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Structural studies on a sulfated polysaccharide from an *Arthrobacter* sp. by NMR spectroscopy and methylation analysis

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Abstract

Structural characterization of a sulfated polysaccharide peptidoglycan complex (SP–PG) from an *Arthrobacter* sp. was performed by NMR spectroscopy and methylation analysis. In order to simplify the analyses, the desulfated SP–PG was used. NMR spectroscopy revealed the presence of a trisaccharide repeating unit and a disaccharide repeating unit. The trisaccharide unit was composed of two galactofuranosides and one glucopyranoside, and the disaccharide unit was of two galactopyranosides, as shown below. The methylation analysis showed that the polysaccharide consists mainly of a 4-linked galactopyranoside, a 6-linked galactopyranoside, a 6-linked galactofuranoside, a 2,6-linked galactofuranoside, a terminal galactopyranoside and a terminal glucopyranoside. These findings confirmed the structure indicated by the NMR spectroscopy. The repeating units determined in this study are novel.

Keywords: *Arthrobacter* sp.; Cell wall; Sulfated glucogalactan; 2D NMR; Methylation analysis

1. Introduction

The sulfated polysaccharide peptidoglycan complex (SP–PG) produced by an *Arthrobacter* sp. [1,2] has antitumor activity due to its suppressant action on vascular neogenesis [3]. This complex was shown to be derived from the bacterial cell wall [1]. A few cell-wall polysaccharides from Gram-positive bacteria were reported [4,5], but no sulfated polysaccharide is known except for a few polymers of the capsules and cell envelopes of Gram-negative bacteria. The SP–PG was divided into three fractions, all of which have the same components but different molecular weights [3]. The SP–PG with the lowest molecular weight (SP–PG-L) has the most potent antitumor activity.

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Chemical characterization of SP–PG and SP–PG-L which have been previously carried out [2,3,6,7], showed that the SP–PG-L is composed of a sulfated glucogalactan and a peptidoglycan. The sulfated glucogalactan is linked to the peptidoglycan through phosphodiester linkage, probably between (1 → 3)-linked *N*-acetylglucosamine 1-phosphate and muramic acid [6]. The sulfated glucogalactan contains galactose and glucose at a ratio of 5:1 and approximately one sulfate group per sugar. The peptide is composed of Ala, Glu, Gly and 2*S*,6*S*-diaminopimelic acid (DAP) in a molar ratio of 2:1:1:1, which shows that it belongs to the A3γ type in Schleifer's classification [8]. The glycan moiety of the PG is proposed to be MurNAc and GlcNAc, which are found in ordinary cell walls. The molecular weight of the SP–PG-L was estimated by MALDI–TOF mass spectrometry to be 14,000 [7].

However, the precise chemical structure of the polysaccharide is still undetermined. In this paper, the structural features of the SP moiety of the SP–PG-L obtained by NMR spectroscopy and methylation analyses are revealed.

2. Results and discussion

The total yield of the desulfated SP–PG-L was approximately 10%, and the content of sulfur was reduced to 0.7% from 11%. The low yields are due to the severe reaction conditions to complete desulfation.

