ELSEVIER

Carbohydrate Polymers 23 (1994) 165–173

0144-8617/94/$07.00

Isolation and partial characterization of a novel β-D-galactan sulfate from the brown seaweed *Laminaria angustata* var. *longissima*

Takashi Nishino,* Yuuzou Takabe & Terukazu Nagumo

Department of Biophysics, School of Hygienic Sciences, Kitasato University, 1-15-1 Kitasato, Sagamihara, Kanagawa 228, Japan

(Received 8 June 1993; revised version received 23 November 1993; accepted 25 November 1993)

The fastest-moving component (LA-5) on electrophoresis was isolated from a sulfated polysaccharide fraction of the brown seaweed *Laminaria angustata* var. *longissima* by anion-exchange chromatography, fractional precipitation with ethanol, and gel filtration. LA-5 consisted of galactose, fucose, glucuronic acid and sulfate (100:2:3:74) and had an apparent average molecular weight of 400 000. The results of enzymatic digestion, optical rotation and ^{13}C-NMR suggested that galactose is present predominantly as the β-D-form in LA-5. By IR, ^{13}C-NMR and methylation analyses, LA-5 has been shown to have a backbone of →3)-β-D-galactosyl-(1→6)-β-D-galactosyl-(1→. Most sulfate groups may be attached to position 6 of 3-linked galactosyl residues. This indicates that the polysaccharide is a new type of galactan sulfate different from the well known galactans from red seaweeds and ascidians. This is the first report on the presence of galactan sulfate in the brown seaweeds.

INTRODUCTION

The brown seaweeds of *Laminaria* species such as *L. angustata*, *L. angustata* var. *longissima* and *L. japonica* ('Mitsuishikonbu', 'Nagakonbu', and 'Makonbu' in Japanese, respectively) have been used from ancient times as a Japanese food. Brown seaweeds have also been used as a decoction for cancer in Chinese herb medicine. A sulfated polysaccharide fraction from *Laminaria angustata* var. *longissima* has been studied for biological activities. Yamamoto *et al.* (1974, 1984) have reported that a crude sulfated polysaccharide fraction extracted from the above seaweed with boiling water showed antitumor activity against Sarcoma-180 and L-1210 leukemia in mice. In a previous paper (Nishino & Nagumo, 1987), we have reported that a crude sulfated polysaccharide fraction (LA-SPS), which was obtained from the above seaweed by extraction with dilute hydrochloric acid and then partial purification with cetylpyridinium chloride and calcium chloride, showed considerable anticoagulant activity with respect to activated partial thromboplastin time (APTT) using human plasma. However, an electrophoretic study indicated that the LA-SPS fraction consisted of more than five sulfated polysaccharide components. Therefore, in order to identify the biologically active component(s), we attempted to isolate each of the polysaccharide components from the fraction. We first tried to isolate the fastest-moving component (LA-5) by electrophoresis of the fraction as it was expected that this component would have the highest content of sulfate groups and thus would show the highest anticoagulant activity among the components in the fraction (Nishino & Nagumo, 1991, 1992). LA-5 was obtained from the LA-SPS fraction by anion-exchange chromatography, fractional precipitation with ethanol and gel filtration. The results of enzymatic, chemical and instrumental analyses indicated that LA-5 is a novel galactan sulfate being composed mainly of D-galactose residues. The galactan sulfate obtained differed from all the previously reported galactose-rich glycans such as carrageenans, agar and porphyran of red seaweeds (Painter, 1983) and sulfated L-galactan from the tunic of ascidians (Pavão *et al.*, 1989). This is the first report on the presence of galactan sulfate in polysaccharides from brown seaweeds. We describe herein the isolation and preliminary characterization of the galactan sulfate from the brown seaweed *L. angustata* var. *longissima*.

*To whom correspondence should be addressed.

MATERIALS

The brown seaweed *Laminaria angustata* Kjellman var. *longissima* Miyabe was collected off the coast of Nemuro in Hokkaido, Japan in May, 1982. The freshly collected seaweed fronds were washed with water, air-dried, milled and stored at −20°C until 1987.

Heparin (167 units/mg) from porcine intestinal mucosa was purchased from Wako Pure Chemical Industries, Ltd, Japan. Normal human plasma was obtained from Boxter Healthcare Co., actin as APTT reagent from American Hospital Supply del Caribe Inc. and human thrombin (500 NIH units) from Green Cross, Ltd, Japan. D-Galactose oxidase (EC 1.1.3.9, 450 units, from *Dactylium dendroides*) and peroxidase (EC 1.11.1.7, 2000 units, RZ: 3.1, from horseradish) were obtained from Sigma Chemical Co. Sephadex G-10, Sepharose 4B and CL-4B were purchased from Pharmacia Fine Chemicals, and ECTEOLA-cellulose from Serva FeinBiochemica and DEAE-cellulose (DE-23) from Whatman Int. Ltd.

METHODS

General

The carbohydrates in column eluates were monitored by the phenol–sulfuric acid method (Hodge & Hofreiter, 1962). Nitrogen content was determined with an elemental analyzer, Shimadu Analyzer Model 240; 6-deoxysugars content by the method of Gibbons (1955) using L-fucose as a standard; uronic acid content by a modified carbazol method (Bitter & Muir, 1962) using D-glucuronic acid as a standard and sulfate content by the modified method (Kawai *et al.*, 1969) of Dodgson and Price (1962). The 3,6-anhydro-galactose content was determined by the resorcinol method (Yaphe & Arsenault, 1965). Neutral sugars were determined by GLC of the corresponding alditol trifluoroacetate derivatives (Imanari *et al.*, 1969) after acid hydrolysis of a sulfated polysaccharide sample (Mian & Percival, 1973) as described previously (Nishino *et al.*, 1989). GLC was carried out with a Hitachi gas chromatograph Model 063 equipped with a flame-ionization detector and a glass column (3 mm × 2 m) of 2% XF-1105 on Gaschrom P at 125°C.

