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Funoran from the red seaweed, *Gloiopeltis complanata*: polysaccharides with sulphated agarose structure and their precursor structure

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Funoran extracted from the red seaweed, *Gloiopeltis complanata*, was fractionated based on the solubility of its cetylpyridinium salt in KCl solution to give the fractions PS1 and PS2. PS2 was further fractionated into PS2G that formed a gel in 0.5 N KCl, and PS2S that did not form a gel in saturated KCl. PS1, PS2G and PS2S were different in composition and properties showing that the funoran was heterogeneous. On the basis of the results from composition analyses, partial methanolysis studies, methylation studies, ^{13}C-NMR spectrum measurements, and regioselective desulphation studies employing a silylating reagent, *N,O*-bis(trimethylsilyl)acetamide, PS2G was shown to consist chiefly of 6-*O*-sulphated agarose, repeats of →3)6-SO_3-β-D-Gal(1→4)3,6-anhydro-α-L-Gal(1→; and PS2S to contain repeats of →3)6-SO_3-β-D-Gal(1→4)6-SO_3-α-L-Gal(1→ as well as smaller amounts of →3)2,6-di-SO_3-β-D-Gal(1→ and →3)2-SO_3-β-D-Gal(1→ residues.

INTRODUCTION

Although sulphated polysaccharides from red seaweeds vary in structure and properties depending on their origin, most of them are galactan sulphates, the main chain of which consists of a common backbone with an underlying repeat of (1→3)-linked β- and (1→4)-linked α-Gal (or its 3,6-anhydride) residues (Painter, 1983). The former residue always occurs as the D-enantiomer while the latter as D- or L-enantiomer depending on the origin. Thus the polysaccharides are classified into carrageenan and agarose types, respectively. Funoran, one of such sulphated galactans from the red seaweeds, *Gloiopeltis* spp. (Hirase *et al.*, 1957), is employed as an adhesive in several Japanese industries such as pottery and textiles. The funoran from *G. furcata* consists of several components which can be fractionated in terms of the solubility of their quaternary ammonium salts in aqueous potassium chloride (Hirase & Watanabe, 1972). The main polysaccharide fraction thereby obtained is an agarose-type polysaccharide consisting of a repeat of:

→3)6-SO_3^--β-D-Gal(1→4)3,6-anhydro-α-L-Gal(1→

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In addition to this main structure, an unfractionated funoran from a closely related species, *G. cervicornis*, (Lawson *et al.*, 1973; Penman & Rees, 1973) has been proposed to possess a precursor moiety that is converted into the agarose sulphate under alkaline conditions. The present paper describes fractionation and structural investigation of the fractions from a funoran from *Gloiopeltis complanata*.

MATERIALS AND METHODS

General methods

Colorimetric determination of galactose (Gal) and 3,6-anhydrogalactose (AGal) contents in polysaccharides were carried out by the method of Yaphe (1960) and expressed as wt.% of their anhydrohexose units. Sulphate contents expressed as wt.% of SO_3K were estimated by titration. A mixture of sulphated polysaccharides and an aliquot of glycolchitosan was titrated with potassium polyvinylsulphate using toluidine blue as indicator. Optical rotation was measured using a Laurent's saccharimeter equipped with a 1 dm cell. Gas–liquid chromatography (GLC) was carried

out with a chromatograph (GC-7A, Shimadzu Corp.) equipped with a flame ionization detector using the following fused silica capillary columns at the respective operating temperatures unless otherwise mentioned; column a, PEG-20M bonded (GL-Science Co.) at 200°C; column b, CP-Sil 88 (Chrompak Co.) at 205°C; column c, OV-1 bonded (GL-Science Co.) at 170°C. Nitrogen was used as carrier gas at a flow rate of 20 ml/min and in a split ratio of 20:1. Combined gas–liquid chromatography–electron impact mass spectrometry (GC/MS) was carried out using a mass spectrometer (GC/MS QP-1000, Shimadzu Corp.) at 70 eV ionization potential. The chromatographic conditions were identical to those mentioned above except that helium was used as the carrier gas. ^{13}C-NMR spectra in D_2O were recorded by spectrometers (XL-200, Varian Instruments and GE-300, General Electric Co.) at 80°C. In calculating for Fourier transformation, apodization using 4 Hz line broadening followed by zero filling was executed. Chemical shifts were measured relative to internal methanol, the chemical shift value of which was taken as 49.30 ppm.