The content of PG in the SP–PG-L is below 4% [7]. Therefore, NMR signals of PG cause no interference in the analysis of the NMR spectrum. Fig. 1 shows a 1D ^{1}H NMR spectrum of the desulfated SP–PG in which five relatively strong signals and two weak signals are indicated for the anomeric region. The residues with strong intensities were marked from A to E according to the anomeric signals from downfield to upfield. Table 1 shows the assignments of the signals achieved by the 2D HOHAHA spectrum (Fig. 2) and the DQF–COSY spectrum. An anomeric resonance of residue C at 4.64 ppm was determined to be an H-1 of a glucose residue because of its strong HOHAHA connectivity, which is observed from H-2 to H-6 [9]. The coupling constant $J_{1,2}$ (7.9 Hz) suggests that the glucose has a β-pyranoside configuration [9]. Anomeric resonances of residues A and B at 5.23 and 5.09 ppm were considered to be due to galactose because they show weak spin connectivities between their H-4 and H-5 in the 2D HOHAHA spectrum, which are characteristic features [10]. These anomeric signals, which are designated residues A and B, give the unresolved singlets with small coupling constants $J_{1,2}$ (< 2 Hz) belonging to β-galactofuranoside configuration [10,11] (e.g., $J_{1,2} = 8$ Hz for β-D-Gal*p*, $J_{1,2} = \sim 3$ Hz for α-D-Gal*f* [10,12]). The chemical shift values also support the resonances having a β-galactofuranoside configuration [13]. The other two anomeric signals (residues D and E) at 4.60 and 4.47 ppm were judged from their coupling constants, $J_{1,2}$ (7.8 Hz), and the chemical shift values, to be derived from galactopyranoside. Assignments of signals except H-1 of each sugar were carried out by analyses of the 2D HOHAHA spectrum and the DQF–COSY. However, the assignment of the H-5 and H-6 protons of Gal*f*-A and the H-6 protons of Gal*f*-B was difficult because of their overlapping and poor coherence transfers in the HOHAHA spectrum. The NOESY spectrum was also used to make their assignments clear.

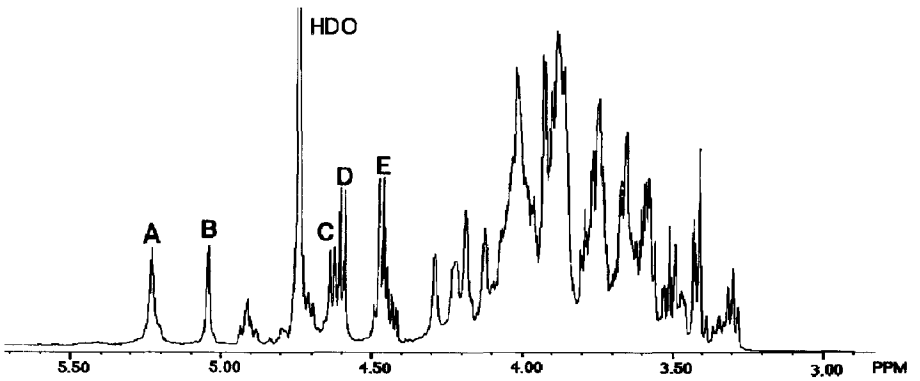


Fig. 1. Partial 1D ^{1}H NMR spectrum of desulfated SP–PG-L in deuterium oxide at 35 °C. Anomeric resonances with strong intensities are labeled. A, B, C, D and E in the spectrum refer to corresponding residues in the structure.