Electrophoresis was performed on a cellulose acetate membrane (6 × 11 cm and 9 × 12 cm, Fuji Film Co. Ltd, Japan) using 0.1 M pyridine–acetic acid buffer (pH 3.5), 0.1 M zinc acetate (pH 6.6), and 0.1 M hydrochloric acid. Densitometry was performed using a Gelman DCD-16 densitometer at 575 nm after treatment of the toluidine blue-stained cellulose acetate membrane with decahydronaphthalene. Gel-filtration chromatography was carried out on a column (1.2 × 94.5 cm) of Sepharose CL-4B with 0.2 M NaCl as an eluant. To estimate the average molecular weight of polysaccharide, Blue dextran (type 2000, Pharmacia) and several molecular weight fractions of pullulan (Shodex standard P-82, Showa Denko, Japan) were used as the molecular weight standards.

Optical rotation was measured for an aqueous solution (0.1%) of a polysaccharide sample at room temperature with a Jasco DIP-4 polarimeter at 589nm. Infrared spectra (IR) were recorded as a KBr pellets with a Perkin–Elmer 983 infrared spectrophotometer. ^{1}H-(400 MHz) and ^{13}C-(100 MHz) NMR spectra of polysaccharides were obtained for a solution in D_2O at 80°C using a Varian XL-400 F.t. spectrometer. Chemical shifts (δ) were expressed in ppm relative to that of sodium 3-(trimethylsily)-1-propane-sulfonate-d_4.

Assay for anticoagulant activity

Activated partial thromboplastin time (APTT) and thrombin time (TT) were performed by the methods of Andersson *et al.* (1976) and Denson and Bonnar (1973), respectively, using normal plasma as described previously (Nishino *et al.*, 1991*a*). The activity was expressed as units/mg in relation to that of heparin (167 units/mg) as a standard.

Isolation and purification of a galactan sulfate (LA-5) from the brown seaweed *L. angustata* var. *longissima*

(1) Preparation of the crude sulfated polysaccharide (LA-SPS) fraction

The crude sulfated polysaccharide (LA-SPS) fraction (11 g) was prepared from the seaweed fronds (500 g) by extraction with dilute hydrochloric acid (pH 2.0), after pretreatment of the fronds with methanol, followed by fractionation with cetylpyridinium chloride and $CaCl_2$ according to the method described previously (Nishino & Nagumo, 1987).

(2) Anion-exchange chromatography

LA-SPS fraction (1.44 g) was applied to a DEAE-cellulose (Cl^-) column (5 × 38 cm) equilibrated with water. The column was developed by eluting stepwise with 0.7 M (Fraction 1) and 1.5 M (Fraction 2) NaCl successively until the eluates were free from carbohydrate. All the eluates of each fraction were combined, dialyzed against water, and lyophilized. The yields of Fraction 1 and Fraction 2 were 15 and 59% of the LA-SPS fraction, respectively. Fraction 2 (0.8 g) was further chromatographed on a column (2.6 × 40 cm) of ECTEOLA-cellulose (Cl^-) by eluting stepwise with 1.6 M (Fraction 2a), 1.8 M (Fraction 2b) and 1.9 M (Fraction 2c) NaCl successively until the eluates were free from carbohydrate. Each eluate was collected, dialyzed, and lyophilized. The respective yields of Fraction 2a, Fraction 2b and Fraction 2c were 59, 11 and 5%.

(3) Fractional precipitation with ethanol

Fraction 2b and Fraction 2c were each dissolved in water (1%). Ethanol containing 0.3% calcium acetate was added to the solution of Fraction 2b until the final concentration of ethanol became 52%, followed by centrifugation. The supernatant and the precipitate were dialyzed, and then lyophilized. The respective yields of the supernatant and the precipitate were 20 and 80%. Fractional precipitation of Fraction 2c was also carried out in the same manner as described above, except that the final concentration of ethanol was 48%, to give the supernatant and the precipitate with yields of 54 and 46%, respectively.

(4) Purification by gel-filtration

Both supernatant fractions of Fractions 2b and 2c were combined and a solution of the fraction (91.2 mg) in 0.2 M NaCl (20 ml) was applied to a column (5 × 84 cm) of Sepharose 4B. The column was developed with 0.2 M NaCl and fractions (approx. 13.5 ml each) were collected. The major fractions (Fraction 3, fraction numbers 45–82) were combined, dialyzed, and lyophilized to give LA-5 (64.6 mg).

Determination of absolute configuration of galactose in LA-5 with D-galactose oxidase

The absolute configuration of galactose in LA-5 was determined by using D-galactose oxidase (Avigad *et al.*, 1962; Mourão & Perlin, 1987). LA-5 was hydrolyzed (Mian & Percival, 1973) and the hydrolyzate (galactose content, 100 μg) was incubated with peroxidase (4 units) and D-galactose oxidase (9 units) in 2 ml of 50 mM sodium acetate buffer (pH 7.0) at 37°C for 2 h. Then, the reaction mixtures were boiled for 10 min to stop the reaction, and after the solvent was removed by evaporation, the residue was trimethylsilylated with *N*-trimethylsilylimidazole–pyridine solution (TMSI-C, GL Sciences Inc., Japan). The amount of D-galactose oxidized with the enzyme was determined by measuring the galactose contents in the hydrolyzates before and after the enzyme treatment by GLC (3% SE-30 on Chromsorb W, 3 mm × 2 m, at 125°C) of the trimethylsilylated (TMS-) derivatives (internal standard; TMS-mannitol).