Extraction and fractionation of polysaccharides

The red alga, *Gloiopeltis complanata*, was harvested from the Shirahama coast in the Shizuoka Prefecture. Dried seaweed (50 g) was macerated with water, homogenized with a blender and extracted twice with water (2500 ml) at 100°C. To the combined extract, 5% cetylpyridinium (CP) chloride was added until the resulting CP salt of sulphated polysaccharide was no longer precipitated. The CP salt of the polysaccharide was washed successively with water, methanol and acetone, and extracted twice with 4 N KCl at 18°C. The combined extract was concentrated and poured into 4 vol. of ethanol to give a precipitate, which was dissolved in water, dialysed, concentrated and then poured into 4 vol. of ethanol to recover the potassium salt of the polysaccharide, PS1 (425 mg, $[\alpha]_D^{27}$ + 64.0° (*c* 0.4), Gal 33.6%, AGal 1.1%, sulphate 36.1%). The residue, which was insoluble in 4 N KCl at 18°C, was extracted with 4 N KCl at 100°C, and was immediately poured into 4 vol. of ethanol. The resulting precipitate was dissolved in water, dialysed, and precipitated with 4 vol. of ethanol to give PS2 (3.78 g, $[\alpha]_D^{18}$ − 26.3° (*c* 1.2), Gal 40.2%, AGal 19.9%, sulphate 31.1%).

Subfractionation of PS2

To an aqueous solution of PS2 (3 g in 150 ml) was added 1 N KCl (150 ml) and the solution stood overnight at 4°C. The resulting gel was collected by centrifugation at 4°C, and the precipitate was dialysed, concentrated and poured into 4 vol. of ethanol to give PS2G (1.92 g, $[\alpha]_D^{20}$ − 20.0° (*c* 1.5), Gal 36.8%, AGal 22.8%, sulphate 26.7%). The KCl concentration of the supernatant was adjusted to 1.6 N, to give a weak gel. This was not further investigated because only a small quantity (0.17 g) was obtained. The supernatant, which did not afford gel even in saturated KCl at 4°C, was dialysed, concentrated and precipitated with ethanol to give PS2S (0.57 g, $[\alpha]_D^{18}$ − 18.5° (*c* 1.5), Gal 46.1%, AGal 8.8%, sulphate 30.2%).

Composition analyses of polysaccharides

To identify the component sugars, polysaccharides (10 mg) were methanolysed with 0.5 ml of 3% methanolic hydrogen chloride for 18 h at 70°C. The methanolysate was trimethylsilylated (Sweeley *et al.*, 1963) and analysed by GLC and GC/MS (column *c*). The absolute configuration of component sugars except for AGal was assigned with the aid of GC/MS (column *c*, 210°C) of trimethylsilylated derivatives of L-2-octyl glycosides (Takano *et al.*, 1993). The ratio of the D- to L-enantiomer of Gal was calculated from the peak area corresponding to each enantiomer. The configuration of AGal was assigned to the L-form, because the agarobiose derivatives were detected whereas no carrabiose derivative was found after the partial methanolysis as mentioned later. For quantitative analysis, component sugars were analysed as their alditol acetates (Björndal *et al.*, 1967) by the method suitably modified for estimation of acid-labile AGal derivatives as follows. The polysaccharides isolated were hydrolysed with 0.02 N sulphuric acid for 1 h at 100°C to cleave acid labile 3,6-anhydrogalactosyl linkage, and treated with $NaBH_4$ for 2 h at room temperature to reduce the aldehyde group of the liberated AGal residues at the reducing ends. The resulting partial hydrolysate was further hydrolysed with 1 N H_2SO_4, neutralized, reduced with borohydride, acetylated with acetic anhydride and pyridine, and analysed by GLC and GC/MS (column a, 200°C, and column b, 205°C). The respective total areas of peaks arising from Gal series and that arising from AGal series were converted to the analytical values based on colorimetric determination. The results of the calculation are shown in Table 1.

