Structural Study on a Sulfated Polysaccharide-peptidoglycan Complex Produced by Arthrobacter sp.

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The structure of a sulfated polysaccharide-peptidoglycan complex (SP-PG) produced by Arthrobacter sp. was analyzed by NMR spectroscopy. In addition, oligosaccharide fragments of the SP-PG-L obtained by HF degradation were analyzed by NMR spectroscopy. These findings indicated that the sulfated polysaccharide (SP) contains a repeating unit composed of two galactofuranosides and a glucopyranoside. The main chain of the trisaccharide is [→6]-β-D-Galp(1→6)-β-D-Galp(1→), with β-D-GlcP linked to one of the Galp/s through a (1→2) linkage. The sulfated positions of the trisaccharide were identified as C-3 and C-5 of the β-glucosylated Galp residues, and C-2 or C-3 of the other Galp residue.

Key words: Arthrobacter sp.; sulfated polysaccharide; glucogalactan; NMR spectroscopy; HF degradation

The sulfated polysaccharide-peptidoglycan complex (SP-PG) examined in this study is produced by Arthrobacter sp.1,2 and has antitumor activity due to suppression of vascular neogenesis.3 The sulfated polysaccharide-peptidoglycan complex with the lowest molecular weight (SP-PG-L) was fractioned by gel-permeation chromatography, and had intense activity.4 In previous studies,5,6 the components and molecular weight of the SP-PG-L, as well as the structure of two repeating units in the SP, were reported. The PG of the complex consists of Ala, Glu, L,L-DAP, Gly, MurNAc, and GlcNAc. The SP moiety is composed mainly of glucose and galactose in a ratio of 1:5. Methylation analysis and NMR analysis of the desulfated SP-PG-L found that there are two repeating units, as shown below.5

$\rightarrow$6)-β-D-Galp(1→6)-β-D-Galp(1→),

$\uparrow$

1

β-D-GlcP

$\rightarrow$6β-D-Galp(1→4)-β-D-Galp(1→).

Component analysis indicated that the SP contains approximately one sulfate per sugar,5 however the location of the sulfate groups is undetermined. In this study, we report the presence of a trisaccharide repeating unit in the SP-PG-L and the sulfated positions of the repeating unit as located by NMR spectroscopy and structural characterization of oligosaccharide fragments.

Materials and Methods

Materials. The SP-PG-L used in this study was prepared from the culture supernatant of an Arthrobacter sp. and chromatographed on a Sephadryl S-300 column (Pharmacia Biotech, Uppsala, Sweden) as previously reported.2,5 The sample used in this experiment was the fraction with the lowest molecular weight (M.W. 14,000).6

Degradation of the SP-PG-L by HF. Two mg of the SP-PG-L was hydrolyzed with 47% HF solution (40 ml) at 30°C for 3 h. The resulting hydrolysate was neutralized and passed through a column (2.5×10 cm) of Dowex 50 (NH4+) (Bio-Rad Laboratories, California, U.S.A.) after concentration to about 25 ml. The eluent was lyophilized to obtain a very hygroscopic powder. The powder was dissolved in 0.1 M CH3COONH4 (pH 6.5), and its molecular weight distribution was examined by analytical gel-permeation chromatography (CCPM, Tosoh, Tokyo, Japan) with a GS-220 column (7.6×500 mm, Asahi Chemical Industry Co., Ltd., Kanagawa, Japan). Detection was done with a refractive index (RI) detector (830-RJ, Japan Spectroscopic Co., Ltd., Tokyo, Japan) and an ultraviolet (UV) absorption detector (UV-8000, Tosoh, Tokyo, Japan). The oligosaccharide fraction (HF-2) of the sample was collected by preparative gel-permeation chromatography with a GS220P column (21.5×500 mm, Asahi Chemical Industry Co., Ltd., Kanagawa, Japan), and the column eluent was monitored by a UV detector.

Separation of the oligosaccharide fraction. For separation and easy detection, the HF-2 fraction (2–3 mg) was pyridylaminated with 20 ml of acetic acid solution of 2-aminopyridine (Takara Shuzo, Kyoto, Japan, 300 mg/100 ml of acetic acid) at 90°C for 60 min, and

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Abbreviations: SP, sulfated polysaccharide; SP-PG-L, sulfated polysaccharide-peptidoglycan complex with the lowest molecular weight; DQF-COSY, double quantum filtered COSY; TOCSY, total correlation spectroscopy; HMQCC, heteronuclear multiple quantum coherence; HMBC, heteronuclear multiple bond correlation; MurNAc, N-acetylmuramic acid
Sulfated Polysaccharide-peptidoglycan Complex

results and discussion

Structural characterization of the SP-PG-L by NMR spectroscopy

The $^1$H-NMR spectrum of the SP-PG-L (Fig. 1) had an abundance of signals due to the heterogeneity of the SP-PG-L. The major signals of the $^1$H-NMR spectrum were assigned from the TOCSY spectrum (Fig. 2) and the DQF-COSY spectrum. The TOCSY spectrum had four major series of sugar signals. Anomeric protons were easily recognizable due to their downfield chemical shifts, and the other protons were assigned by a combination of DQF-COSY and TOCSY spectra. The assignments of the $^1$H-NMR spectrum are summarized in Table 1. An anomeric resonance at 4.71 ppm was assigned to be an H-1 of a glucose residue due to its strong TOCSY connectivity, which was observed through from H-2 to H-5. The coupling constant $J_{1,2}$ was 8.8 Hz, suggesting that the glucose has a $\beta$-pyranose configuration. The anomeric proton resonances at 5.37, 5.29, and 5.18 ppm were assumed to be due to galactose residues, which were designated as residues Gal-A, B, and C, respectively, because their correlation peaks between the anomeric and other protons were very small in the TOCSY spectrum. In addition, these anomeric protons gave broad singlets belonging to the $\beta$-galac-