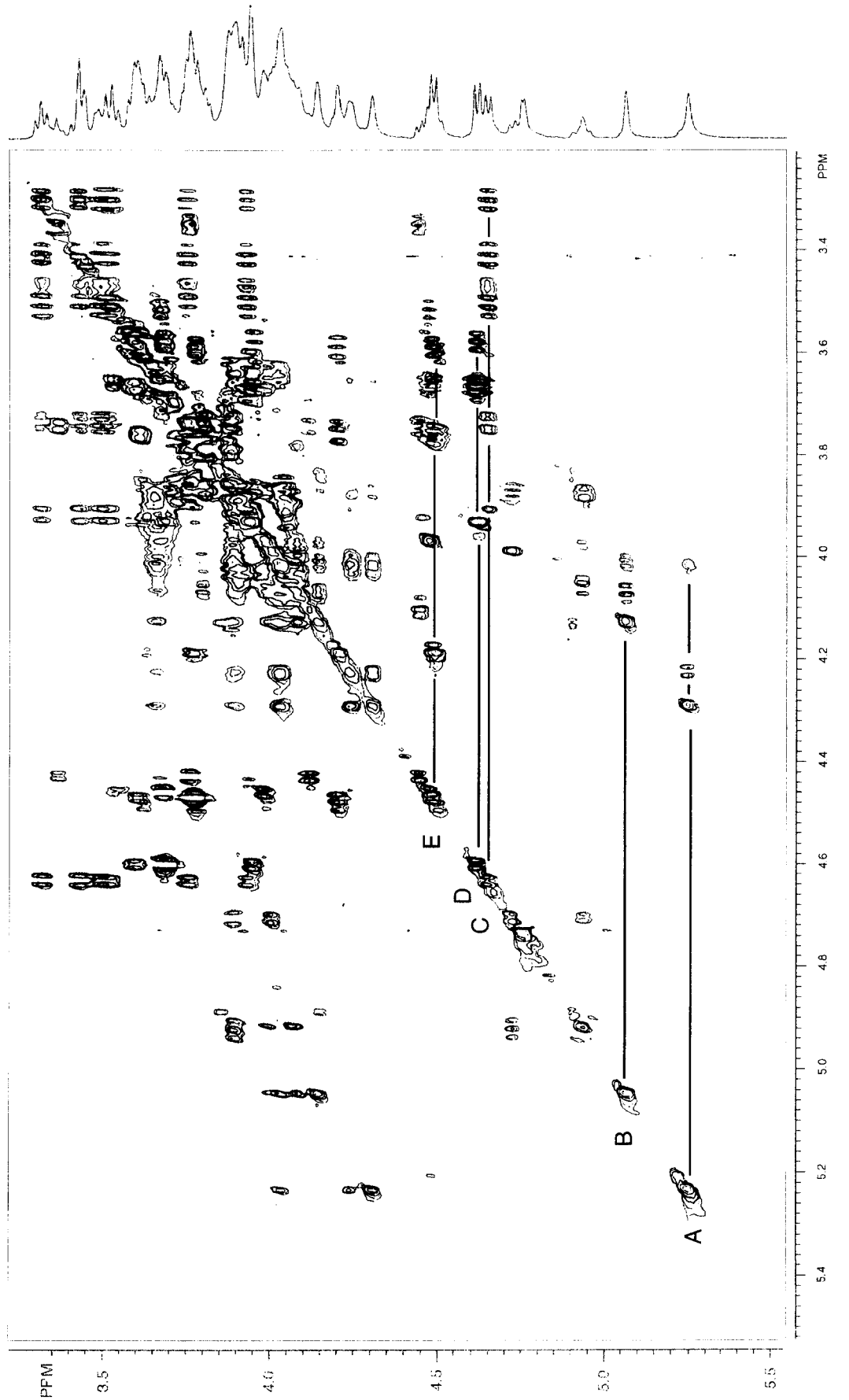


Fig. 2. A region of 2D HOHAHA spectrum of SP–PG-L in deuterium oxide at 35 °C.

Table 1
^{1}H NMR chemical shifts for the desulfated SP–PG-L from *Arthrobacter* sp. in deuterium oxide at 35 °C[a]

Residue no.	Sugar type	1	2	3	4	5	6
A	β-D-Gal*f*	5.23	4.29	4.22	4.02	3.87	3.63
B	β-D-Gal*f*	5.09	4.12	4.07	4.00	3.64	3.88, 4.01
C	β-D-Glc*p*	4.64	3.30	3.50	3.41	3.47	3.92, 3.74
D	β-D-Gal*p*	4.60	3.58	3.66	3.93	3.88	4.03, 3.93
E	β-D-Gal*p*	4.47	3.59	3.75	4.19	3.74	3.79, 3.87

[a]Chemical shifts are referenced to internal TSP.