Solvolysis

Desulfation of LA-5 was performed as described by Nagasawa *et al.* (1977). LA-5 (58 mg) in water (1%) was applied on a column (5 × 13 cm) of AG 50W × 8 (H^+, 200–400 mesh) for decationation with water at 4°C and the eluate was neutralized with pyridine at 4°C and lyophilized. The resultant pyridinium salt was dissolved in 6 ml of dimethyl sulfoxide–methanol (9:1). The mixture was heated at 80°C for 3 h, and the products were chromatographed on a Sephadex G-10 column (2.64 × 94 cm) equilibrated with water after the reaction mixture was adjusted to pH 9.0 with 0.1 N NaOH. The major fraction was collected and lyophilized. The fraction obtained was applied to an ECTEOLA–cellulose (Cl^-) column (1.5 × 28 cm) equilibrated with water. The column was washed with water to give the non-adsorbed fraction (DS-LA-5; 12.8 mg) and then developed by a linear gradient with 0 → 2 M NaCl (500 ml) to yield the adsorbed fraction. The degree of desulfation was confirmed by the infrared spectrum.

Methylation analysis

The poly- and oligo-saccharides were methylated by the method of Hakomori (1964). Completeness of the formation of alkoxide was checked by using triphenylmethane (McNeil *et al.*, 1980). Each methylated product was purified by using a Sep-pak C_{18} cartridge (Waters Association) according to the procedure of Waeghe *et al.* (1983) as described previously (Nishino *et al.*, 1991*b*). Methylated polysaccharide from LA-5 was fractionated into a non-adsorbed (hydrophilic) fraction and an adsorbed (hydrophobic) fraction by this procedure. The non-adsorbed fraction was dialyzed against distilled water and lyophilized. Methylation was repeated three times. The carboxyl groups in the fully methylated polysaccharides were reduced with sodium borodeuteride in 90% ethanol–tetrahydrofuran (27:73) for 18 h at room temperature, followed by incubation at 80°C for 1 h, and the samples were desalted with AG 50W-X8 (H^+) cation-exchange resin (Waeghe *et al.*, 1983; Dutton *et al.*, 1978). The products were hydrolyzed with 90% formic acid at 100°C for 6 h, followed by hydrolysis with 1 M trifluoroacetic acid at 100°C for 2 h. The hydrolyzates were reduced to the corresponding alditols with sodium borohydride in ethanol containing 1 M ammonium hydroxide, and then the resultant alditols acetylated with acetic anhydride at 121°C for 3 h in the presence of sodium acetate (Dutton *et al.*, 1978). The partially *O*-methylated alditol acetate derivatives were analyzed by GLC and GLC-MS (Lindberg, 1972). GLC was performed on a Hitachi G-3000 gas chromatograph equipped with a flame-ionization detector and with a SPB-1 capillary column (0.25 μm film thickness, 0.25 mm i.d. × 25 m, SUPELCO) in splitless mode. The temperature program was 60–150°C at 18°C/min and then 150–210°C at 2°C/min. GLC-MS was performed on a JEOL DX-300 instrument equipped with a SPB-1 column, an ionization voltage of 70 eV, helium as the carrier gas at 0.9 ml/min, and temperature program 120–210°C at 2°C/min. Peaks were identified on the basis of the relative retention time to that of 2,3,4,6-tetra-*O*-methyl galactitol acetate and fragmentation patterns. The molar ratios for each sugar were calibrated using the peak areas and response factors of the flame-ionization detector in GLC on SPB-1 (Sweet *et al.*, 1975).

RESULTS AND DICUSSION

Isolation and purification of a galactan sulfate (LA-5) from the crude sulfated polysaccharide (LA-SPS) fraction

The crude sulfated polysaccharide (LA-SPS) fraction obtained from the fronds of *L. angustata* var. *longissima* was subjected to electrophoresis (0.1 M zinc acetate, pH 6.6) with a cellulose acetate membrane and its electrophoretic pattern was traced with a densitometer. Electrophoresis showed that the LA-SPS fraction consisted of five sulfated polysaccharide components at least, which were named tentatively, in order of increasing mobility as LA-1, -2, -3, -4 and -5 (Fig. 1). The concentration ratios of LA-1, -2, -3, -4 and -5 were densitometrically estimated at 6:16:27:37:14 (data not shown). In order to identify the anticoagulant polysaccharide(s) in the LA-SPS fraction, we first attempted to isolate the fraction (LA-5) having the highest mobility as it was expected that the fraction containing the largest amount of sulfate groups would have the highest anticoagulant activity (Nishino & Nagumo, 1991, 1992). For separation of each component, the LA-SPS fraction was first fractionated into Fraction 1 and Fraction 2 by DEAE–cellulose chromatography. Electrophoresis indicated that Fraction 2 contained LA-5 (Fig. 1). Fraction 2 was further chromatographed on an ECTEOLA–cellulose column to give three fractions 2a, 2b and 2c. Electrophoresis indicated that both Fractions 2b and 2c contained LA-5 as the minor and the major components, respectively (Fig. 1). Fractions 2b and 2c were fractionated by fractional precipitation with ethanol and the respective supernatant fractions containing LA-5 (data not shown) were combined and further purified by gel chromatography (data not shown). Electrophoresis showed that the purified fraction (Fraction 3) behaved as a single band and contained LA-5 alone (Fig. 2). The gel filtration chromatography of LA-5 on a Sepharose CL-4B column gave a single and almost symmetrical elution curve (Fig. 3). The yield of LA-5 obtained was 0.04% on a basis of weight of the seaweed fronds.

