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# Structural characterization of a new anticoagulant fucan sulfate from the brown seaweed *Ecklonia kurome**

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ABSTRACT

Methylation analysis of a fucose-containing, sulfated polysaccharide (C-II), which was isolated from the brown seaweed *Ecklonia kurome* and has a potent anticoagulant activity, showed the presence of 3-*O*- and 3,4-*O*-disubstituted fucopyranosyl residues in addition to small proportions of nonreducing, terminal fucofuranosyl and fucopyranosyl groups, and 2,3-di-*O*- and 2,3,4-tri-*O*-substituted fucopyranosyl and galactopyranosyl residues with various glycosidic linkages. Methanolysis of C-II gave several neutral oligosaccharide fractions in small proportions and two high-molecular-weight acidic fractions in large proportions. Methylation analysis of the low-sulfated acidic fraction showed that the proportion of 3-*O*-linked fucosyl residues increases and that of 3,4-*O*-disubstituted decreased as compared to C-II. Methylation and g.l.c.–m.s. analysis of the neutral oligosaccharide fractions showed the presence of Fuc-(1→3)-Fuc and a fucosyl trisaccharide, in addition to small proportions of Gal-(1→4)-Fuc, Fuc-(1→2)-Fuc, Fuc-(1→4)-Fuc, Fuc-(1→2)-Gal, and Fuc→Gal→Fuc. Methylated C-II was also desulfated by methanolysis, followed by remethylation with ($^2H_3$)methyl iodide, and most of ($^2H_3$)methyl groups were linked to O-4 of the 3-*O*-linked fucosyl residues. These results suggested a highly branched, new type of fucan sulfate containing a backbone of (1→3)-linked L-fucosyl residues having sulfate groups mainly attached to C-4.

## INTRODUCTION

Since a fucan sulfate (fucoidan), which was isolated from the brown seaweed *Fucus vesiculosus*, showed[2] a potent anticoagulant activity, various fucan sulfates having this activity have been isolated[3–7] from several brown seaweeds. The structural analysis[8–12] of fucan sulfates has not been extensive, except that of anticoagulant fucan sulfates. Recently, we have found[13] a potent anticoagulant activity in the polysaccharide fractions from several species of brown seaweeds, and a very active sulfated polysaccharide (C-II) was purified from *Ecklonia kurome*[1]. We report herein the structure characterization of this polysaccharide.

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EXPERIMENTAL

*Materials.* — The fucose-containing, sulfated polysaccharide (Polysaccharide C-II) was purified from *E. kurome* as described previously[1]. Bio-Gel P-2 (200–400 mesh), AG 50W-X8, and AG 1-X8 ion-exchange resins were obtained from Bio-Rad Laboratories; Sephadex G-25 and LH-20, and Sepharose 4B and CL-6B from Pharmacia LKB Biotechnology Inc.; Ecteola-cellulose from Serva Biochemicals; and Sep-Pak $C_{18}$ cartridge from Waters Associates Inc. ($^2H_3$)Methyl iodide was purchased from Merck Sharpe & Dohme/Isotopes.

*General.* — The carbohydrate content of column eluates was monitored by the phenol–$H_2SO_4$ method[14]. Acetyl content was determined by the method of McComb and McCready[15] using 1,2,3,4,6-penta-*O*-acetyl-D-glucose as a standard. Sulfate content was determined by the method of Dodgson and Price[16]. Sulfated polysaccharides were hydrolyzed[17] with 90% formic acid for 6 h at 100°, followed by addition of water (5 vols.) and heating for 2 h at 100°. The acid hydrolyzates were analyzed by t.l.c. on cellulose F (Merck) in 5:5:1:3 ethyl acetate–pyridine–acetic acid–water. Reducing sugars were detected with alkaline silver nitrate[18], and uronic acids with 4-anisidine hydrochloride[19]. Sugars were converted conventionally into the alditol acetates by the method of Albersheim *et al.*[20]. G.l.c. of alditol acetates was performed[21] with a Hewlett–Packard model 5840A gas chromatograph equipped with a DB-1 capillary column (0.25-$\mu$m film thickness, 30m × 0.25mm i.d.; J & W Scientific) in splitless mode; the temperature program used was 60–180° at 30°/min and then 180–220° at 3°/min. The molar ratios of sugars were calculated from the peak areas and the molecular weights of the corresponding alditol acetaes. Gel filtration chromatography was carried out in a column (1.2 × 98.8 cm) of Sepharose CL-6B with 0.2M NaCl as an eluent. To estimate the molecular weight of polysaccharides, Blue Dextran (Type 2000) and several sizes of pullulans (Showa Denko, Tokyo) were used as standards.

*N.m.r. spectroscopy.* — $^1$H- (400 MHz) and $^{13}$C-(100 MHz) n.m.r. spectra of Polysaccharide C-II were obtained for a solution in $D_2O$ at 80° with a Varian XL-400 F.t. spectroscope. Chemical shifts ($\delta$) are expressed in $\delta$ values relative to the signal of sodium 4,4-dimethyl-4-sila-(2,3-$^2H_4$)pentane sulfonate.

