated fucan from *F. vesiculosus* is composed mainly of 4-sulfated and 2-linked α-L-fucopyranosyl units [1,2] and has only recently been superseded by a revised structure in which the α-L-fucopyranosyl units are 1-3-linked [26]. This structure resembles that determined for a fucoidan from *Ecklonia kurome*, another brown seaweed [3,27]. The structure of the *Cladosiphon fucoidan* has not yet been described in detail. The relationships between the chemical structures of these sulfated fucans and their biological activities therefore remain to be established. The present paper reports the results of a structural study of a fucoidan from *Cladosiphon okamuranus* TOKIDA.

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Materials and methods

Extraction of the polysaccharide from *Cladosiphon okamuranus* TOKIDA

The brown seaweed *Cladosiphon okamuranus* TOKIDA was cultivated in Okinawa, Japan. This seaweed was purchased from Tropical Technology Center Co. (Okinawa, Japan) as salted food. The pickled seaweed (1 kg) was dipped in tap water for desalting. The seaweed was then washed three times with de-ionized water. The washed seaweed was suspended in de-ionized water (3 l). The suspension was adjusted to pH 3.0 with 30% HCl and heated at 100 °C for 15 min. The suspension was centrifuged (10,000 × g, 20 min) at 4 °C. The supernatant was collected and neutralized with NaOH. The extraction was concentrated by ultrafiltration (MW cut off 5,000, Prep/scale UF cartridge, Millipore Co., Bedford, MA, USA) to 700 ml. Calcium chloride (500 mg) and ethanol (1 l) were added to the solution, and the mixture was left standing for 20 h at 4 °C. The precipitate was collected and dissolved in 400 ml de-ionized water. The solution was dialyzed against distilled water (MW cut off 6,000, Spectrapor dialysis membrane, Spectrum Co., Gardena, CA, USA). The dialysate was collected and lyophilized. The yield of the fraction was 15.0 g. This fraction used as the crude polysaccharide.

Purification of the acidic polysaccharide by anion-exchange chromatography

The crude polysaccharide (200 mg) was applied to an Econo-Pac high Q cartridge (5 ml × 2, Bio-Rad Laboratories, Heroules, CA, USA), equilibrated with 50 mM sodium acetate buffer (pH 4.6) containing 10 mM EDTA. The column was eluted in five different steps. Initially the column was eluted by 30 ml of the same buffer. Then the column was eluted by a linear gradient prepared by mixing 10 ml of 50 mM sodium acetate buffer (pH 4.6) containing 10 mM EDTA with 10 ml of 0.15 M NaCl in the same buffer. Thereafter, the column was eluted by a linear gradient prepared by mixing 10 ml of 50 mM sodium acetate buffer (pH 4.6) containing 0.15 M NaCl and 10 mM EDTA with 10 ml of 0.45 M NaCl in the same buffer. The column was then eluted by a linear gradient prepared by mixing 20 ml of 0.45 M NaCl with 20 ml of 1.5 M NaCl, both in the same sodium acetate buffer. Finally, the column was eluted by 30 ml of sodium acetate buffer containing 1.5 M NaCl and 10 mM EDTA. The flow rate of the column was 2 $mlmin^{-1}$, and 2ml fractions were collected. The fractions were checked for the neutral sugar and uronic acid by the phenol-H_2SO_4 [28] and the carbazole reaction [29], respectively. The eluate was dialyzed and lyophilized (130 mg). This procedure was repeated, and finally 2.3 g of purified acidic polysaccharide was obtained. The purity and molecular mass of this polysaccharide were estimated by gel filtration. Gel filtration chromatography was performed on a column of Asahipack GS620HQ (7.6 mm I.D × 300 mm Shoko Co, Tokyo, Japan) with 10 mM EDTA solution in a high-performance liquid chromatography (HPLC) system (flow rate 0.5 $mlmin^{-1}$, 50 °C). The eluate was monitored with a differential thermal analyzer. In order to estimate the molecular mass of the acidic polysaccharide, Dextran T-2000 (Sigma chemicals, St. Louis, MO, USA), pullulan (Shodex standard P-10, P-20, P-50, P-100, P-200, P-400, P-800, Shoko Co.) and glucose were used as the molecular weights standards.