Table 2
^{13}C NMR chemical shifts for the desulfated SP–PG-L from *Arthrobacter* sp. in deuterium oxide at 35 °C

Residue No.	Sugar type	1	2	3	4	5	6
A	β-D-Gal*f*	106.22	88.38	75.43	82.90	70.38	68.87
B	β-D-Gal*f*	107.50	80.66	76.55	82.40	72.28	68.64
C	β-D-Glc*p*	101.49	72.74	75.23	69.16	75.65	60.33
D	β-D-Gal*p*	103.89	70.94	72.32	68.32	73.43	69.06
E	β-D-Gal*p*	102.99	71.04	72.64	76.90	73.86	60.28

The assignment of ^{13}C NMR signals (Fig. 3) was achieved by the HMQC spectrum on the basis of assignments of ^{1}H NMR signals. Signals that were not assigned by this method were assigned collectively from all the spectra, including the HMBC spectrum. The results are shown in Table 2. The chemical shift values of anomeric carbons support the above characterization of sugar configurations [9,13,14]. The other striking characteristic of the chemical shift values is that there are downfield shifts in C-2, C-6 of Gal*f*-A, C-6 of Gal*f*-B, C-6 of Gal*p*-D and C-4 of Gal*p*-E that indicate their participation to glycosidic linkages.

Linkage assignment was achieved by analysis of the HMBC spectrum (Fig. 4). In this spectrum, there are six cross-peaks that define glycosidic linkages clearly. The anomeric proton of the Gal *p*-D residue showed a long-range correlation to C-4 of Gal*p*-E, defining the linkage as a Gal*p*-D-(1 → 4)-Gal*p*-E, and a correlation between H-4 of Gal*p*-E and C-1 of Gal*p*-D indicated the same Gal *p*-D-(1 → 4)-Gal*p*-E linkage. Also, the anomeric proton of Gal*p*-E shows

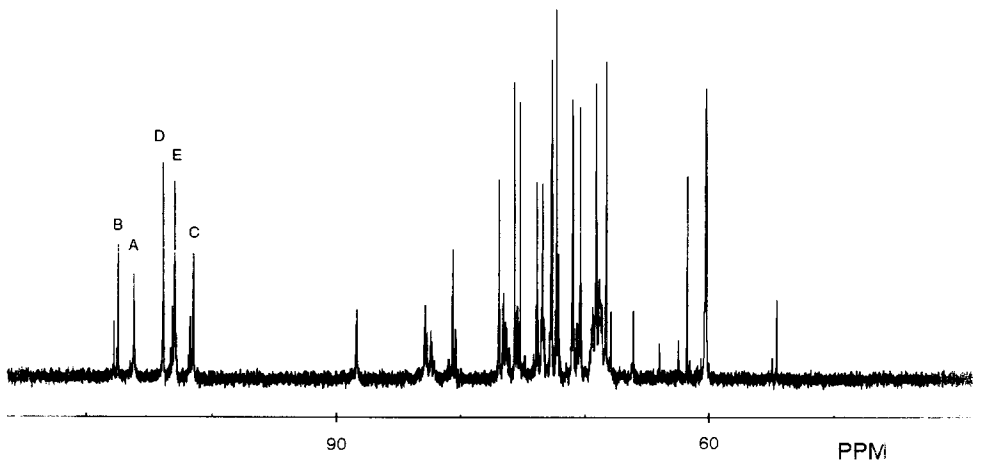


Fig. 3. ^{13}C NMR spectrum of desulfated SP–PG-L in deuterium oxide at 35 °C. Anomeric resonances are labeled. A, B, C, D and E in the spectrum refer to corresponding residues in the structure.