*Methanolysis of C-II.* — A suspension of Polysaccharide C-II (44.9 mg) in 91mM methanolic HCl (3.4 mL) was stirred for 24 h at room temperature[22]. The reaction mixture was diluted with water (1.7 mL) and then neutralized with 0.5M NaOH. After the insoluble material (negative reaction with the phenol–$H_2SO_4$ assay) had been centrifuged off, the supernatant solution was applied to a column (1.9 × 143 cm) of Sephadex G-25; a major Fraction I (15.9 mg) was eluted in the void volume and a minor, lower-molecular-weight Fraction II (6.1 mg) was obtained (Fig. 1A). Fraction I was further fractionated by anion-exchange chromatography on a column (1.5 × 26 cm) of Ecteola-cellulose ($Cl^-$) (Fig. 1B). After the neutral fraction had been eluted with water, acidic fractions were eluted with a linear gradient of 0–2M NaCl (300 mL). The acidic fractions, fractions Ia (4.1 mg) and Ib (11.6 mg), were obtained in the concentrations 0–0.4 and 0.4–1.7M NaCl, respectively, and each fraction was desalted in a column (2.6

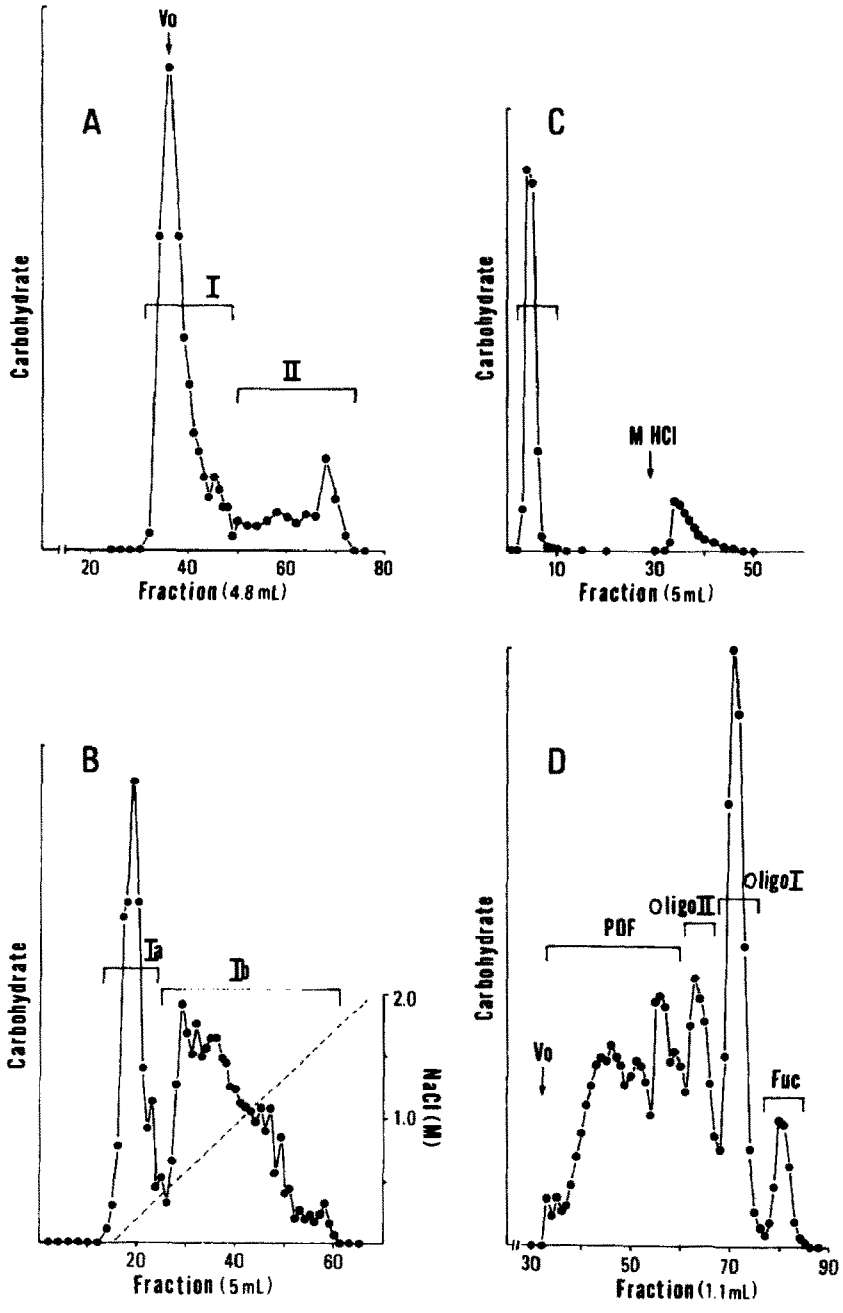



Fig. 1. (A) Gel filtration on Sephadex G-25 of the methanolyzate of Polysaccharide C-II. (B) Fractionation of Fractions I from Fig. 1A by Ecteola-cellulose ($Cl^-$) chromatography. (C) Fractionation of Fraction II from Fig. 1A on AG 1-X8 ($AcO^-$) anion-exchange resin. (D) Fractionation of neutral fraction from Fig. 1C by gel filtration on Bio-Gel P-2. $V_0$, void volume. Carbohydrate content was monitored by the phenol–sulfuric acid test.