Reduction of the carboxyl group of hexuronic acid of acidic polysaccharide

The carboxyl group of hexuronic acid of acidic polysaccharide was reduced by the method of Taylor and Conrad [30]. In brief, polysaccharide (1 g) was dissolved in deionized water (50 ml). Five grams of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was added to the solution, and the solution was maintained at pH 4.7 with 6 M HCl. The reaction was allowed to proceed for 2 h. Sodium borohydride solution (500 $mgml^{-1}$, 10 ml) was added slowly. The pH was maintained at pH 7.0 with 6 M HCl for 1 h. The reaction mixture was dialyzed against de-ionized water and lyophilized (678 mg). This polysaccharide was perfectly reduced, which was confirmed by the loss of the carbonyl signal (δ176.2) in ^{13}C-NMR spectra. The molecular mass reduction of the native polysaccharide was not observed in this procedure, which was monitored by gel filtration chromatography.

Smith degradation

Carboxyl-reduced polysaccharide (116 mg) was oxidized with 0.2 M $NaIO_4$ in 5 ml of 50 mM sodium acetate buffer (pH 5.0) in the dark at 4 °C for 72 h. After the addition of an excess amount of ethylene glycol, the product was reduced with $NaBH_4$ in 0.1 M sodium borate buffer (pH 8.0) at 4 °C for 20 h. The resulting material was dialyzed against deionized water and then treated with 0.3 M HCl at 20 °C for 20 h. The hydrolysate was dried in vacuo and then dissolved in a small amount of water. The solution was subjected to gel filtration on a Sephadex G-25 (4 × 90 cm). Smith degradation was also carried out on the native polysaccharide (50mg), and the resulting material was dialyzed against distilled water. The dialysate was lyophilized and subjected to a nuclear magnetic resonance (NMR) analysis.

Partial acid hydrolysis

Acidic polysaccharide (1.0 g) was hydrolyzed with 2 M H_2SO_4 for 30 min at 100 °C. The material was neutralized with 6 M NaOH and subjected to gel filtration on a column of Sephadex G-25 (4 × 100 cm). Part of the hexuronic

acid-containing fraction was applied to the carboxyl group reduction by using the same conditions described in the above section, "Reduction of the carboxyl group of hexuronic acid of acidic polysaccharide". The carboxyl-reduced material was subjected to gel filtration on a Sephadex G-25 column.

Desulfation

Desulfation of the Smith degradation product was performed by solvolysis in dimethyl sulfoxide (DMSO). The conditions of desulfation was those described Ribeiro et al. [31].

Analytical methods

Unless otherwise indicated, the analytical methods were the same as those described previously [32,33]. Glycerol, fucose, glucose were analyzed by gas liquid chromatography (GLC) after acid hydrolysis and trimethylsilylation. After the acid hydrolysis of the polysaccharides (4.0 M HCl at 100 °C for 4 h), total sulfate was measured by the $BaCl_2$-gelatin method [34]. The presence of contaminant-free sulfate in the polysaccharide solution was excluded by the negative $BaCl_2$-gelatin reaction previous to the acid hydrolysis of the polysaccharide. The absolute configurations of component sugars were established by conversion to the corresponding trimethylsilylated R-(-)-2-butylglycosides, followed by GLC [35]. ^1H NMR and ^{13}C NMR spectra were recorded (with acetone as the internal standard, δ_H 2.22 and δ_C 30.5) on D_2O solutions at 30 °C or 50 °C with a spectrometer (FX400, Jeol, Tokyo, Japan). HSQC, COSY and HMBC spectra were recorded as described in a previous paper [33]. Reference samples of methyl-α-D-glucopyranoside, methyl-α-L-fucopyranoside and fucitol were purchased from Sigma. The chemical shifts of these standard sugars were assigned by HSQC, COSY and HMBC spectra.