Partial methanolysis of PS2G

PS2G (400 mg) in 0.1 N methanolic hydrogen chloride was heated at 70°C for 2 h, and then the solution was neutralized with Ag_2CO_3. A portion of the dried methanolysate was trimethylsilylated and analysed by GLC and GC/MS (column *c* at 250°C). TMS derivatives of 4-*O*-β-D-galactopyranosyl-3,6-anhydro-L-galactose dimethylacetal (agarobiose dimethylacetal) and 4-*O*-(6′-*O*-methyl-β-D-galactopyranosyl)-3,6-anhydro-L-galactose dimethylacetal (6′-*O*-methylagarobiose dimethylacetal) were identified.

Alkaline treatment of polysaccharides

To an aqueous solution of PS2G (100 mg/70 ml) was added 20 mg of KBH_4 and the solution stood for 18 h at

15°C. Then, KOH (2.8 g) and additional KBH_4 (50 mg) were added and heated to 80°C for 2 h. The solution was neutralized with acetic acid, dialysed against tap water, concentrated and precipitated with 4 vol. of ethanol to give alkali-treated PS2G, (designated as PS2GA, 80 mg, $[\alpha]_D$ − 26.8° (*c* 0.9), Gal 35.3%, AGal 26.6% sulphate 23.2%). PS2S was also treated similarly to give alkali-treated PS2S (PS2SA, $[\alpha]_D$−14.4° (*c* 1.0), Gal 43.8%, AGal 20.1%, sulphate 19.9%).

Methylation analysis of polysaccharides

Polysaccharide samples were methylated by powdered NaOH and iodomethane based on the methods of Isogai *et al.* (1985) and Ciucanu & Kerek (1984) except for the use of the triethylammonium salt of polysaccharide according to the recommendation by Stevenson and Furneaux (1991). The methylated polysaccharide was successively hydrolysed with 0.1 N H_2SO_4, treated with $NaBH_4$ and further hydrolysed with 1 N H_2SO_4 as described above. The hydrolysis products were analysed by GLC and GC/MS (column a, 200°C, and column b, 205°C) as partially methylated alditol acetates (Jansson *et al.*, 1976). The results are summarized in Table 2. Absolute configuration of the partially methylated sugar obtained after total hydrolysis of methylated polysaccharide was assigned as described elsewhere (Takano *et al.*, 1993).

Partial methanolysis of PS2S

PS2S (80 mg) was partially methanolysed similarly to PS2G. The sediment was methanolysed again, and the remained residue was recovered as PS2SR (33 mg). The combined methanolysate was analysed similarly to that of PS2G by GLC to detect the agarobiose derivative. PS2SR was methylated and analysed as already described.

BTSA treatment of polysaccharides

Polysaccharide (50 mg) was converted into the pyridinium salt, and treated with BTSA (4 ml) in dry pyridine (20 ml) as described elsewhere, (Matsuo *et al.*, 1993) to give the 6-desulphated polysaccharide (PS2SB, 37 mg).

RESULTS AND DISCUSSION

Extraction and fractionation of funoran

A funoran extracted from *Gloiopeltis complanata* was fractionated similarly to that from *G. furcata* (Hirase & Watanabe, 1972) based on a method for mucopolysaccharides (Schiller *et al.*, 1961). A sulphated polysaccharide was precipitated from the algal extract as its insoluble cetylpyridinium (CP) salt, which was dissolved at 18°C with 4 N KCl, to recover the potassium salt of the polysaccharide designated as PS1. The insoluble CP salt was extracted at 100°C with 4 N KCl to give the potassium salt of a sulphated polysaccharide, PS2. Besides the fractionation method employed above, carrageenans are fractionated into κ- and λ-fractions based on the difference in their gelling properties in dilute potassium chloride solution. Fujiki (1973) has indicated that a funoran from the closely related *G. tenax* can be fractionated into gel-forming and non-gel-forming fractions using higher levels of potassium chloride solution concentration than employed for the fractionation of carrageenans. On this basis, PS2 was further fractionated with 0.5 N potassium chloride at 4°C to give a gel-forming subfraction designated as PS2G. The supernatant was recovered as a subfraction PS2S, which did not form a gel even in saturated potassium chloride solution at 4°C.