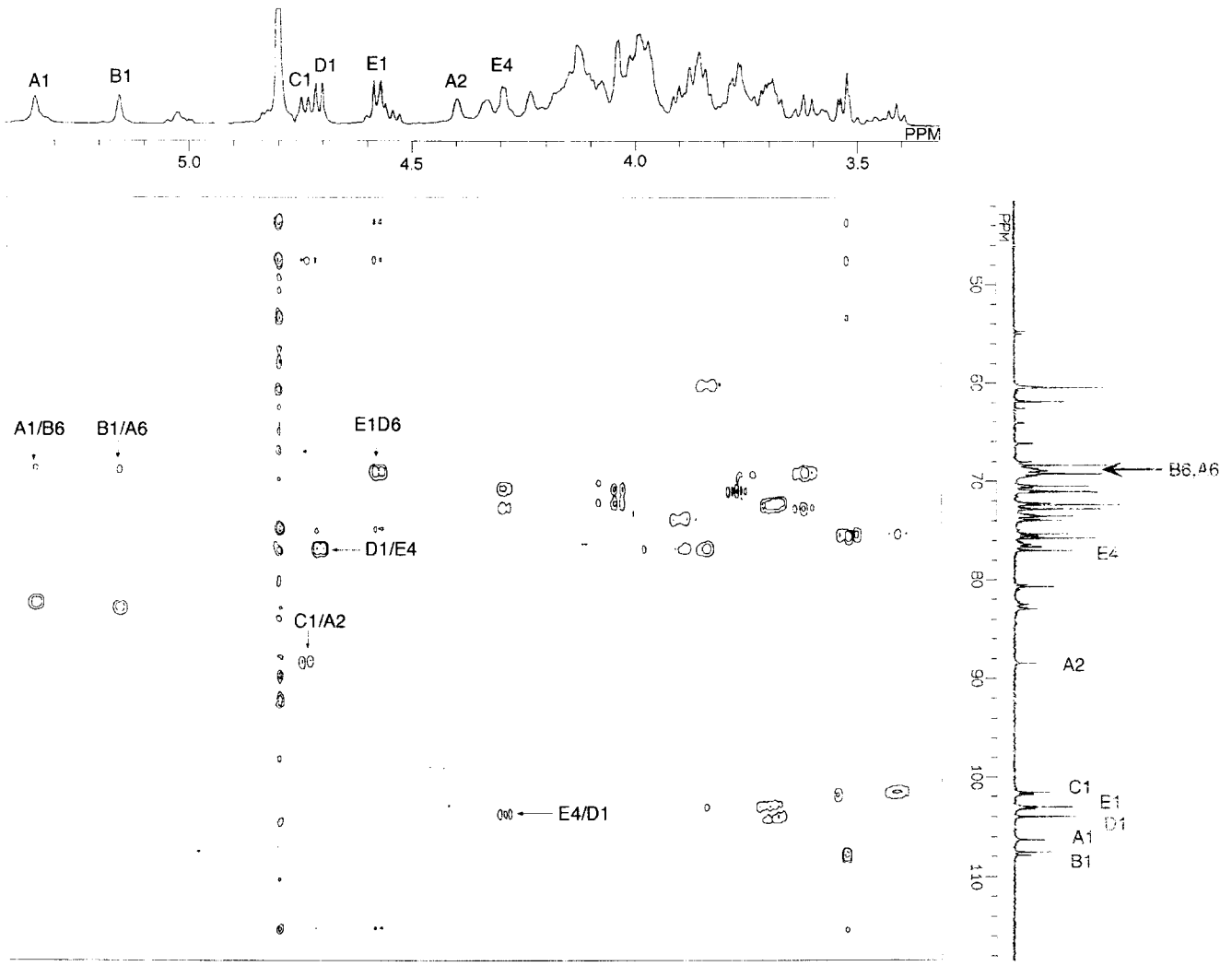
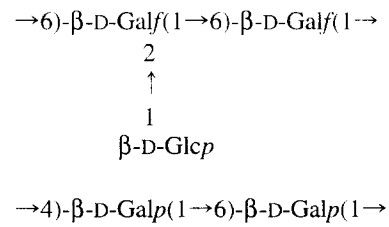
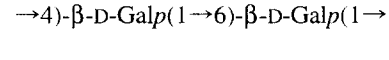


Fig. 4. HMBC spectrum of desulfated SP–PG-L in deuterium oxide at 35 °C. Cross-peaks for linkage assignment are labeled.