Methylation analysis

The carboxyl-reduced, Smith-degraded and desulfated products were methylated as follows. Each polysaccharide (10 $mgml^{-1}$) was passed through a Dowex 50 H^+ column (1 ml). The solution was neutralized with pyridine and then dialyzed against de-ionized water. The dialysates were lyophilized. To a solution of the dry polysaccharides (5 mg) in DMSO (2 ml) were added powdered NaOH (50 mg) and CH_3I (2 ml) [36]. The mixture was stirred for 30 min at room temperature, cooled, mixed with 1 M acetic acid (1 ml) and distilled water (2 ml), dialyzed against de-ionized water, and lyophilized. This procedure was repeated two times. Each methylated polysaccharide was hydrolyzed with 2 M trifluoroacetic acid for 2 h at 100°C. The products reduced with $NaBH_4$ and acetylated. The resulting alditol acetates were analysed by GLC-MS (M80b, Hitachi, Tokyo, Japan) [37]. The oligosaccharide obtained from the partial acid hydrolysis was also methylated by the same condition. The methylated material was partitioned by $CHCl_3/H_2O$ (1 : 1). The chloroform layer was collected and back-washed with H_2O. The $CHCl_3$ layer was then dried by flash evaporation. This methylated material was hydrolyzed and analyzed as alditol acetate by GLC-MS.

Other materials and methods

Unless otherwise indicated, the materials and methods were the same as those described in previous papers [32,33,37]. The *O*-acyl group was removed from the native polysaccharide and Smith degradation product by treatment at 37°C for 1h in 0.01 M NaOH [38]. These solutions were neutralized with HCl and dialyzed against de-ionized water. The dialysates were lyophilized and used as deacetylated materials.

Results

Purification of acidic polysaccharide from *Cladosiphon okamuranus* TOKIDA

The polysaccharide extracted from *Cladosiphon okamuranus* was purified by anion exchange chromatography on an Econopack High Q column. The acidic polymer fraction was obtained as a single peak (Figure 1). The purity and molecular mass of this fraction were determined by gel filtration on a GS620HQ column. From the elution position of this polysaccharide, the molecular mass was calculated

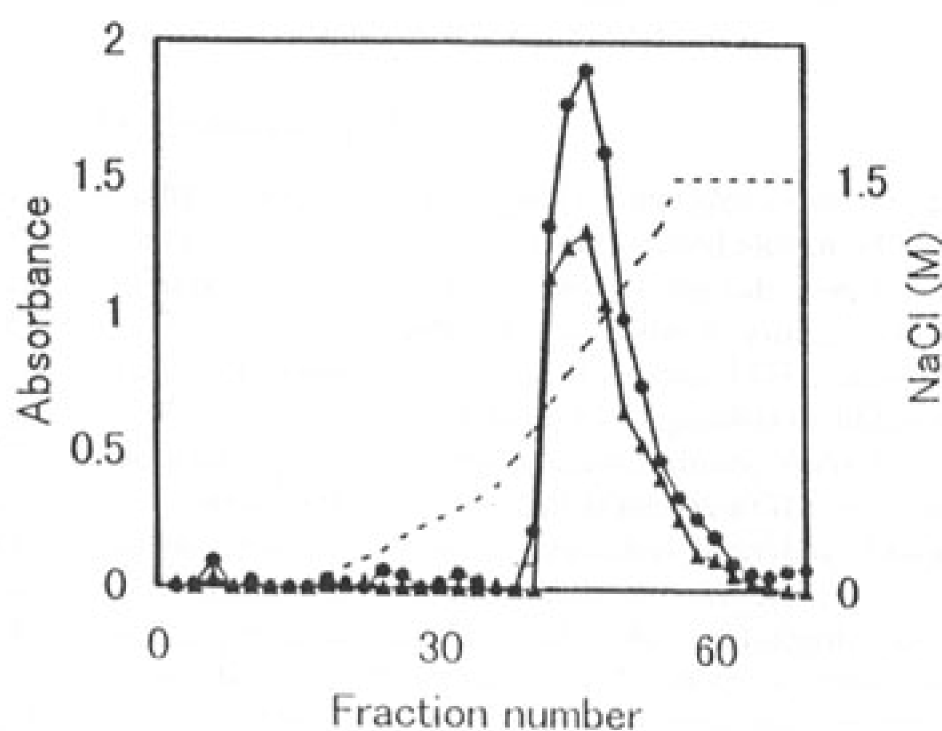

Figure 1. Purification of the acidic polysaccharide from *Cladosiphon okamuranus* on Econopak High Q column (5ml × 2). Fractions were checked by phenol-H_2SO_4 (●) and carbazole (▲). Fractions (40 60) were pooled and used as purified polysaccharide.

to be about 56 kDa. The purity of this polysaccharide was confirmed by this procedure.