As summarized in Table 1, the total amount of D-Gal and its methyl ether in PS2G and PS2S was nearly equal to that of L-Gal, 3,6-anhydro-L-Gal (L-AGal), and the respective methyl ethers. In contrast, PS1 consisted of a large amount of D-Gal, while it contained only small amounts of L-AGal and D-Gal. PS1 was also distinguished from PS2G and PS2S by positive optical rotation. PS2G and PS2S were substantially different in their AGal content. Funoran thus appeared to be heterogeneous, consisting of at least three components

Table 1. Yield, composition[a], and specific optical rotation of PS1, PS2G and PS2S from the red alga, *Gloiopeltis complanata* and their derivatives

	Yield (%)[b]	D-Gal	6M-D-Gal[c]	L-Gal	2M-L-Gal[c]	L-AGal[d]	$-SO_3^-$	$[\alpha]_D$
PS1	0.9	87.7	12.3	4.3	1.7	3.6	158.8	+64°
PS2G	4.8	97.4	2.6	8.9	1.3	76.0	109.0	−20°
PS2GA	–	98.0	2.0	0.1	0	85.9	89.0	−27°
PS2S	1.4	95.8	4.2	54.5	2.0	33.4	139.7	−19°
PS2SA	–	96.5	3.5	25.5	0	52.4	77.5	−14°

[a]Molar ratio. Total of D-series galactoses is taken as 100.
[b]Based on the dry seaweed.
[c]6M-D-Gal = 6-*O*-methyl-D-galactose, etc.
[d]3,6-anhydro-L-galactose.

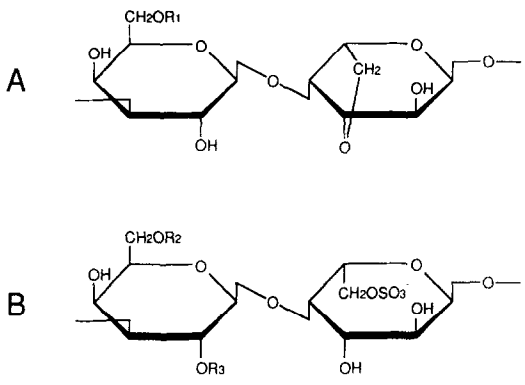

Fig. 1. Idealized structural moieties in the funorans from *Gloiopeltis complanata*. (A) Agarose sulphate moiety: $R_1 = SO_3^-$, CH_3. (B) 'Precursor' moiety: $R_2 = SO_3^-$, CH_3, H; $R_3 = H$, SO_3^-.

corresponding to PS1, PS2G, and PS2S. In the present paper, PS2G and PS2S were further investigated.

Occurrence of the agarose 6-sulphate structure

On partial methanolysis to selectively cleave 3,6-anhydrogalactosyl linkages, PS2G and PS2S gave derivatives of a repeating disaccharide, 4-*O*-β-D-galactopyranosyl-3,6-anhydro-L-galactose (agarobiose) and a small amount of 4-*O*-(6′-*O*-methyl-β-D-galactopyranosyl)-3,6-anhydro-L-galactose (6′-*O*-methylagarobiose) attributable to backbones of agarose structure and its methylated structure, respectively (Fig. 1A). Since 2,4-di-*O*-methyl-D-Gal and 2-*O*-methyl-AGal were the principal products in the methylation analysis of PS2G (Table 2), the major part of the (1→3)-linked residue is most likely to carry 6-sulphate, while the (1→4)-linked AGal residue has no sulphate. This was supported by the ^{13}C-NMR spectra (Fig. 2), in which the 12 signals had chemical shift values (Table 3) identical to those from the agarose 6-sulphate moiety of the polysaccharide from a red alga, *Gracilaria dominguensis* (Fernández *et al.*, 1989). As reported previously (Matsuo *et al.*, 1993), when 6-*O*-specifically desulphated by using a silylating reagent, *N*,*O*-bis(trimethylsilyl)acetamide (BTSA), PS2G yielded a non-sulphated polysaccharide with a ^{13}C-NMR spectra identical to that of agarose. This result also confirms the agarose 6-sulphate structure in PS2G. In the ^{13}C-NMR spectrum of PS2S, the identical set of 12 signals attributed to the agarose sulphate structure was also observed (Fig. 3A) in addition to the peaks indicated by the arrows. These were attributed to another structure as discussed below.