a correlation to C-6 of Gal*p*-D that indicates a Gal*p*-E-(1 → 6)-Gal*p*-D linkage. These linkage assignments show the presence of a repeating unit, [→ 6)Gal*p*-D-(1 → 4)-Gal*p*-E-(1 →]$_n$. The other cross-peak is a long-range connectivity between H-1 of Glc*p*-C and C-2 of Gal*f*-A, which indicates that the linkage is a Glc*p*-C-(1 → 2)-Gal*f*-A. The assignments of H-6 of residues Gal*f*-A and Gal*f*-B were not clear because of an overlapping of both proton and carbon signals in this region. However, the anomeric protons of residues Gal*f*-A and Gal*f*-B show long-range correlations to the different C-6 that are not assigned. Because a correlation between H-1 and C-6 of a same furanoside residue must be too weak to observe, it is probable that Gal*f*-A is linked to Gal*f*-B through a (1 → 6) glycosidic linkage and Gal*f*-B to Gal*f*-A in the same manner. A NOESY spectrum of the SP–PG-L provided additional evidence for the above interpretation. These linkage assignments show the presence of a repeating unit composed of [→ 6)-Gal*f*-A-(1 → 6)-Gal*f*-B-(1 →]$_n$ and Glc*p*-C linked to Gal*f*-A through a (1 → 2) glycosidic linkage. Therefore, the two kinds of repeating units in the SP–PG-L are determined as described below.





The mass fragmentograms of the partially methylated alditol acetates suggested the presence of seven types of sugars, summarized in Table 3. Terminal

Table 3
Methylation analysis of the desulfated SP–PG-L from *Arthrobacter* sp.

Partially methylated alditol acetate	Abbreviated name of glycosyl residue	Molar ratio (%)
1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl Galol	terminal Gal*p*	5.9
1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl Glcol	terminal Glc*p*	8.1
1,4,6-tri-*O*-acetyl-2,3,5-tri-*O*-methyl Galol	6-linked Gal*f*	7.0
1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl Galol	6-linked Gal*p*	38.5
1,4,5-tri-*O*-acetyl-2,3,5-tri-*O*-methyl Galol	4-linked Gal*p*	34.9
1,2,4,6-tetra-*O*-acetyl-3,5-di-*O*-methyl Galol	2,6-linked Gal*f* or 3,6-linked Gal*p*	5.3

Glc*p* and terminal Gal*p* were differentiated by their GLC retention times.

The results of NMR spectroscopy showed the presence of one each of trisaccharide and disaccharide repeating units. The constituents of the trisaccharide, → 2,6)-Gal*f*-β-(1 → , → 6)-Gal*f*-β-(1 → and Glc*p*-β- (1 → agreed with 1,2,4,6-tetra-*O*-acetyl-3,5-di-*O*-methyl Galol, 1,4,6-tri-*O*-acetyl-2,3,5-tri-*O*-methyl Galol and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl Glcol, respectively. Similarly, in the case of the disaccharide unit, → 6)-Gal*p*-β-(1 → and → 4)-Gal*p*-β-(1 → are compatible with 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl Galol and 1,4,5-tri-*O*-acetyl-2,3,5-tri-*O*-methyl Galol, respectively. 1,2,4,6-Tetra-*O*-acetyl-3,5-di-*O*-methyl Galol (is equal to 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl Galol) can be derived from 2,6-linked Gal*f* and 3,6-linked Gal*p*. In the NMR spectrum, there is no strong signal corresponding to 3,6-linked Gal*p*, which indicates that the Galol is from 2,6-linked Gal*f*. Therefore, there is no or little 3,6-linked Gal*p*.

Although the presence of terminal Gal*p* was shown by the methylation analysis, there is no strong signal corresponding to the terminal Gal*p* in the NMR spectrum. The reason is that the amount of the terminal Gal*p* is too small to assign in the NMR spectrum. However, there is a possibility that signal around the anomeric proton of Gal*p* is derived from the terminal Gal*p*.

The amounts of the alditol acetates in each unit are nearly equimolar. However, the amounts of the alditol acetates from the trisaccharide unit are less relative to those from the disaccharide unit. On the other hand, the NMR spectrum showed that their amounts are nearly equal. The discrepancy is caused by the lability of galactofuranoside under acidic conditions during hydrolysis. It is also known that permethylated –6-Gal*f*– moieties can form large amounts of 1,6-anhydrogalactofuranoside during hydrolysis, and the anhydrogalactofuranoside escapes reduction.