Analysis of the carboxyl-reduced acidic polysaccharide

A uronic acid residue was detected in this polysaccharide by carbazole reaction. Since the uronic acid residue was resistant to acid hydrolysis, it is difficult to analyze the component sugars [39]. Therefore, a carboxyl-reduced polysaccharide was used for the analysis of the component sugars. The results of the GLC analysis indicated that fucose and glucose were present in a molar ratio of 6.1 : 1.0. The absolute configurations of fucose and glucose residue were found been to be an L- and D-configuration, respectively. The glucose residue might be derived from the glucuronic acid residue of the native polysaccharide, because a glucose residue had not been detected in the native polysaccharide but was detected after the reduction of the carboxyl group. The molar ratio of fucose and uronic acid in native polysaccharide was estimated to be about 5.7 : 1.0 by colorimetric assay. Considering these results and the results of the assay for the sulfate group, the native polysaccharide was composed of fucose, glucuronic acid and sulfate in a molar ratio of about 6 : 1 : 3. Thus, this acidic polysaccharide was fucoidan.

Smith degradation

The Smith degradation product of the carboxyl-reduced fucoidan was chromatographed on a column of Sephadex G-25. A fucose-containing fraction was eluted at void volume, and glycerol was detected near the Vi fraction. This polymer fraction consisted of fucose and sulfate. The glucose residue was perfectly oxidized. Thus, the glucose (glucuronic acid) residue might be present as a branch in the native polysaccharide, which was oxidized and generated to glycerol by Smith degradation.

Partial acid hydrolysis of fucoidan

Partial acid hydrolysis was carried out on native fucoidan. The products were separated into three fractions by gel filtration on a Sephadex G-25 column (Figure 2). Fraction I contained hexuronic acid and fucose. The analysis of fractions II and III by GLC and colorimetric methods revealed that these fractions consisted of fucose and small amount of sulfate. Considering the elution positions of fraction II and III and ^{13}C NMR spectral data, these fractions might be mixture of di- and monosaccharide units of fucose and a part of which were sulfated. After the reduction of the carboxyl group and the reducing terminal of fraction I, glucose and fucitol were detected in a molar ratio of 1.17 : 1.00. As a result of the methylation analysis of reduced fraction I, 2,3,4,6-tetra-*O*-methylglucitol and 1,3,4,5-tetra-