Precursor moiety for agarose 6-sulphate

In algal polysaccharides such as porphyran and some carrageenans, the (1→4)-linked L- or D-Gal 6-sulphate residue is converted into the 3,6-anhydride under alkaline conditions (Rees, 1961*a*). Such a residue is known as a 'precursor residue' in the biosynthetic process, where a similar reaction also takes place enzymatically (Rees, 1961*b*; Rees & Conway, 1962). In addition to the established agarose 6-sulphate structure in PS2G and PS2S, another moiety containing L-Gal residue with sulphate at O-6 was suggested from the decrease in L-Gal content accompanied by an increase in AGal content after the alkaline treatment (PS2GA and PS2SA in Table 1). The precursor is enriched in PS2S, because the increase in AGal content and decrease in L-Gal content after the alkaline treatment were much more significant in PS2S than in PS2G. The principal components in the methylation analysis of PS2S (Table 2) were 2,3-di-*O*-methyl-L- and 2,4-di-*O*-methyl-D-galactose derivatives attributable to 6-sulphated (1→4)-linked L- and (1→3)-linked D-Gal residues, respectively. In addition, 4-*O*-methyl- and 4,6-di-*O*-methyl-D-galactose derivatives are likely to have arisen from (1→3)-linked 2,6-disulphated and 2-sulphated (and/or 2-sulphated 6-*O*-methyl-) D-Gal residues, respectively. This was supported by the large increase in

Table 2. Methylation analyses[a] of polysaccharides from *Gloiopeltis complanata*

Product[b]	PS2G	PS2GA[c]	PS2S	PS2SA[c]	PS2SB[d]	PS2SR[e]
2,3,4,6-Me_4-D- and L-Gal	0	0	0	0	0	10
2,4,6-Me_3-D-Gal	7	8	21	10	63	91
2,3,6-Me_3-L-Gal	1	1	15	10	100[f]	100[f]
4,6-Me_2-D-Gal	0	0	12	9	37	1
2,6-Me_2-D- and L-Gal	0	0	0	0	0	3
2,4-Me_2-D-Gal	100[f]	100[f]	100[f]	100[f]	41	21
2,3-Me_2-D-Gal	8	0	98	18	17	12
4-Me-D-Gal	0	0	33	27	2	0
2-Me-L-AGal	95	106	44	95	40	0

[a]Based on peak area of GLC of partially methylated alditol acetates from hydrolysated methylated polysaccharides.
[b]2,3,4,6-Me_4-Gal, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, etc.
[c]Alkaline treated PS2G and PS2S.
[d]BTSA-treated PS2S.
[e]Partial methanolysis residue of PS2S.
[f]The values are taken as 100.