Preliminary characterization of LA-5

The physicochemical properties of LA-5 are summarized in Table 1. Its apparent average molecular weight was estimated at 400,000 by gel-filtration. Unexpectedly, LA-5 contained only a little fucose which is a major constituent sugar in fucan sulfates from the brown seaweeds. The polysaccharide contained galactose and sulfate as the major constituents. 3,6-Anhydro-D-galactose and nitrogen were not detected in the polysaccharide. LA-5 was composed of galactose, fucose, glucuronic acid and ester sulfate in the approximate molar percentages of 55.9:1.2:1.7:41.1, indicating that the polysaccharide may be a galactan sulfate. The molar ratio of sulfate groups to the sugar residues was about 0.7. The infrared spectrum showed a strong absorption band of S=O stretching vibration at about 1240 cm^{-1} in addition to a strong band of C–O–S at 818 cm^{-1}. Lloyd *et al.* (1961) and Lloyd and Dodgson (1961) reported that infrared absorption bands at about 820 cm^{-1} were due to a stretching vibration of C–O–S

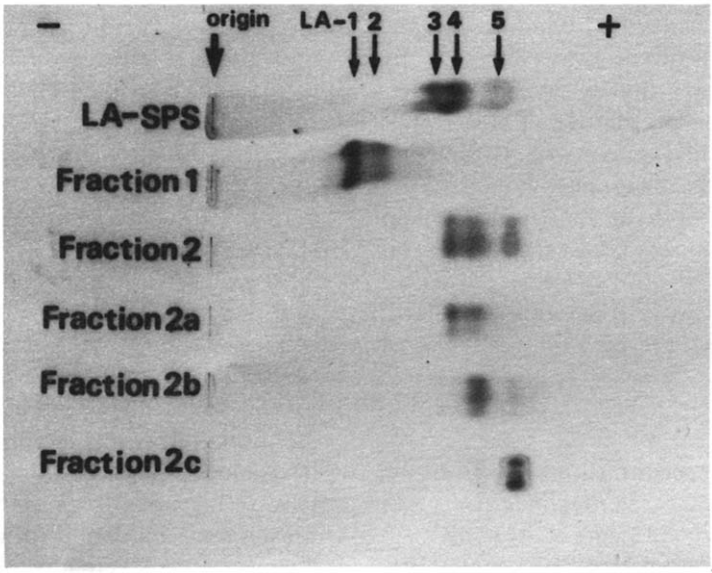


Fig. 1. Electrophoresis of the sulfated polysaccharide fractions from *L. angustata* var. *longissima*. Electrophoresis was performed on a cellulose acetate membrane (9 × 12 cm) for 1 h at 200 V in 0.1 M zinc acetate (pH 6.6). Staining was performed with 0.5% toluidin blue in 3% acetic acid. Fractions 1 and 2 were fractionated from the LA–SPS fraction, which was extracted from the *L. angustata* var. *longissima* with dilute acid, by stepwise elution with 0.7 M and 1.5 M NaCl on a DEAE–cellulose column, respectively. Fractions 2a, 2b and 2c were fractionated from Fraction 2 by stepwise elution with 1.6 M, 1.8 M and 1.9 M NaCl on a ECTEOLA–cellulose column, respectively. The arrows indicate the component polysaccharides in the LA–SPS fraction.

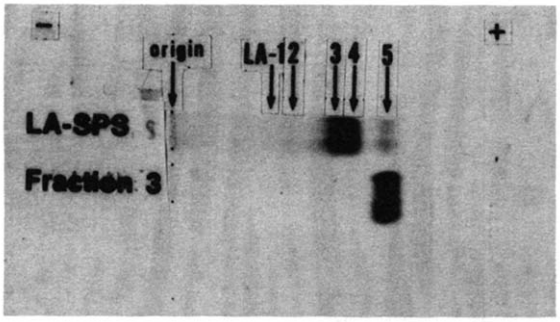


Fig. 2. Electrophoresis of the purified sulfated polysaccharide fraction from *L. angustata* var. *longissima*. The electrophoresis conditions are described in the legend of Fig. 1. Fraction 3 was purified by gel filtration chromatography of the supernatants obtained by fractional precipitation with ethanol of Fractions 2b and 2c.

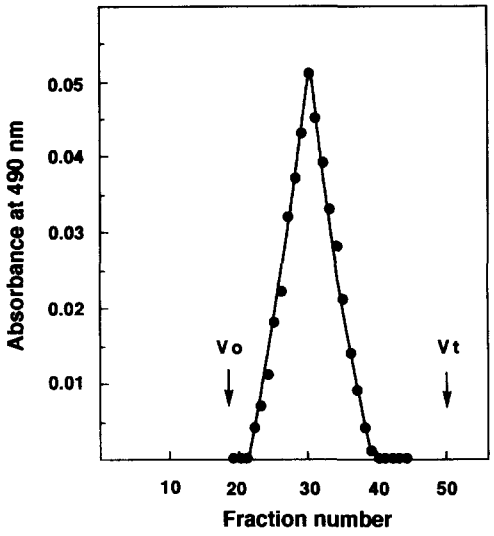


Fig. 3. Gel filtration chromatogram of LA-5 on Sepharose CL-4B. LA-5, which was purified as described in the legends of Figs 1 and 2, was chromatographed on a Sepharose CL-4B column (1.2 × 94.5 cm) and eluted with 0.2 M NaCl. The flow rate of the column was 4.1 ml/h and fractions of 2.13 ml were collected. The carbohydrates in column eluate were monitored by the phenol–sulfuric acid method (490 nm).

for the sulfate groups of primary and/or secondary equatorial hydroxyl groups. Archbald *et al.* (1981) also showed that D-galactose 6-sulfate had an IR absorption band at 818 cm^{-1}. Therefore, most sulfate groups in LA-5 may be attached at position 6 of the galactose residues.