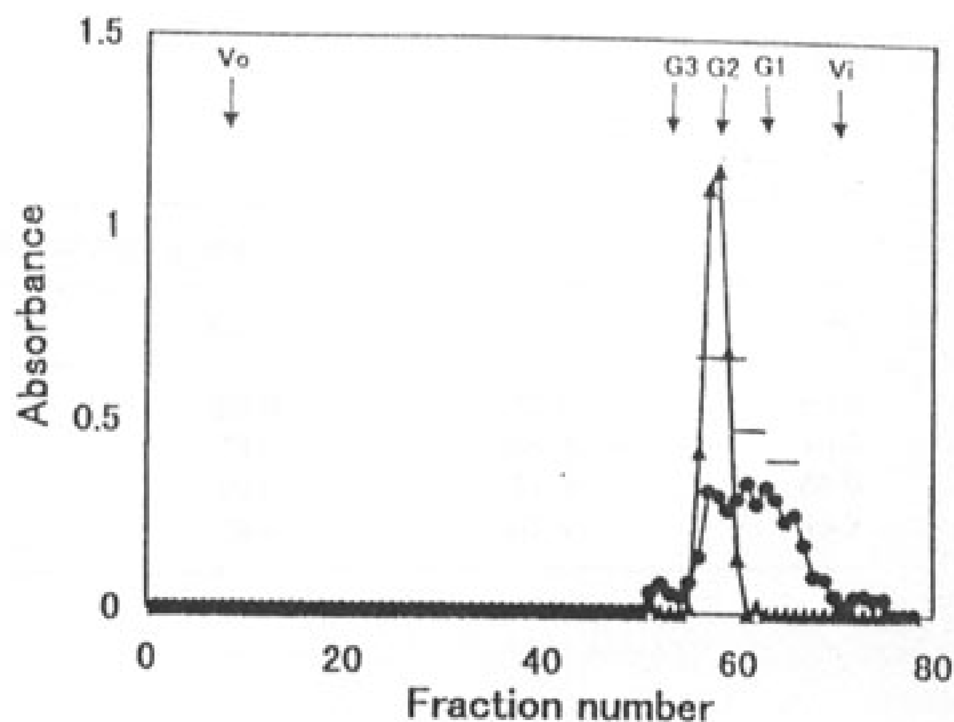

Figure 2. Gel filtration of the partial acid hydrolysis products of fucoidan. Fractions (10 ml) were collected and checked by phenol-H_2SO_4 (●) and carbazole (▲). Fractions 55–59, 60–62 and 63–65 were pooled and used as Fractions I, II and III, respectively. Arrows G1, G2 and G3 indicate the elution positions of the monomer, dimer and trimer of glucose, respectively.

O-methylfucitol were detected. Therefore, the glucose residue was linked to the C-2 of fucitol. This observation was confirmed by ^{13}C-NMR. Compared with standard fucitol, the signal of the C-2 of the fucitol residue of reduced fraction I was shifted 7.8 ppm downfield (α-effect) and those of the C-1 and C-3 were shifted upfield (β -effect), which indicated that the C-2 of the fucitol residue was *O*-glucosylated (Table 1). The ^{1}H spectrum of reduced fraction I exhibited the signal of an anomeric proton (δ 5.08, $J_{1,2}$ = 3.9 Hz). This doublet signal was assigned to the α-linked glucose residue. Thus, the structure of reduced fraction I was Glc(α1→2)Fuc-ol. On the basis of these results, the structure of fraction I was GlcA(α1→2)Fuc.

Methylation analysis

Methylation of sulfated polysaccharides does not always yield reliable proportions of methylated alditols [40,41], but sulfated polysaccharides in the pyridine salt form have given good yields and results. To avoid the β -elimination of the glycosidic bond of uronic acid residue [42], a methylation analysis was performed on Smith-degraded fucoidan, its desulfated form and the carboxyl-reduced fucoidan, all of which were in the pyridine salt-form. By a GLC-MS spectrometric analysis of Smith-degraded fucoidan, 2-mono-*O*-methylfucitol and 2,4-di-*O*-methylfucitol were identified in a peak area ratio of about 1.00 : 0.96. After desulfation of the Smith-degraded fucoidan, only 2,4-di-*O*-methylfucitol was detected. Thus, the Smith-degraded fucoidan was composed of 1→3 linked fucose units, half of which were sulfated at the

Table 1. ¹³C NMR chemical shifts of Fraction I

	Chemical shift (ppm)					
	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
Glc(α→	100.3	72.0	73.2	69.9	72.3	60.8
→ 2)Fucitol	62.0	79.6	70.2	73.1	66.3	19.0
Me-α-Glc*	99.6	71.6	73.4	69.9	71.9	60.9
Fucitol*	64.4	71.8	71.2	74.4	67.4	19.7

*These sugars were used as the standard.

O-4 position. With the methylation of the carboxyl-reduced fucoidan, 2,3,4,6,-tetra-*O*-methylglucitol, 2,4-di-*O*-methylfucitol, 2-mono-*O*-methylfucitol and 4-mono-*O*-methylfucitol were detected in a peak area ratio of about 1.00 : 2.13 : 2.89 : 1.04. Considering the results of the partial acid hydrolysis, Smith degradation and methylation analysis (Table 2), the glucose residue (glucuronic acid) is linked to the C-2 of the 3-*O*-substituted fucose residue. Therefore, this fucoidan is a 1→3 linked fucan and a portion of the fucose residues (1/6) are substituted by glucuronic acid, and half of the fucose residues are sulfated at *O*-4.