Concerning the disaccharide repeating unit, it is probable that the terminal residue is a Gal*p*. If the

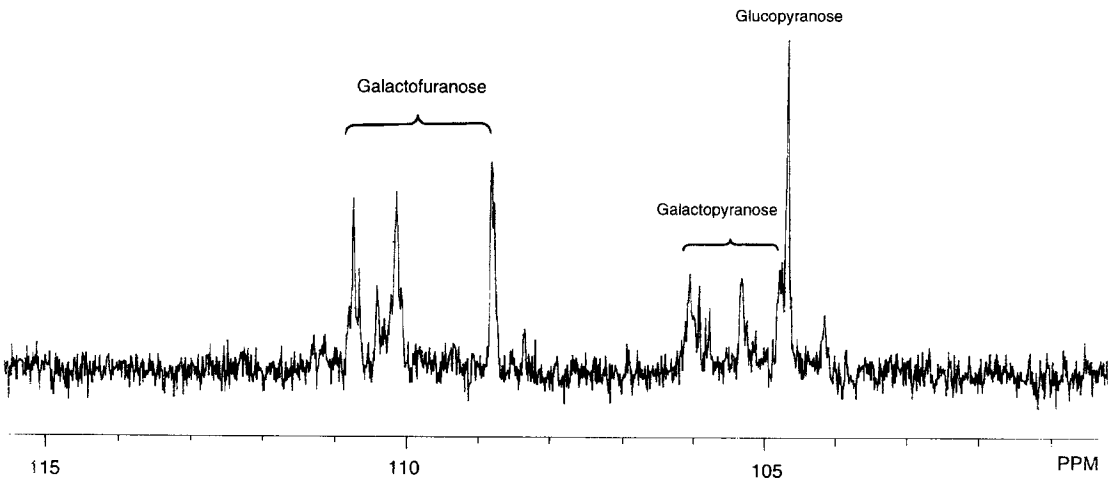


Fig. 5. Part of the ^{13}C NMR spectrum of the original SP–PG-L in deuterium oxide at 35 °C.

1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl Galol is derived only from a terminal of →6)-Gal*p*-β-(1→4)-Gal*p*-β-(1→, the molar ratio of the →6)-Gal*p*-β-(1→4)-Gal*p*-β-(1→ to a terminal Gal*p* is approximately 6. When the Galol is derived from the disaccharide unit and the other terminal, the number is greater than 6. Therefore, the disaccharide repeating unit might be proposed to be β-D-Gal*p*-(1→[→6)-β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→]$_n$ ($n \geq 6$).

As mentioned above, the presence of the trisaccharide unit and the disaccharide unit have been demonstrated by NMR spectroscopy and were supported by the results of methylation analysis. In the ^{1}H NMR spectrum of the original SP–PG-L before desulfation, the signals of Gal*p* have not been clearly observed, probably because a varying degree of sulfation of Gal*p* causes dispersion of signals, and therefore low signal strength in each signal. A few small signals (106.1, 105.9, 105.3, 104.8 and 104.1 ppm etc.) in the anomeric region of the ^{13}C NMR spectrum of the SP–PG-L (Fig. 5) confirm this hypothesis.

Galactofuranan polysaccharides have been found in the cell walls of *Bifidobacterium* [15], *Penicillium* [16], *Aspergillus* [17] and *Eupenicillium* [18], etc. However, the unit structures of the SP–PG-L determined in this report are novel, and no sulfated glucogalactan in cell walls has been reported.

Structural studies of fragments involved in partially hydrolyzed SP–PG-L are now under investigation so as to obtain more information about the structure of the SP–PG-L and the positions of sulfation.