The absolute configuration of galactose in the polysaccharide was determined by using D-galactose oxidase (Avigad *et al.*, 1962; Mourão & Perlin, 1987). After the enzyme treatment, the galactose residues in the hydrolyzates of LA-5 disappeared, indicating that the galactose residues were oxidized completely by the enzyme (Table 2). This result showed that the galactose residues occurred as the D-isomer in LA-5.

The low negative specific rotation (−1.5°) of LA-5 suggested that the residues in the polysaccharide have mainly the β-D-galacto configuration, since the specific rotation of methyl β-D-galactopyranosyl residues is +0.61° (Collins, 1987).

Table 1. Properties of LA-5. LA-5 was purified from the sulfated polysaccharide fraction, which was obtained from the brown seaweed *L. angustata* var. *longissima*, by anion-exchange chromatography, fractional precipitation with ethanol and gel-filtration

Yield (%)[a]	0·04
$[\alpha]_D$ (water) (degree)	−1·5
Apparent molecular weight[b]	400,000
IR (cm^{-1})	1240,818
6-Deoxy-sugar (% as fucose)	~2·0
Uronic acid (% as glucuronic acid)	~3·3
Sulfate (%)	38·4
Molar (%)	
Galactose	55·9
Fucose	~1·2
Glucuronic acid	~1·7
Sulfate	41·1
Anticoagulant activity (units/mg)[c]	
APTT[d]	16
TT[e]	6

[a] Expressed on a basis of weight of the dried seaweed fronds.
[b] Estimated by gel filtration on a Sepharose CL-4B column in 0·2 M NaCl.
[c] Expressed as units/mg in relation to the activity of heparin (167 units/mg).
[d] Activated partial thromboplastin time.
[e] Thrombin time.

Methylation studies

Three separate methylated fractions were analyzed, two from LA-5 and one (DS-LA-5) obtained after solvolytic desulfation. Methylated LA-5 was fractionated into a hydrophobic component (low sulfation) and a hydrophilic component (high sulfation). The results are given in Table 3. The methylation products of DS-LA-5 indicated that the main component was a linear trisaccharide repeat consisting of alternating 3- and 6-linked galactosyl residues but comparison with the low-sulfate (hydrophobic) LA-5 showed that the latter was more complex and contained substantial additional proportions of 4-linked, 3,6-linked and 4,6-linked galactose. The high-sulfate (hydrophilic) LA-5 was also complex and showed the occurrence of additional 4-linked, 3,4-linked, 2,4-linked, 3,6-linked and 3,4,6-linked galactose as well as a reduced number of non-reduced terminal galactosyl residues. The methylation analysis does not allow the proposal of a unique partial structure for any of these fractions but it appears that LA-5 must have a highly branched structure in which 3,6-linked and/or 3,6-sulfated galactose residues are a feature. It may be significant that the hydrophobic LA-5 fraction contained all the minor components (fucose and glucuronic acid) and these may form a linking region between the low and high sulfate regions. No evidence was found for the occurrence of galactofuranose residues.

NMR studies

The ^{1}H-NMR spectrum (data not shown) of LA-5 was very complex and not interpretable, suggesting also that the structure of the polysaccharide is complex. The spectrum of DS-LA-5 showed signals for three β-anomeric protons at $\delta = 4.47$, 4.63 and 4.68 ppm, suggesting that DS-LA-5 is a fraction containing mainly a trisaccharide. This result agreed with that from methylation analysis.

The ^{13}C-NMR spectrum of LA-5 (Fig. 4(A)) showed the signals for the anomeric carbons at $\delta = 105.2$–106.9 ppm and a large variety of signals attributable to glycosidic linkages or sulfated secondary carbons which resonate at $\delta = 68.8$–85.7 ppm. These results showed that the main anomeric carbons of LA-5 resonate in the range expected for β-galactopyranoside (cf. $\delta = 106.58$ ppm for C-1 of methyl β-D-galactopyranoside (Yamada *et al.*, 1987)). The signals for C-3 of β-D-galactose 3-sulfate and C-6 of β-D-galactose 6-sulfate appeared in

Table 2. Oxidation of galactose in LA-5 by D-galactose oxidase. The absolute configuration of galactose in LA-5 was determined by using D-galactose oxidase. The amount of D-galactose oxidized with the enzyme was determined by measuring the galactose contents in the hydrolyzates of LA-5 by GLC before and after the enzyme treatment

	Gal (%)[a]	Residual Gal (%)[b]	Proportion of Gal consumed by the oxidase (%)	D-Gal (%)[c]
D-Gal (standard)				
before oxidation	53·0	100		
after oxidation	9·5	18·0	82·0	100·0
LA-5				
before oxidation	98·6	100		
after oxidation	n.d.[d]	n.d.	100·0	121·9

[a] The proportion of area of Gal peak to that of the internal standard one (mannitol).
[b] Expressed as the relative peak area (Gal/internal standard) before oxidation by the oxidase as 100%.
[c] Expressed as the proportion of Gal consumed by the oxidase in the standard D-Gal sample as 100%.
[d] Not detected.
Gal, galactose.