NMR spectra

Six main anomeric carbons were shown by the ¹³C-NMR spectrum of native fucoidan. These signals were observed in the range of δ 93–100 (Figure 3). The anomeric signals of native fucoidan by ¹H-NMR were detected in the range of δ 5.0–5.5. The accurate values of the coupling constants of these anomeric protons were not obtained but under 4 Hz. Therefore, all of the fucose and glucuronic acid residues of fucoidan were linked α-glycosidically [7]. Two carbonyl signals (δ 173.9 and δ 176.2) were observed in ¹³C NMR spectra. Since the signal of δ 176.2 disappeared with the Smith degradation of native fucoidan, this signal was assigned to the C-6 of GlcA. The signals of δ 173.9 and δ 20.5 were assigned to acetyl ester. These signals were also observed in the Smith degradation product of native fucoidan but disappeared by mild alkaline treatment. Therefore, this acetyl group was present in the backbone chain as the *O*-acyl group. In the light of the relative intensity of the methyl proton of the acetyl group to that of the fucose residue shown by ¹H NMR spectra, one acetyl ester was present in every 6 fucose residues.

Structure of fucoidan

On the basis of these results, the most probable structure of this fucoidan is as indicated in Figure 4.

Discussion

The average structure of *Cladosiphon* fucoidan was determined in this experiment. Considering the ¹³C-NMR spec-

Table 2. Methylated sugars obtained from carboxyl reduced fraction I, Smith degradation product, Smith degradation and desulfated fucoidan and carboxylreduced fucoidan.

	Peak area ratio			
	Carboxyl reduced fraction I[a]	*Smith degradation product*[b]	*Smith degradation and desulfated fucoidan*[b]	*Carboxyl reduced fucoidan*[a]
2,3,4,6-Me_4-Glc	1.00			1.00
1,3,4,5-Me_4-Fuc	0.82			
2,4-Me_2-Fuc		1.00	1.00	2.13
2-Me-Fuc		0.96		2.89
4-Me-Fuc				1.04

[a]2,3,4,6-Me_4-Glc taken to be 1.00.
[b]2,4-Me_2-Fuc taken to be 1.00.