× 95 cm) of Sephadex G-25. Fraction II was further fractionated by anion-exchange chromatography in a column (1.5 × 22 cm) of AG 1–X8 ($AcO^-$) anion-exchange resin to give a large proportion of neutral and a small proportion of acidic fractions by elution with water and M HCl, respectively (Fig. 1C). The neutral fraction was further fractionated by gel filtration in water in a column (2.5 × 50 cm) of Bio-Gel P-2 at 55° to give a monosaccharide fraction, two oligosaccharide fractions (Oligosaccharides I and II) which were eluted in the di- and tri-saccharide regions, respectively, and a high-mol.-wt. oligosaccharide fraction (Oligosaccharide POF) (Fig. 1D).

*Methylation analysis.* — *(a)*. Polysaccharide C-II, and fractions Ia and Ib were methylated by the method of Hakomori[23]. Completeness of the formation of alkoxide was tested by use of triphenylmethane[24], and the methylation was performed once to prevent the β-elimination reaction[25]. The per-*O*-methylated samples were recovered from the reaction mixture by use of a Sep-pak $C_{18}$ cartridge according to the procedure of Waeghe *et al.*[26], except that ethanol alone was used as the eluent. The carboxyl groups in the fully methylated polysaccharides were reduced[26,27] with $NaBD_4$ in 27:73 (v/v) 90% ethanol–oxolane for 18 h at room temperature, followed by incubation at 80° for 1 h, and the samples were desalted with AG 50W-X8 ($H^+$) cation-exchange resin. The products were hydrolyzed with 90% formic acid for 6 h at 100°, followed by hydrolysis with M trifluoroacetic acid for 2 h at 100°, and then reduced to give the corresponding alditols with $NaBH_4$ in 95% ethanol containing M $NH_4OH$, and acetylated[26] with acetic anhydride for 3 h at 121° in the presence of sodium acetate. The partially *O*-methylated alditol acetate derivatives were analyzed[28] by g.l.c. and g.l.c.–m.s. G.l.c. was performed in a DB-1 capillary column and the temperature program was 60→150° at 18°/min, and then 150→210° at 2°/min. G.l.c.–m.s. was performed[29] with a Jeol DX-300 instrument, equipped with a SPB-1 capillary column (0.25-μm film thickness, 25 m × 0.25 mm i.d., Supelco), and operated under voltage of 70eV with He as the carrier gas at 0.9 mL/min, and a temperature program of 120→210° at 2°/min.

*(b)*. Oligosaccharides I and II, obtained by methanolysis of Polysaccharide C-II, were each reduced with $NaBD_4$ in M NaOH for 18 h at room temperature and then for 2 h at 80° to give the oligosaccharide alditols. After desalting with AG 50W-X8 ($H^+$) cation-exchange resin, they were methylated as described above.

*(c)*. For desulfation, fully methylated Polysaccharide C-II was methanolyzed with 91mM methanolic hydrogen chloride at room temperature for 24 h. After the solvent had been removed by an air stream, the products were dissolved in 50% dimethyl sulfoxide and applied to a Sep-pak $C_{18}$ cartridge. Methyl sulfates were eluted with water and then the methanolyzates with ethanol. These were remethylated with $(^2H_3)CH_3I$, followed by purification in a Sep-pak $C_{18}$ cartridge as just described, and then fractionated[29] on a column (1.5 × 26 cm) of Sephadex LH-20 with 1:1 chloroform–methanol. Fractions of high (HMF) and low mol. wt. were obtained by monitoring with the 1-naphtol–sulfuric acid reagent[18]. The carboxyl groups of the high-mol.-wt. fraction were reduced, and the product was hydrolyzed[21] with 2M trifluoroacetic acid and derivatized into alditol acetates as described above.

*G.l.c.–m.s. of the methylated oligosaccharide-alditols.* — G.l.c.–m.s. of per-*O*-methyloligosaccharide-alditols was carried out as described by Yamada *et al.*[30]. The solution of *O*-methyloligosaccharide-alditols in acetone was injected into a SPB-1 capillary column in the splitless mode under a temperature program of 180° to 310° at 4°/min. Mass spectra were obtained with a Jeol DX-300 mass spectrometer, a e.i.–m.s. at 70 eV with an ionization current of 300 $\mu$A, and c.i. (isobutane) at 250 eV and an accelerating voltage of 3 kV. C.i.[31] and e.i.[32] fragment ions [A, J, and alditol (ald)] were used to determine the structures of the per-*O*-methyloligosaccharide-alditols.