Table 3. Methylation analysis

Glycosyl residues	Position of *O*-methyl groups	t_R[a]	Major mass spectral fragment ions (*m/z*)	Composition (mol. %)[b]		
				LA-5		DS-LA-5[c]
				Hydrophobic fraction[c]	Hydrophilic fraction[c]	
Fucosyl	2, 3, 4	0·80	175, 161, 131, 117, 101, 89, 43	0·8		2·9
	2, 3	0·91	203, 143, 117, 101, 43	0·8		0·7
	2, 4	0·93	233, 173, 159, 131, 117, 101, 89, 43	1·1		0·3
	2	1·01	275, 173, 129, 117, 113, 99, 87, 43	0·4		0·9
	4	1·06	261, 201, 131, 127, 89, 43	0·6		0·9
Galactosyl	2, 3, 4, 6	1·00	205, 161, 145, 129, 117, 101, 45	12·6	4·3	27·5
	2, 3, 6	1·12	233, 173, 161, 117, 113, 101, 45	7·8	13·7	6·9
	2, 4, 6	1·15	233, 161, 129, 117, 101, 45	19·3	15·3	29·4
	2, 3, 4	1·21	233, 189, 161, 129, 117, 101	18·3	9·6	25·6
	2, 6	1·23	305, 231, 203, 143, 129, 117, 45	4·2	6·4	
	4, 6	1·26	261, 201, 161, 129, 101, 45	3·6		0·5
	3, 6	1·27	233, 189, 129, 113, 45	2·0	6·7	
	2, 3	1·34	261, 201, 127, 117, 101	9·1	3·5	4·0
	2, 4	1·38	233, 189, 129, 117, 87	12·1	27·7	0·8
	2	1·44	333, 259, 139, 117	2·9	11·3	
	3	1·50	261, 201, 189, 159, 129	4·5	1·1	
Glucosyl uronic acid[d]	2, 3, 4	1·17	235, 191, 161, 131, 117, 101	0·4		

[a] Retention time (t_R) on SPB-1 capillary column relative to 2, 3, 4, 6-tetra-*O*-methyl galactitol.
[b] The molar percentages are based on the area of each peak compared with total area and response factors (Sweet *et al.*, 1975).
[c] See experimental section.
[d] Detected as partially methylated (6-$^2H_2$) glucitol acetates.

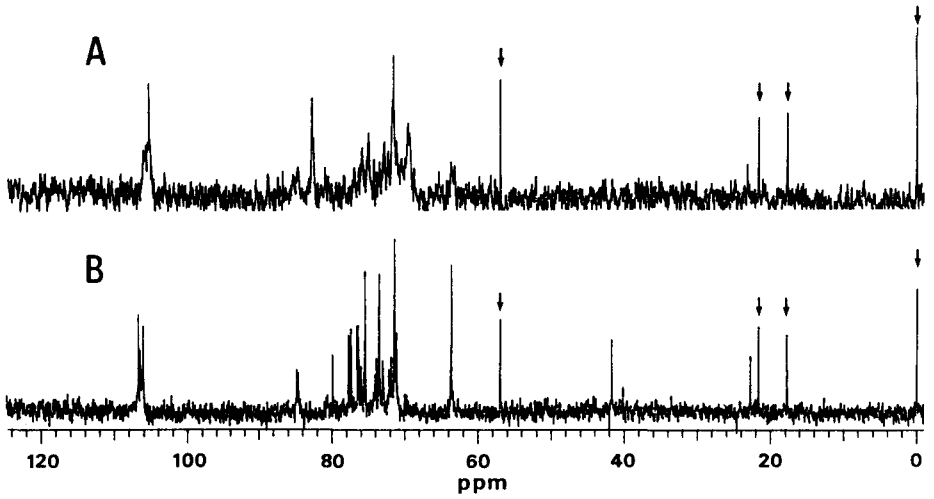


Fig. 4. ^{13}C-NMR spectra of intact LA-5 and the fraction (DS-LA-5) obtained from LA-5 by solvolysis. The spectra were recorded with D_2O as the solvent at 80°. Chemical shifts (δ) were expressed in ppm relative to that of sodium 3-(trimethyl)-1-propane-sulfonate-d_4. The signals marked corresponded to carbons of an internal standard. (A) LA-5; (B) DS-LA-5.

the vicinity of δ = 81.2 ppm and 68.1 ppm, respectively (Archbald *et al.*, 1981; Lupescu *et al.*, 1991). Thus, the signal in the vicinity of δ = 82.8 ppm and 69.5 ppm in LA-5 may be attributable to sulfated C-3 and C-6, respectively.

As shown in Fig. 4(B), the signals in the vicinity of δ = 82.8 ppm and 69.5 ppm disappeared and the signal at δ = 63.7 ppm appeared in desulfated trisaccharide fraction (DS-LA-5). The signal at δ = 63.7 ppm is attributable to the non-substituted C-6 of the galactose residues. These results also suggest that a part of the sulfate groups may be located at C-3 and C-6 of the galactose residues. However, since the pattern of the displacements is obscured by the overall complexity of the spectra, this result does not afford reliable information about the sites of sulfation of secondary carbons other than C-3 in this polysaccharide. Craigie and Jurgens (1989) reported that the signals for C-6 and C-3 of the 3-linked β-D-galactose residues with side branch at position 6 resonated at δ = 71.4 ppm and 82.0 ppm, respectively. Collins *et al.* (1981) also reported that the signal for C-3 of the 3-linked β-D-galactose residues appeared in the vicinity of δ = 83 ppm. From these results and the methylation data (Table 3), the signals in the vicinity of 84.7 ppm and 71.3 ppm in DS-LA-5 may be attributable to the glycosidically substituted C-3 and C-6, respectively.