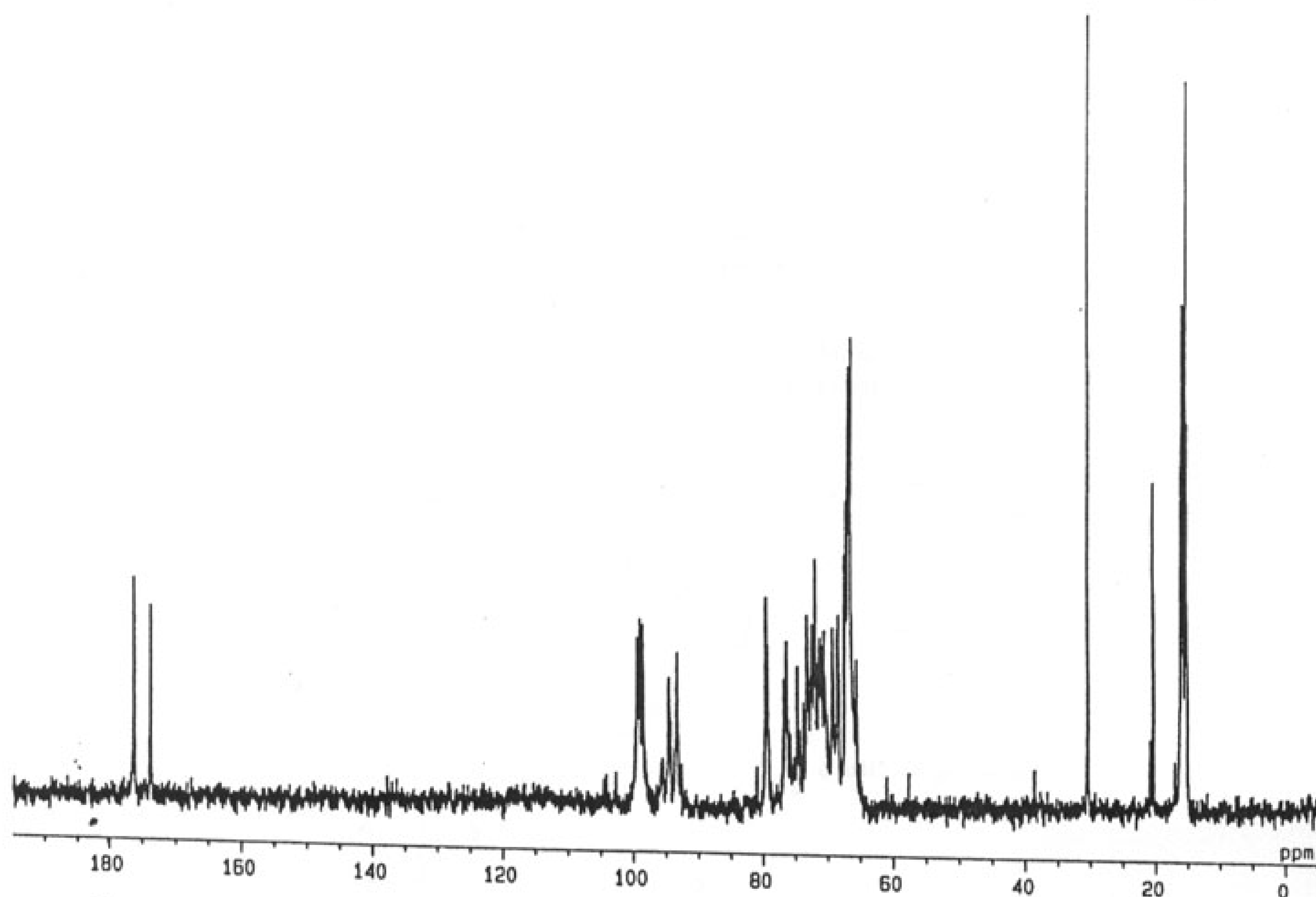

Figure 3. ^{13}C NMR spectrum of *Cladosiphon* fucoidan. The signal of δ_C 30.5 was acetone as the internal standard.

tra, this polysaccharide seems to be composed of a heptasaccharide as a repeating unit. To obtain clear information regarding the sulfation, *O*-acetylation and branching points of the backbone chain, the HMBC, HSQC and COSY spectra of the native polysaccharide were measured. The desired signals were not detected due to the heterogeneity of fucoidan, which may relate to differential branching or sulfation of regions within the fucan chains and viscosity. In this experiment, we could not determined to the *O*-acetyled position of backbone chain. On the basis of the basic structure of this fucoidan, acetyl ester might be present in the *O*-2 or *O*-4 of the fucose residue.

In previous studies, *Cladosiphon* fucoidan was shown to have an anti-ulcer effect and anti-adhesion activity for *H. pylori*. These biological activities were higher than *Fucus vesiculosus* fucoidan [24,25]. The structure of *Fucus* fucoidan was composed mainly of 4-sulfated and 2-linked α-fucopyranosyl units [1], and a revised structure was recently proposed in which the α-fucopyranosyl units are 1 → 3 linked [26]. This revised structure is similar to that of *Cladosiphon* fucoidan (Figure3). The differences in these fucoidans are in their sulfate contents and branched sugars. *Cladosiphon* fucoidan has one sulfate group for every 2 mol of fucose, and *Fucus* fucoidan has 3–4 mol of fucose. Furthermore, *Cladosiphon* fucoidan has one glucuronic acid residue for every 6 mol of fucose as a branched chain, and *Fucus*-derived fucoidan has fucose branches. Anti-ulcer activity was also observed in the deacylated Smith degradation product of *Cladosiphon* fucoidan [24]. Thus, the backbone chain of *Cladosiphon* fucoidan is important in the anti-ulcer effect. The basic structure of the backbone chains of these fucoidans is the same. The sulfation ratio and fucose branch of *Fucus* fucoidan might be reflected by its biological activities. This possibility is currently being investigated.