## RESULTS

*Methylation analysis of Polysaccharide C-II.* — Methylation analysis (Table I) showed that Polysaccharide C-II contains mainly 3- and 3,4-*O*-linked α-L-fucopyranosyl residues, in addition to small proportions of terminal fucopyranosyl and fucofuranosyl groups, and 2,3-di- and 2,3,4-tri-substituted fucopyranosyl, and galactosyl residues substituted at various locations. However, the linkage of the glucopyranosyluronic acid residue could not be deduced because of the small amount of material available. The proportion of terminal fucosyl group to branched fucosyl residue was very small. It may have resulted from the high volatility of the tri-*O*-methylfucitol derivatives or the presence of terminal fucosyl groups substituted with some sulfate groups.

*N.m.r analysis of Polysaccharide C-II.* — The $^1$H-n.m.r. spectrum of Polysaccharide C-II showed signals for the anomeric protons at $\delta \sim 5$–5.6, for the methyl protons of 6-deoxy sugars at $\delta$ 1.1–1.4, and for acetyl protons at $\delta \sim 2.1$. The acetyl content was found to be 0.9% by the hydroxamic acid test[15]. The $^{13}$C-n.m.r. spectrum (Fig. 2) of Polysaccharide C-II showed the signals for the anomeric carbon atoms at $\delta \sim 93$–104, for the ring carbon atoms at $\delta$ 83–69, and for the methyl groups of 6-deoxy sugars at $\delta$ 18.5. Two major anomeric-carbon signals at $\delta$ 99.16 and 95.17 were assigned to C-1 of the 3-*O*-linked α-L-fucopyranosyl (*cf.* $\delta$ 99.1 for C-1 of the 3-*O*-linked α-D-fucopyranosyl residue[33]) and 3,4-di-*O*-substituted fucopyranosyl residue (downfield shift by β-effect), respectively. These results agreed with those of the methylation analysis.

*Methanolysis of Polysaccharide C-II.* — Desulfation of fucan sulfate by methanolysis was shown earlier[12,22], but cleavage of its glycosidic linkages by this reaction has

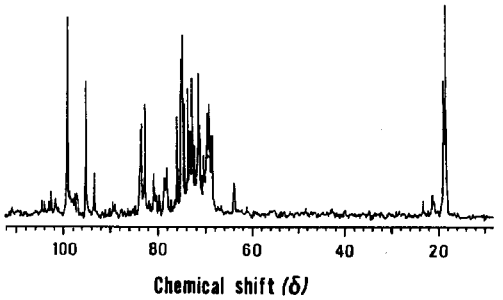
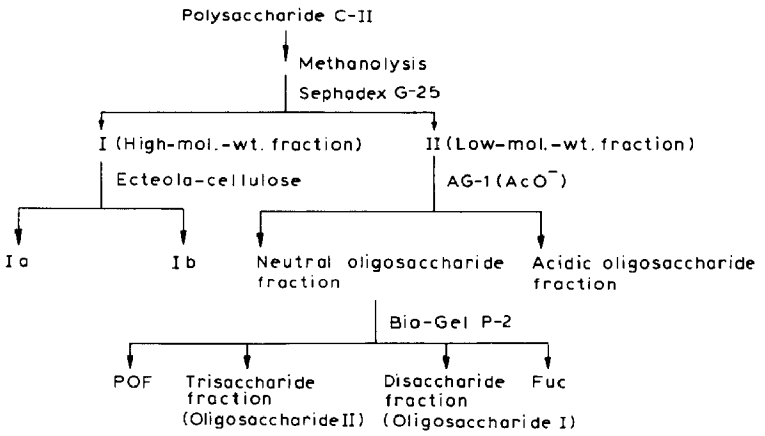



Fig. 2. $^{13}$C-N.m.r. spectrum of Polysaccharide C-II.

never been discussed. The methanolyzates of Polysaccharide C-II were fractionated into two large acidic fractions of high molecular-weight (Fractions Ia and Ib), three small fractions of neutral oligosaccharides (Oligosaccharides POF, I, and II), a monosaccharide fraction, and a fraction of acidic oligosaccharide in trace amount, as shown in Scheme 1 and Fig. 1. The monosaccharide fraction contained only fucose. These results suggested that methanolysis causes the partial cleavage of the glycosidic linkages of Polysaccharide C-II, together with the desulfation of the polysaccharide.




Scheme 1.

*Analysis of high-molecular-weight Fractions Ia and Ib.* — Fractions Ia and Ib were composed of fucose and galactose in the molar ratios of ~25:2 and ~5:1, respectively, with a respective sulfate content of 14.2 and 31.9%; Fraction Ib also contained glucuronic acid. Fractions Ia and Ib gave each a single, symmetrical elution peak by gel filtration on Sepharose CL-6B. The average mol. wts. of Fractions Ia and Ib were estimated at ~5000 and 10 500, respectively (data not shown).

Methanolysis under the present conditions could not achieve complete desulfation of Polysaccharide C-II as Fractions Ia and Ib still contained sulfate groups, although in decreased proportions when compared with Polysaccharide C-II. The incomplete desulfation of the polysaccharide may have been caused by a heterogeneous reaction, as the polysaccharide was insoluble in methanol.