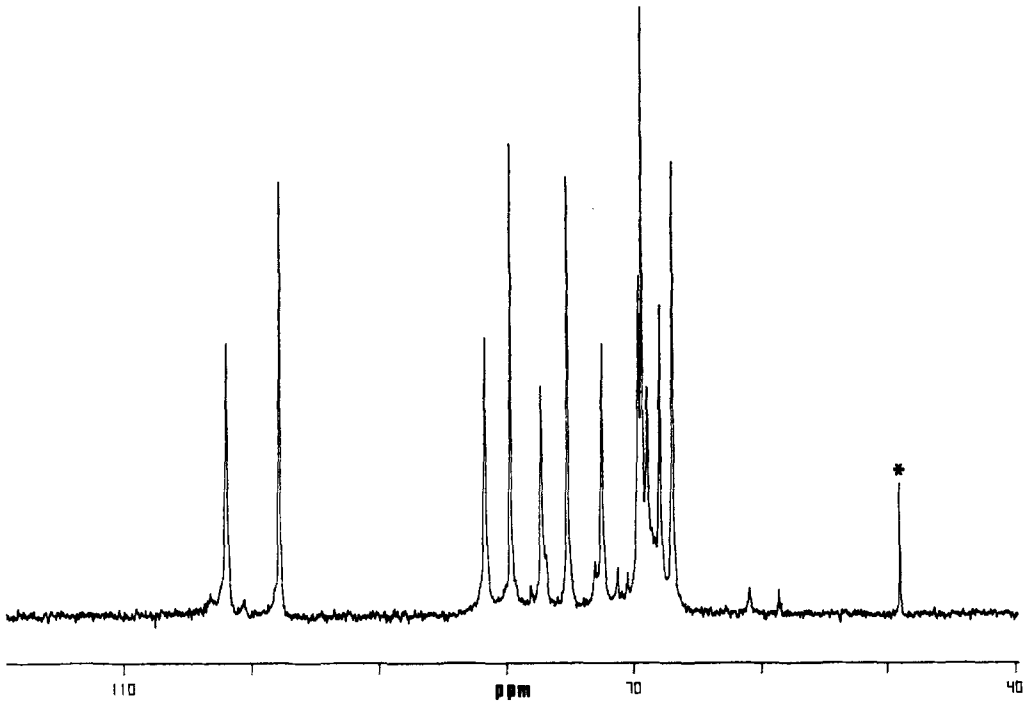

Fig. 2. ^{13}C-NMR spectrum of PS2G. The asterisked signal arises from internal methanol.

Table 3. ^{13}C-NMR chemical shift values and peak assignments of PS2G, PS2S and other structurally related polysaccharides

	C1	C2	C3	C4	C5	C6
→3)β-D-Gal-6-SO_3^-(1→ (agarose sulphate moiety in PS2G and PS2S)	102.35	69.91	81.99	68.21	72.80	67.22
→3)β-D-Gal-6-SO_3^-(1→ (*Gracilaria dominguensis* agar)[a]	102.5	70.1	82.1	68.4	72.9	67.4
→3)β-D-Gal(1→ (agarose)[b]	102.26	70.07	82.04	68.59	75.29	61.24
→3)β-D-Gal-6-SO_3^-(1→ (precursor moiety in PS2S)	103.59	69.92	80.55	68.71	73.23	67.21
→3)β-D-Gal(1→ (porphyran)[c]	103.48	69.96	80.90	69.02	75.65	61.46
→4)3,6-anhydro-α-L-Gal(1→ (agarose sulphate moiety in PS2G and PS2S)	98.16	69.64	79.96	77.58	75.51	69.23
→4)3,6-anhydro-α-L-Gal(1→ (agarose)[b]	98.14	69.69	79.95	77.21	75.46	69.26
→4)α-L-Gal-6-SO_3^-(1→ (precursor moiety in PS2S)	100.94	68.96	70.85	79.55	69.64	67.36
→4)α-L-Gal-6-SO_3^-(1→ (porphyran)[c]	101.09	69.60	70.87	78.91	69.96	67.55

[a]Chemical shift values and assignment based on Fernández *et al.* (1989).
[b]Assignment based on Nicolaisen *et al.* (1980).
[c]Assignment based on Lahaye & Yaphe (1985).

2-*O*-methyl-AGal which was accompanied by a decrease in the 2,3-di-*O*-methylgalactose derivative in the methylation analysis after the alkaline treatment of PS2S (PS2SA in Table 2). This was further confirmed by a treatment of PS2S with BTSA, which regioselectively removed the 6-sulphate group (Takano *et al.*, 1992; Matsuo *et al.*, 1993). In the methylation analysis of the BTSA-treated PS2S (PS2SB in Table 2), decreases in

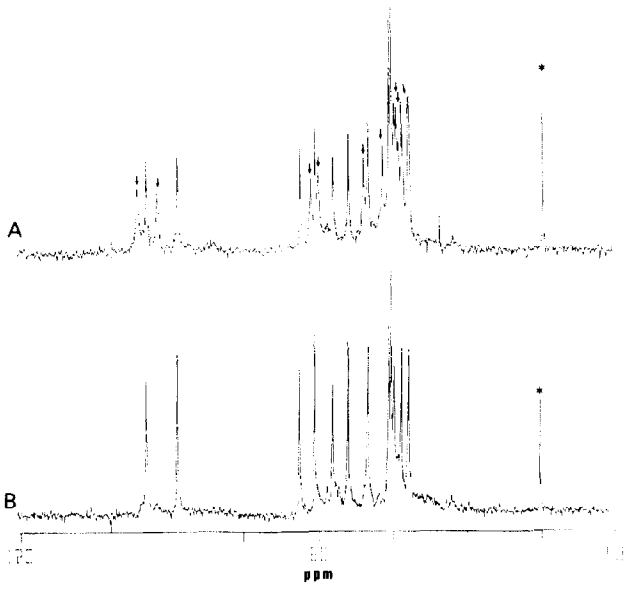

Fig. 3. ^{13}C-NMR spectra of PS2S. The asterisked signals arise from internal methanol. (A) Native PS2S. The arrows indicate the signals attributable to 'precursor' moiety. (B) Alkali-treated PS2S (PS2SA).