Acknowledgment

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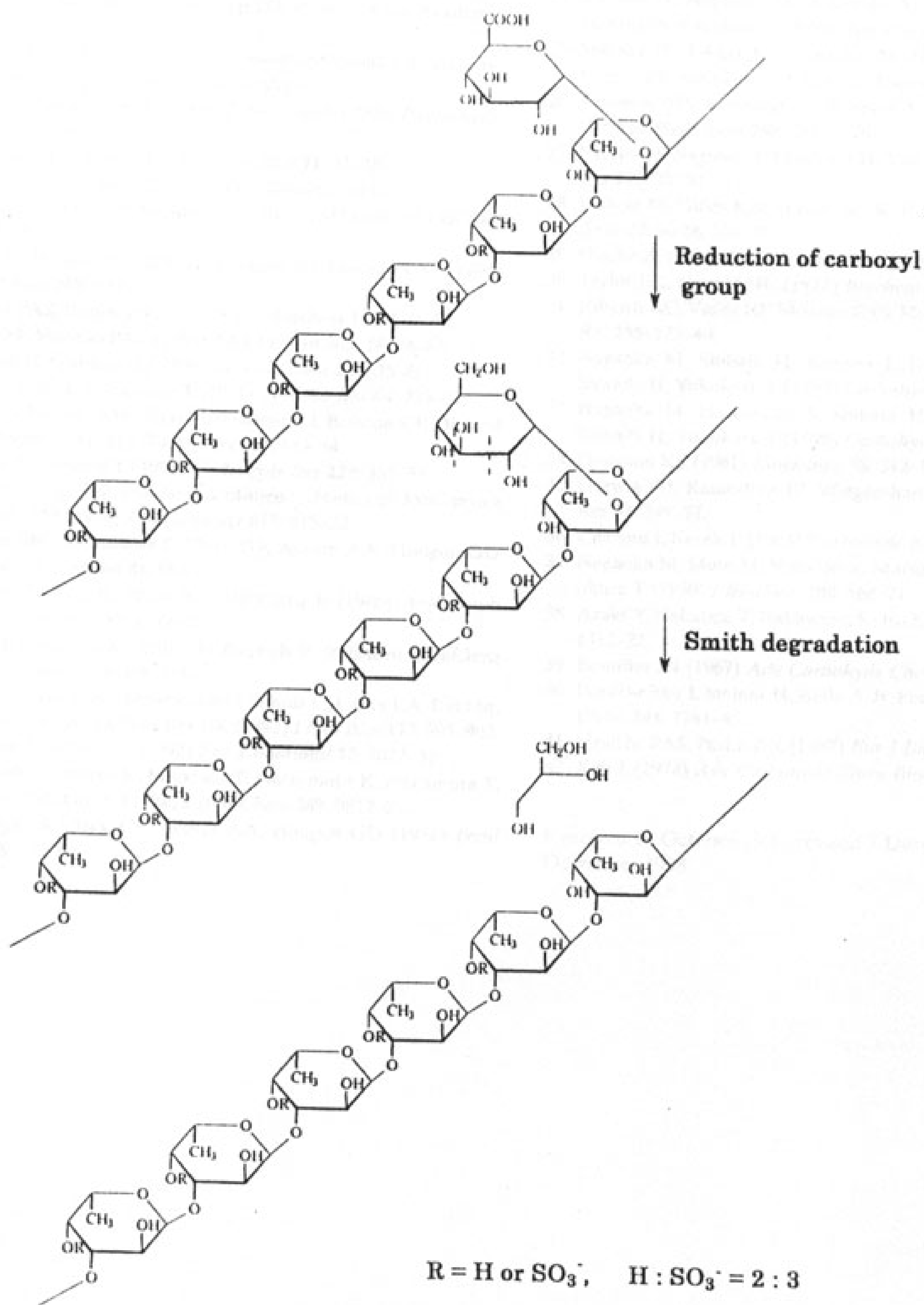

Figure 4. Schematic interpretation of the structure of the fucoidan, carboxyl-reduced fucoidan and Smith degradation product.------:oxidation position by Smith degradation. Parts of the fucose residues were *O*-acetylated. The ratio of acetyl group and fucose residues was about 1 : 6.

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