Methylation analysis (Table I) showed that the proportion of 3-*O*-linked fucosyl residues had increased in Fraction Ia, and that of 3,4-*O*-disubstituted fucosyl residues in Fractions Ia and Ib had decreased as compared to that of Polysaccharide C-II. Although a 2,4-*O*-disubstituted fucosyl residue was not detected in Polysaccharide C-II, it was present in Fraction Ib. In both Fractions Ia and Ib, the content of terminal fucofuranosyl groups was found to be much lower than that in Polysaccharide C-II, suggesting that the glycosidic linkage of these groups may be very labile to acid conditions. Fractions Ib also contained 4-*O*- and 3,4-*O*-linked glucopyranosyluronic acid residues.

TABLE I

Methylation analysis of Polysaccharide C-II, and Fractions Ia and Ib

| *Glycosyl residue or group* | *Position of O-methyl groups* | *Deduced position of glycosyl linkages* | *Major mass spectral fragment ions (m/z)* | *Composition (mol%)* | | |
|---|---|---|---|---|---|---|
| | | | | *C-II* | *Ia* | *Ib* |
| Fucosyl | 2,3,5 | Terminal(*f*) | 175, 117, 101, 59 | 8.3 | 0.3 | 0.3 |
| | 2,3,4 | Terminal (*p*) | 175, 161, 131, 117, 101, 89 | 5.5 | 6.1 | 3.6 |
| | 2,3 | 4 | 203, 143, 117, 101 | 6.5 | 6.5 | 4.8 |
| | 2,4 | 3 | 233, 173, 159, 131, 117, 101, 89 | 25.0 | 44.8 | 25.6 |
| | 2 | 3,4 | 275, 173, 129, 117, 113, 99, 87 | 31.1 | 22.6 | 24.7 |
| | 3 | 2,4 | 203, 189, 143, 129, 101, 87 | | | 5.3 |
| | 4 | 2,3 | 261, 201, 131, 127 | 2.5 | 7.3 | 4.1 |
| | | 2,3,4 | 231, 201, 187, 170, 157, 145, 128, 115, 103 | 4.4 | 1.0 | 3.2 |
| Galactosyl | 2,3,4,6 | Terminal(*p*) | 205, 161, 145, 129, 117, 101, 45 | | [b] | 0.9 |
| | 2,3,4 | 6 | 233, 189, 161, 129, 117, 101, 99 | | 0.3 | 1.7 |
| | 2,3,6 | 4 | 233, 173, 161, 117, 113, 101, 45 | | [b] | [b] |
| | 2,4,6 | 3 | 233, 161, 129, 117, 101, 45 | 2.5 | 2.6 | 3.5 |
| | 3,4,6 | 2 | 189, 161, 129, 45 | | 0.8 | 1.0 |
| | 2,3 | 4,6 | 261, 201, 127, 117, 101 | 1.4 | | |
| | 2,4 | 3,6 | 233, 189, 129, 117, 87 | 1.4 | 2.1 | 2.5 |
| | 2,6 | 3,4 | 305, 231, 203, 143, 129, 117, 45 | | 0.2 | 1.5 |
| | 3,6 | 2,4 | 233, 189, 129, 113, 99, 45 | 5.1 | | 3.7 |
| | 4,6 | 2,3 | 261, 201, 161, 129, 101, 45 | 4.1 | 3.5 | 3.0 |
| | 2 | 3,4,6 | 333, 259, 139, 117 | | | 1.4 |
| | 3 | 2,4,6 | 261, 201, 189, 159, 129 | 1.2 | 0.3 | 0.4 |
| | 6 | 2,3,4 | 184, 157, 139, 129, 115 | 1.2 | 1.2 | 4.0 |
| Glucosyl-uronic acid[c] | 2,3 | 4 | 263, 203, 161, 129, 117, 101 | | | 6.0 |
| | 2 | 3,4 | 335, 261, 157, 141, 117 | | | 1.2 |

[a] Calculated from peak areas and response factors[37] of hydrogen-flame-ionization detector on g.l.c. [b] Trace. [c] Detected as partially methylated (6-$^2H_2$)glucitol acetates.

TABLE II

Diagnostic ions of c.i.m.s. of O-methyldisaccharide alditols derived from Oligosaccharide I

| *Fragment* | *Oligosaccharide* | *C.i.m.s. fragment ions*[a] (m/z) | | | |
|---|---|---|---|---|---|
| | | $(M+H)^+$ | $aJ_2OH_2$ | $aJ_2$ | $bA_1$ |
| **a** (major) | 6-Deoxyhexosyl→6-deoxy-(1-$^2$H)hexitol | 412 (50.4) | 224 (100) | 206 (1.8) | 189 (100) |
| **b** | 6-Deoxyhexosyl→6-deoxy-(1-$^2$H)hexitol | 412 (19.3) | 224 (100) | 206 (1.8) | 189 (36.8) |
| **c** | 6-Deoxyhexosyl→6-deoxy-(1-$^2$H)hexitol | 412 (19.3) | 224 (100) | 206 (15.8) | 189 (98.2) |
| **d** (medium) | Hexosyl→6-deoxy-(1-$^2$H)hexitol | 442 (33.3) | 224 (61.4) | 206 (1.8) | 219 (64.9) |
| **e** | 6-Deoxyhexosyl→(1-$^2$H)hexitol | 442 (28.1) | 254 (100) | 236 (14.0) | 189 (59.6) |

[a] Relative abundance in parentheses.