Biological activity

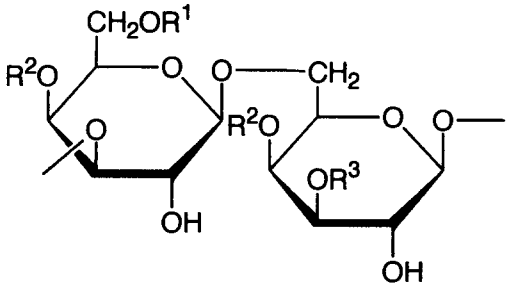
The anticoagulant activities of LA-5 were assayed with respect to activated partial thromboplastin time (APTT) and thrombin time (TT), which are related to intrinsic coagulation pathways and antithrombin activity, respectively, using normal human plasma. Unexpectedly, the anticoagulant activities of LA-5 were very low for both APTT (10% of that of heparin) and TT (4%) as compared with the LA-SPS fraction (19% for APTT and 16% for TT (Nishino & Nagumo, 1987)) (Table 1). This result showed that LA-5 was not an anticoagulant-active polysaccharide in the sulfated polysaccharide fraction from the brown seaweed *L. angustata* var. *longissima*. Despite the fact that LA-5 is highly sulfated, its low anticoagulant activity may be caused by high molecular weight and sugar components of the polysaccharide.

However, from a different point of view in the biological activity, LA-5 might be expected to have a potent anti-HIV activity. Because it was reported that many natural and semi-synthetic sulfated polysaccharides showed anti-HIV activity *in vitro* (Nakashima *et al.*, 1987, 1989; Baba *et al.*, 1988; Ito *et al.*, 1989; Itoh *et al.*, 1990; Kaneko *et al.*, 1990; Hatanaka *et al.*, 1991), and also that highly sulfated polysaccharides with high anti-HIV activity and low anticoagulant activity are useful as a potent anti-HIV agent.

CONCLUSION

A component of the dilute-acid extract of *L. angustata* var. *longissima*, LA-5, has been shown by physical and chemical analyses to be a high molecular weight galactan sulfate (molar ratio of sulfate to sugar residues, 0.7) containing traces of fucose and glucuronic acid. This is in great contrast to the commonly occurring sulfated polysaccharides (fucoidan) in brown algae which have been previously reported (Bernardi & Springer, 1962; Usui *et al.*, 1980; Nishino *et al.*, 1989). Galactofucan sulfates have been isolated from the brown seaweed *Undaria pinnatifida* (Mori *et al.*, 1982), but galactan sulfates, such as LA-5, have not been reported previously.

Studies on the biosynthesis of fucan sulfates in brown



$R^1 = SO_3^-$ or H, R^2 = Galactosyl or H, R^3 = H or SO_3^-

Fig. 5. Possible partial structure of LA-5.

seaweeds are few except for those on incorporation of sulfate into the fucan portion (Lestang & Quillet, 1973, 1979; Quatrano & Crayton, 1973). This finding may induce new developments in the studies on the biosynthesis of sulfated polysaccharides in brown seaweeds.

The main structure of LA-5 was studied by enzymatic, chemical and instrumental analyses. The results of D-galactose oxidation, optical rotation, ^{13}C-NMR, and methylation analyses of LA-5 suggested that galactose residues are present predominantly in the β-D-pyranosyl form in the polysaccharide. By IR, ^{13}C-NMR and methylation analyses, it was estimated that LA-5 consisted mainly of repeating units of →3)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→ with 4-linked galactosyl side chains branched at the respective position 4, and also that most sulfate groups are attached to position 6 of 3-linked galactosyl residues (Fig. 5). However, in the present study, further study of LA-5 could not be made and so the complete structure of the polysaccharide could not be determined, because of the very small amount of the material available. Nevertheless, LA-5 appears to be a new type of galactan sulfate unlike the well known galactan sulfates from other sources (Painter, 1983; Mourão & Perlin, 1987; Pavão *et al.*, 1989).

REFERENCES

Abdel-Fattah, A.F., Hussein, M.M. & Salem, H.M. (1974). *Carbohydr. Res.*, **33**, 9–17.

Andersson, L.-O., Barrowcliffe, T.W., Holmer, E., Johnson, E.A. & Sims, G.E.C. (1976). *Thromb. Res.*, **9**, 575–83.

Archbald, P.J., Fenn, M.D. & Roy, A.B. (1981). *Carbohydr. Res.*, **93**, 177–90.

Avigad, G., Amaral, D., Asensio, C. & Horecker, L. (1962). *J. Biol. Chem.*, **237**, 2736–43.

Baba, M., Snoeck, R., Pauwels, R. & De Clercq, E. (1988). *Antimicrob. Agents Chemother.*, **32**, 1742–5.

Bernardi, G. & Springer, G.F. (1962). *J. Biol. Chem.*, **237**, 75–80.

Bitter, T. & Muir, H.M. (1962). *Anal. Biochem.*, **4**, 330–4.

Collins, J.G., Bradbury, J.H., Trifonoff, E. & Messer, M. (1981). *Carbohydr. Res.*, **92**, 136–40.

Collins, P.M. (ed.) (1987). In *Carbohydrates*. Chapman & Hall, London, New York, p. 358.

Craigie, J.S. & Jurgens, A. (1989). *Carbohydr. Polym.*, **11**, 265–78.

Denson, K.W.E. & Bonnar, J. (1973). *Thromb. Diath. Haemorrh.*, **30**, 471–9.

Dodgson, K.S. & Price, R.G. (1962). *Biochem. J.*, **84**, 106–10.

Dutton, G.G.S., Mackie, K.L., Savage, V. & Stephenson, M.D. (1978). *Carbohydr. Res.*, **66**, 125–31.

Gibbons, M.N. (1955). *Analyst*, **80**, 268–76.

Hakomori, S. (1964). *Biochem. J. (Tokyo)*, **55**, 205–8.