3. Experimental

Sample.—Solvolytic desulfation of the SP–PG-L was performed by the methods of Inoue and Nagasawa [19]. The complex (Na salt, 2.0 g) was passed through a column of Dowex 50-X2 (H^+ form) at 4 °C and neutralized with pyridine to give a pyridinium salt. The pyridinium salt was treated with dimethyl sulfoxide containing 10% methanol at 80 °C for 10 h, and ethanol was added into the solution to precipitate the desulfated complex. After the precipitate was dissolved in water, the solution was dialyzed against water using a cellulose tube (molecular weight cut off, 12,000) and lyophilized. Its sulfur content was determined using ion chromatography. The sample was dissolved in water, and formic acid was added to the solution. The sample was hydrolyzed at 100 °C for 24 h, and the formic acid was removed by centrifugation. The sample solutions were diluted with water and put in a TOSOH Ion Chromatography System with a column of IC-Anion-PW (4.6 × 50 mm, TOSOH). TSKeluent IC-Anion-A was used as the mobile phase at a flow rate of 1.2 mL/min. Detection was by a conductivity detector CM-8000. The column temperature was kept at 40 °C.

NMR spectrometry.—The 1D ^{1}H NMR spectrum, 1D ^{13}C NMR spectrum, DQF–COSY, 2D HOHAHA, NOESY, HMQC and HMBC were recorded on a JEOL GSX-500 spectrometer and a Varian Unity 500plus spectrometer at 35 °C. TSP (sodium trimethylsilylpropionate-2,2,3,3-d_4) was used as the internal standard. The proton-decoupled ^{13}C NMR spectrum was recorded with 28,000 scans and a spectral width of 40,000 Hz. All 2D spectra were measured in a phase-sensitive mode using the method of States et al. [20].

A standard pulse sequence [21] was used to obtain a DQF–COSY spectrum with a spectral width of 4000 Hz in both dimensions. The data size was 256 × 2 K points, and 16 scans were used per increment. Zero-filling was carried out in the F_1 dimension so as to give a 1 K × 4 K data matrix.

The 2D HOHAHA spectrum [22] was recorded with a mixing time of 150 ms. Sixteen scans were accumulated. In total, 256 t_1 points were sampled with 4 K complex points in t_2 and with a spectral width of 4000 Hz. Zero-filling was carried out in the F_1 dimension so as to give a 1 K × 4 K data matrix.

HMQC [23] and HMBC [24] spectra were recorded with spectral widths of 4000 Hz in ^{1}H dimension and 15,000 Hz in the ^{13}C dimension. The WALTZ [25] sequence was adopted for ^{13}C-decoupling in HMQC, and the delay after a BIRD pulse [26] was 500 ms. The data matrix 2 K × 256 points with 80 scans for HMQC and 400 scans for HMBC. Zero-filling was carried out in the F_1 dimension so as to give a 1 K × 4 K data matrix.

Methylation analysis.—The desulfated SP–PG-L was methylated by treatment with a prepared methylsulfinyl carbanion and iodomethane according to the method of Hakomori [27]. The completeness of the reaction was monitored by measuring an infrared spectrum to check for the disappearance of a hydroxyl group. The methylated sample was hydrolyzed with 0.5 M H_2SO_4–90% CH_3COOH at 80 °C for 10 h, followed by hydrolysis with water at 80 °C for 5 h. The hydrolysate was neutralized by being passed through a column of Amberlite IRA-45 (CH_3COO^- form, 10 mm i.d. × 600 mm) and dissolved in 7% ammonium hydroxide. The resulting partially meth-

ylated monosaccharides were converted into their corresponding alditol acetates by reduction with $NaBH_4$ at room temperature for 10 h. The reduced polysaccharide was acetylated with Ac_2O and pyridine at room temperature for 10 h in accordance with the procedure of Bjorndal et al. [28].

The partially methylated alditol acetates were analyzed by GLC–MS. The GLC used was a Hewlett–Packard 5840 gas chromatograph fitted with a fused silica capillary column (30 m × 0.25 mm i.d.) of DB-210 (J&W Scientific). The column oven was cooled to 80 °C for 2 min, and the temperature was then raised rapidly to 220 °C at a speed of 70 °C/min and maintained at this temperature for 27 min. Mass spectra were obtained by using a JEOL AMX-AX505W connected to the JEOL data system, COMPLEMENT.

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