*Analysis of neutral oligosaccharides.* — Each Oligosaccharide I and II was reduced with sodium borodeuteride, and then methylated. Per-*O*-methyl-oligosaccharide-alditols were analyzed by g.l.c.–m.s. C.i.–m.s. of Oligosaccharide I gave five fragments [**a** (major), **b** (minor), **c** (minor), **d** (medium), and **e** (minor)] (see Table II). Fragments **a**, **b**, and **c** corresponded to a 6-deoxyhexosyl→6-deoxy(1-$^2$H)hexitol, fragment **d** to a hexosyl→6-deoxy(1-$^2$H)hexitol, and fragment **e** to a 6-deoxyhexosyl→(1-$^2$H)hexitol structure. E.i.–m.s. and g.l.c. data indicated that fragment **a** was Fuc-(1→3)-(1-$^2$H)-Fucol, fragment **b** Fuc-(1→4)-(1-$^2$H)Fucol, fragment **c** Fuc-(1→2)-(1-$^2$H)Fucol, fragment **d** Gal-(1→4)-(1-$^2$H)Fucol, and fragment **e** Fuc-(1→2)-(1-$^2$H)Galol (see Table III). C.i.–m.s. data showed that Oligosaccharide II gave two fragments [**f** (major) and **g** (minor)]. Fragment **f** gave a protonated molecular ion at *m/z* 586, indicating a 6-deoxyhexosyltrisaccharide-(1-$^2$H)alditol structure, whereas fragment **g** did not show a molecular ion. Fragment **g** gave ions at *m/z* 393 due to $cbA_1$ 224 to $aJ_2OH_2$, and 189 to $cA_1$, suggesting that fragment **g** possesses a 6-deoxyhexosyl→hexosyl→6-deoxy(1-$^2$H)-hexitol structure (see Table IV). E.i.–m.s. data also suggested that fragments **f** and **g** may have a Fuc→Fuc→(1-$^2$H)Fucol and a Fuc→Gal→(1-$^2$H)Fucol structure, respectively (Table V). However, specific fragment ions of the ald series were not observed for fragments **f** and **g** and, therefore, the glycosidic linkages of fragments **f** and **g** could not be deduced. Further study of the acidic oligosaccharides was not possible, because of the small amount of material available.

*Methanolysis of methylated Polysaccharide C-II.* — Methylated Polysaccharide C-II was soluble in methanol (homogeneous reaction) and, therefore, it was subjected to methanolysis, followed by remethylation with ($^2H_3$)methyl iodide in order to confirm the locations of sulfate groups as it was expected that *O*-deuteriomethylation occurs at the position of desulfation. Methanolysis under the present conditions may cause not only desulfation, but also partial degradation of glycosidic linkages as described above;

TABLE III

Diagnostic ions of e.i.m.s. of *O*-methyldisaccharide alditols derived from Oligosaccharide I

| *Fragment* | *Oligosaccharide* | *E.i.m.s. fragment ions* (m/z)[a] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $aJ_1$ | $aJ_2$ | $bA_1$ | $bA_2$ | *ald* | | | | | |
| **a** | Fuc-(1→3)-(1-$^2$H)Fucol | 266 | 206 | 189 | 157 | 365 | 352 | 308 | 276 | 103 | |
| (major) | | (35.1) | (10.5) | (100) | (52.6) | (0.3) | (2.2) | (1.2) | (1.0) | (100) | |
| **b** | Fuc-(1→4)-(1-$^2$H)Fucol | 266 | 206 | 189 | 157 | 365 | 352 | 277 | 134 | | |
| | | (21.1) | (5.3) | (77.2) | (71.2) | (1.0) | (4.1) | (1.3) | (8.8) | | |
| **c** | Fuc-(1→2)-(1-$^2$H)Fucol | 266 | 206 | 189 | 157 | 352 | 308 | | | | |
| | | (12.3) | (59.6) | (100) | (61.4) | (0.8) | (0.7) | | | | |
| **d** | Gal-(1→4)-(1-$^2$H)Fucol | 266 | 206 | 219 | 187 | 382 | 350 | 319 | 307 | 134 | 102 |
| (medium) | | (29.8) | (8.8) | (24.6) | (86.0) | (1.7) | (0.6) | (0.6) | (0.8) | (6.1) | (50.8) |
| **e** | Fuc-(1→2)-(1-$^2$H)Galol | 296 | 236 | 189 | 157 | 397 | 352 | 308 | 177 | 145 | 133 |
| | | (14.0) | (75.4) | (100) | (56.1) | (0.2) | (4.0) | (2.3) | (14.0) | (82.5) | (12.3) |

[a] Relative abundance in parentheses.