Hatanaka, K., Kurihara, Y., Uryu, T., Yoshida, O., Yamamoto, N., Mimura, T. & Kaneko, Y. (1991). *Carbohydr. Res.*, **214**, 147–54.

Hodge, J.E. & Hofreiter, B.T. (1962). *Methods Carbohydr. Chem.*, **1**, 380–94.

Imanari, T., Arakawa, Y. & Tamura, Z. (1969). *Chem. Pharm. Bull. Tokyo*, **17**, 1967–9.

Ito, M., Baba, M., Hirabayashi, K., Matsumoto, T., Suzuki, M., Suzuki, S., Shigeta, S. & De Clercq, E. (1989). *Eur. J. Clin. Microbiol. Infect. Dis.*, **8**, 171–3.

Itoh, W., Sugawara, I., Kimura, S., Tabata, K., Hirata, A., Kojima, T., Mori, S. & Shimada, K. (1990). *Int. J. Immunopharm.*, **12**, 225–33.

Kaneko, Y., Yoshida, O., Nakagawa, R., Yoshida, T., Date, M., Ogiwara, S., Shioya, S., Matsuzawa, Y., Nagashima, N., Irie, Y., Mimura, T., Shinkai, H., Yasuda, N., Matsuzaki, K., Uryu, T. & Yamamoto, N. (1990). *Biochem. Pharm.*, **39**, 793–7.

Kawai, Y., Seno, N. & Anno, K. (1969). *Anal. Biochem.*, **32**, 314–21.

Larsen, B., Haug, A. & Painter, T.J. (1966). *Acta Chem. Scand.*, **20**, 219–30.

Lestang, G.De & Quillet, M. (1973). *C.R. Acad. Sci. Paris Ser. D*, **277**, 2005–8.

Lestang, G.De & Quillet, M. (1979). *Physiol. Vég.*, **12**, 199–227.

Lindberg, B. (1972). *Enzymol. Methods*, **28**, 178–95.

Lloyd, A.G. & Dodgson, K.S. (1961). *Biochim. Biophys. Acta*, **46**, 116–20.

Lloyd, A.G., Dodgson, K.S., Price, R.G. & Rose, F.A. (1961). *Biochim. Biophys. Acta*, **46**, 108–15.

Lupescu, N., Arad, S.M., Geresh, S., Bernstein, M.A. & Glaser, R. (1991). *Carbohydr. Res.*, **210**, 349–52.

McNeil, M., Darvill, A.G. & Albersheim, P. (1980). *Plant Physiol.*, **66**, 1128–34.

Mian, A.J. & Percival, E. (1973). *Carbohydr. Res.*, **26**, 133–46.

Mori, H., Kamei, H., Nishide, E. & Nishizawa, K. (1982). *Mar. Algae Pharm. Sci.*, **2**, 109–21.

Mourão, P.A.S. & Perlin, A.S. (1987). *Eur. J. Biochem.*, **166**, 431–6.

Nagasawa, K., Inoue, Y. & Kamata, T. (1977). *Carbohydr. Res.*, **58**, 47–55.

Nakashima, H., Kido, Y., Kobayashi, N., Motoki, Y., Neushul, M. & Yamamoto, N. (1987). *Antimicrob. Agents Chemother.*, **31**, 1524–8.

Nakashima, H., Yoshida, O., Baba, M., De Clercq, E. & Yamamoto, N. (1989). *Anti-viral. Res.*, **11**, 233–46.

Nishino, T. & Nagumo, T. (1987). *Nippon Nogeikagaku Kaishi* (in Japanese), **61**, 361–3.

Nishino, T. & Nagumo, T. (1991). *Carbohydr. Res.*, **214**, 193–7.

Nishino, T. & Nagumo, T. (1992). *Carbohydr. Res.*, **229**, 355–62.

Nishino, T., Yokoyama, G., Dobashi, K., Fujihara, M. & Nagumo, T. (1989). *Carbohydr. Res.*, **186**, 119–29.

Nishino, T., Aizu, Y. & Nagumo, T. (1991*a*). *Agric. Biol. Chem.*, **55**, 791–6.

Nishino, T., Kiyohara, H., Yamada, H. & Nagumo, T. (1991*b*). *Carbohydr. Res.*, **211**, 77–90.

Painter, T.J. (1983). In *The Polysaccharides*, Vol. 2, ed. G. O. Aspinall. Academic Press, New York, pp. 195–285.
Pavão, M.S.G., Albano, R.M., Lawson, A.M. & Mourão, P.A.S. (1989). *J. Biol. Chem.*, **264**, 9972–9.
Quatrano, R.S. & Crayton, M.A. (1973). *Dev. Biol.*, **30**, 29–41.
Sweet, D.P., Shapiro, R.H. & Albersheim, P. (1975). *Carbohydr. Res.*, **40**, 217–25.
Usui, T., Asari, K. & Mizuno, T. (1980). *Agric. Biol. Chem.*, **44**, 1965–6.
Waeghe, T.J., Darvill, A.G., McNeil, M. & Albersheim, P. (1983). *Carbohydr. Res.*, **123**, 281–304.
Yamada, H., Kiyohara, H., Cyong, J.-C. & Otsuka, Y. (1987). *Carbohydr. Res.*, **167**, 221–37.
Yamamoto, I., Nagumo, T., Yagi, K., Tominaga, H. & Aoki, M. (1974). *Japan J. Exp. Med.*, **44**, 543–6.
Yamamoto, I., Takahashi, M., Tamura, E., Maruyama, H. & Mori, H. (1984). *Hydrobiologia*, **116/117**, 145–8.
Yaphe, W. & Arsenault, G.P (1965). *Anal. Biochem.*, **13**, 143–8.