2,3-di-*O*-, 2,4-di-*O*-, and 4-*O*-methylgalactose derivatives were accompanied by increases in the 2,3,6-tri-*O*-, 2,4,6-tri-*O*-, and 4,6-di-*O*-methylgalactose derivatives. The covalent structure of the 'precursor' moiety thus can be illustrated as shown in Fig. 1B. The peaks indicated with arrows in the NMR spectrum of PS2S (Fig. 3A) can be attributed to the major 'precursor' moiety →3)β-D-Gal-6-SO_3^-(1→4)α-L-Gal-6-SO_3^-(1→, by referring to the published spectral data and their assignment for porphyran (Table 3) consisting of a repeating unit, →3)β-D-Gal(1→4)α-L-Gal-6-SO_3 (1→ (Lahaye & Yaphe, 1985; Usov *et al.*, 1983). The spectrum obtained after the alkaline treatment (Fig. 3B) was almost identical to that of PS2G, which corresponds very closely to the idealized agarose 6-sulphate structure. However, signals attributable to the minor repeating structure carrying 2-sulphate at the (1→3)-linked residues were not clearly observed possibly due to their low intensity both before and after alkaline treatment.

Although PS2S yielded agarobiose derivatives on partial methanolysis as already described, this polysaccharide was not completely degraded. A substantial amount of methanol-insoluble material termed PS2SR remained. This is likely to have arisen from a moiety that consists of consecutive repeating units containing the precursor residues, because approximately the same amount of (1→3)-linked D- and (1→4)-linked L-Gal residues appeared to occur as based on the methylation analysis of PS2SR (Table 2).

Penman and Rees (1973), who had investigated an unfractionated funoran from a closely related alga, *Gloiopeltis cervicornis*, have proposed (1→4)-linked 2,6-disulphate residue as the precursor unit. This was because there was an increase in 3,6-anhydride content on alkaline treatment even after periodate oxidation. This should have decomposed (1→4)-linked Gal residue with free hydroxyl groups at C-2 and C-3 such as (1→4)-linked Gal mono-6-*O*-sulphate residue. The proposed structure does not agree, however, with the established structure of PS2S based on the results from the methylation studies and the NMR measurement. It is unclear whether this disagreement is due to an abnormal periodate-resistant property of the sulphated residue, or to a difference in the source of the polysaccharides. When treated with alkali after the periodate oxidation and borohydride reduction, however, PS2S yielded, on hydrolysis, 1,4-anhydrothreitol, which is most likely to have arisen from the (1→4)-linked 6-monosulphated Gal residue.

The treatment of PS2S with BTSA appeared to remove regioselectively 6-sulphate groups. However, the removal of the sulphate was incomplete, while the main fraction of funoran (PS2G in the present paper), porphyran, chondroitin sulphate, and dermatan sulphate are almost completely 6-desulphated as described elsewhere (Matsuo *et al.*, 1993). Such incomplete removal of the 6-sulphate group was also found in BTSA-treatment of heparin (unpublished results) which is highly sulphated and contains conformationally flexible L-iduronic acid residues (Casu, 1985).

The biosynthetic route and biological role of the funoran is unknown. A funoran from another source, *G. furcata* (Hirase & Watanabe, 1972), and a sulphated polysaccharide from *Gracilaria dominguensis* with an agarose sulphate structure (Fernandez *et al.*, 1989) have been reported to possess antitumor activity. Another red algal polysaccharide with a very closely related agarose type structure, porphyran from *Porphyra yezoyensis*, and one of the sulphated agar fractions of *Gracilaria verrculosa*, have been reported to enhance activation and/or increment of macrophage (Yoshizawa *et al.*, 1993). A kind of carrageenan has also been reported to possess anti-HIV activity (Nakashima *et al.*, 1987). The funoran, therefore, would be a potential material with biologically and pharmaceutically useful properties, in addition to several industrial usages.

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