TABLE IV

Diagnostic ions of c.i.m.s. of *O*-methyltrisaccharide alditols derived from Oligosaccharide II

| *Fragment* | *Oligosaccharide* | *C.i.m.s. fragment ions* (m/z)[a] | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | $(M+H)^+$ | $(acJ_2+H)$ | $abJ_2$ | $cbA_1$ | $aJ_2OH_2$ | $aJ_2$ | $cA_1$ |
| **f** | 6-Deoxyhexosyl→6-deoxyhexosyl→6-deoxy-(1-$^2$H)hexitol | 586 | 398 | | 363 | 224 | | 189 |
| (major) | | (4.1) | (3.4) | | (43.1) | (51.7) | | (100) |
| **g** | 6-Deoxyhexosyl→hexosyl→6-deoxy-(1-$^2$H)hexitol | | | 410 | 393 | 224 | 206 | 189 |
| | | | | (4.7) | (72.4) | (42.2) | (6.9) | (37.9) |

[a] Relative abundance in parentheses.

TABLE V

Diagnostic ions of e.i.m.s. of *O*-methyltrisaccharide alditols derived from Oligosaccharide II

| *Fragment* | *Oligosaccharide* | *E.i.m.s. fragment ions* (m/z)[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | $aJ_0$ | $aJ_1$ | $aJ_2$ | $abJ_1$ | $abJ_2$ | $cA_1$ | $cA_2$ | $cbA_1$ |
| **f** (major) | Fuc-(1→?)-Fuc-(1→?)-(1-$^2$H)Fucol | 252 (100) | | 206 (25.5) | 440 (18.1) | 380 (12.8) | 189 (100) | 157 (100) | 363 (83) |
| **g** | Fuc-(1→?)-Gal-(1→?)-(1-$^2$H)Fucol | | 266 (21.6) | 206 (18.1) | 470 (8.1) | | 189 (100) | 157 (70.7) | 393 (5.2) |

[a] Relative abundance in parentheses.

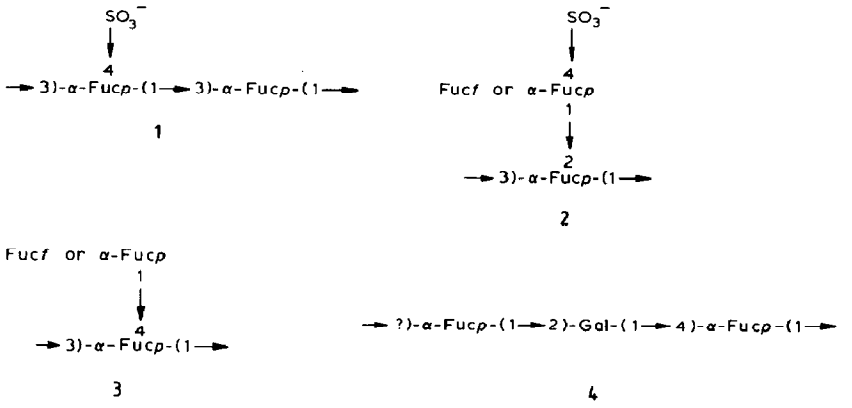
TABLE VI

Methylation analysis of high-mol.-wt. fraction HMF obtained from methylated Polysaccharide C-II

| *Glycosyl residues or group* | *Position of O-methyl groups* | *Position of O-($^2H_3$)methyl groups* | *Deduced positions of linkages* | *Composition mol%*[a] |
|---|---|---|---|---|
| Fucosyl | 2,3,5 | | Terminal (*f*) | 1.5 |
| | 2,3,4 | | Terminal (*p*) | 4.7 |
| | 3,4 | 2 | Terminal (*p*) | 2.6 |
| | 2,3 | 4 | Terminal (*p*) | 7.9 |
| | 3 | 2 | 4 | 1.1 |
| | 2, | 3 | 4 | 2.7 |
| | 2,4 | | 3 | 17.7 |
| | 4 | 2 | 3 | 6.7 |
| | 2 | 4 | 3 | 20.9 |
| | 4 | | 2,3 | 4.8 |
| | 2 | | 3,4 | 4.2 |
| Galactosyl | 2,3,4,6 | | Terminal (*p*) | 3.4 |
| | 3,4,6 | 2 | Terminal (*p*) | |
| | 2,4,6 | 3 | Terminal (*p*) | |
| | 2,3,6 | 4 | Terminal (*p*) | |
| | 2,3,4 | 6 | Terminal (*p*) | 0.8 |
| | 2,3,4 | | 6 | 2.8 |
| | 2,4 | 3 | 6 | 4.8 |
| | 3,6 | 2 | 4 | 0.7 |
| | 2,6 | 3 | 4 | 1.6 |
| | 2,3 | 6 | 4 | 1.6 |
| | 4,6 | 2 | 3 | 0.5 |
| | 2,6 | 4 | 3 | 0.1 |
| | 2,4 | 6 | 3 | 0.5 |
| | 3,4,6 | | 2 | [b] |
| | 2,3 | | 4,6 | 4.9 |
| | 3 | 2 | 4,6 | 1.1 |

[a] Calculated from peak areas and response factors[37] of hydrogen-flame-ionization detector on g.l.c., and from the ratio of the intensities of specific fragment ions. [b] Trace.

therefore, only the high-mol.-wt. fraction (HMF) was further analyzed. This mainly gave 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl- and 1,3,5-tri-*O*-acetyl-2-*O*-methyl-4-*O*-($^2H_3$)methyl-fucitol, in addition to small proportions of 1,5-di-*O*-acetyl-2-*O*-($^2H_3$)methyl-3,4-di-*O*-methyl-, of 1,5-di-*O*-acetyl-2,3-di-*O*-methyl-4-*O*-($^2H_3$)methyl-2,3-di-*O*-methyl-, 1,4,5-tri-*O*-acetyl-3-*O*-methyl-2-*O*-($^2H_3$)methyl-, 1,4,5-tri-*O*-acetyl-2-*O*-methyl-3-*O*-($^2H_3$)methyl-, and 1,3,5-tri-*O*-acetyl-4-*O*-methyl-2-*O*-($^2H_3$)methyl-fucitol; and 1,5-di-*O*-acetyl-tri-*O*-methyl-mono-*O*-($^2H_3$)methyl- and 1,5,6-tri-*O*-acetyl-2,4-di-*O*-methyl-3-*O*-($^2H_3$)methyl-galactitol; in addition, small proportions of nondeuteriomethylated 2,3-di-*O*- and 3,4-*O*-di substituted fucosyl and 4,6-di-*O*-substituted galactosyl derivatives were observed (see Table VI).




## DISCUSSION

An anticoagulant, fucose-containing, sulfated polysaccharide from *E. kurome* was characterized as a new type of fucan sulfate. The results of optical rotation[1] and n.m.r. analysis suggested that fucose is present preponderantly in the α-L form in Polysaccharide C-II. Methylation analysis of Polysaccharide C-II and its partially desulfated, high-molecular-weight fractions (Ia and Ib), and oligosaccharide analysis indicated that Polysaccharide C-II has a backbone of 3-O-linked fucosyl residues.

Methylation analysis of the high-molecular-fraction (HMF), obtained from methylated Polysaccharide C-II by methanolysis, showed that most of the ($^2H_3$)methyl groups were linked to O-4 of the fucosyl residues. This result and a comparative methylation analysis between Polysaccharide C-II and the low-sulfated fraction (Ia) suggested that the sulfate groups may be mainly located at C-4 of the 3-*O*-linked fucan backbone (**1**). Although the present results also assumed that some sulfate groups may be attached to various positions of the fucosyl and galactosyl residues, it is not known whether deuteriomethylation occured at free hydroxyl groups released by desulfation or cleavage of glycosidic linkages during methanolysis. In a previous study[1], the i.r. spectrum of Polysaccharide C-II indicated that most sulfate groups are mainly linked to C-4 and some to C-2 or C-3. Anno *et al.*[34] reported that fucoidan from *Pelvetia wrightii* has sulfate groups at C-4 because fucose 4-sulfate was obtained from a partial hydrolyzate of this fucoidan. Our results are in agreement with Anno *et al.*[34] results, but the molar ratio of sulfate and fucose suggests that some fucosyl residues of Polysaccharide C-II have more than one sulfate group or that partial desulfation may have taken place in the course of the methylation of fucan sulfate by the method of Hakomori[23]. Methylation analysis of the high-mol.-wt. fraction HMF and oligosaccharide analysis also indicated that fucosyl groups were linked to O-2 and O-4 of the backbone 3-*O*-linked fucosyl residues (**2** and **3**, respectively).

The fucan sulfate (fucoidan) isolated from *F. vesiculosus* consisted[8–10] mainly of 2-*O*-linked fucosyl residues with sulfate groups located at C-4, in addition to small proportions of 2,3-*O*- and 2,4-*O*-disubstituted fucosyl residues. Polysaccharide C-II has

some structures similar to those of other polysaccharides having a high fucose sulfate content[17,35] from *Himanthalia lorea* and *Bifurcaria bifurcata*; however these polysaccharides contain mainly 2-*O*-linked fucosyl residues in addition to 3-*O*-linked fucosyl residues. Therefore, Polysaccharide C-II is a new type of fucan sulfate, as the structure of its backbone differs from that of other known fucans. Polysaccharide C-II also contains terminal fucofuranosyl groups. The presence of these groups has been reported only for the extracellular polysaccharide[36] produced by the diatom *Chaetoceros curvisetus*.

Polysaccharide C-II also contains 2-*O*-linked galactosyl residues and 4-*O*-linked and 3,4-di-*O*-substituted glucosyluronic acid residues as minor components. The present results suggested that the galactosyl residues may be interconnected in the fucosyl backbone or may be components of side-chains because several linkages were observed in both Fractions Ia and Ib. A fragment Fuc→Gal→Fuc was found in the methanolyzate of Polysaccharide C-II; it may have the structure Fuc-(1→2)-Gal-(1→4)-Fuc (**4**) because Gal-(1→4)-Fuc and Fuc-(1→2)-Gal were detected by oligosaccharide analysis.

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