NMR spectroscopy. The solution of 40 mg of the SP-PG-L in D$_2$O (99.99% deuterium purity, MSD) was lyophilized, and dissolved in 0.5 ml of D$_2$O again. The pH of the solution was about 6 without any adjustment. In the case of the fragment oligosaccharides of the SP-PG-L, each 1.5 mg was dissolved in D$_2$O respectively and treated as the SP-PG-L solution.

All spectra were recorded on a Jeol EX-400 spectrometer and a Jeol GSX-500 spectrometer at 35°C. TSP (Sodium trimethylsilylpropionate-2,2,3-d$_4$) or acetone was used as an internal standard. All 2D spectra were measured in a phase-sensitive mode using the method of States. A standard pulse sequence was used to obtain COSY spectra with a spectral width of 4,000 Hz in both dimensions. The data size was $256 \times 2$ K points, and 16 scans were used per increment. Zero-filling was done in the F$_1$ dimension to obtain a $1 \times 4$ K data matrix.

TOCSY spectra were recorded with a mixing time of 90 or 150 msec. Sixteen scans were accumulated. In total, 256 $t_2$ points were sampled with 4 K complex points in $t_2$ and with a spectral width of 4,000 Hz. Zero-filling was done in the $F_1$ dimension to obtain a $1 \times 4$ K data matrix.

HMOC and HMBC spectra were recorded with spectral widths of 4,000 Hz in $^1$H dimension and 12,000 Hz in $^1$C. The WALTZ sequence was adopted for $^1$C-decoupling in HMOC and the delay after BIRD pulse was 500 ms. The data matrix was $2 \times 256$ points for HMOC and HMBC. Zero-filling was done in the $F_1$ dimension to obtain a $1 \times 4$ K data matrix.

$^1$H signals were assigned by analyses of COSY and TOCSY spectra and $^1$C signals were assigned based on HMOC and in part based on HMBC spectra. Chemical shifts were measured relative to the signal of the methyl group of the internal standard acetone (2.216 ppm for $^1$H, 29.92 ppm for $^1$C) or TSP (0.0 ppm).

![Fig. 1. $^1$H-NMR Spectrum of the SP-PG-L from Arthrobacter sp. in D$_2$O at 35°C.](image)

<table>
<thead>
<tr>
<th>Residue No.</th>
<th>Sugar type</th>
<th>Chemical shifts (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>$\beta$-Gal</td>
<td>5.37, 4.63, 4.96, 4.49, 4.77, 4.09, 3.89</td>
</tr>
<tr>
<td>B</td>
<td>$\beta$-Gal</td>
<td>5.30, 4.67, 4.30, 4.07, 4.01, 3.92, 3.67</td>
</tr>
<tr>
<td>C</td>
<td>$\beta$-Gal</td>
<td>5.17, 4.41, 4.65, 4.23, 4.09, 3.95, 3.70</td>
</tr>
<tr>
<td>D</td>
<td>$\beta$-Glc</td>
<td>4.71, 3.34, 3.55, 3.47, 3.48, 3.93, 3.78</td>
</tr>
</tbody>
</table>

Chemical shifts are referenced to internal standard TSP.

a: Chemical shifts are downfield shifted relative to those of the desulfated SP-PG-L.

Reduced with 20 ml of acetic acid solution of borane-dimethyamine complex (Takara Shuzo, Kyoto, Japan, 20 mg/100 ml of acetic acid) at 80°C for 60 min in a sealed tube. The reagents and acetic acid were removed by evaporation with toluene and methanol, yielding the product, pyridylaminated HF-2 (HF-2-PA). This procedure was repeated to obtain about 40 mg of HF-2-PA.
Fig. 2. TOCSY Spectrum of the SP-PG-L from *Arthrobacter* sp. in D₂O at 35°C.

tofuranoside configuration. The chemical shifts of these resonances are also evidence that the galactoses have a β-D-galactofuranoside configuration. The signals at 5.02 ppm could not be identified because they appeared to be composed of multiple peaks and had little connectivity. Moreover, the ¹H-NMR spectrum had many minor signals, indicating the presence of heterogeneity in this sample.

Besides sugar components, there are protons derived from amino acids and amino sugars involved in the peptidoglycan of the SP-PG-L. The signals around 1.3–1.4 ppm were assignable to the methyl protons of Ala and MurNAc, and the signals around 2.1–2.2 ppm to the acetyl protons of MurNAc and GlcNAc.

The ¹³C-NMR spectrum (Fig. 3) of the SP-PG-L had four major anomeric carbon resonances at 104.7, 108.8, 110.2, and 110.8 ppm. The carbon signals were assigned by direct correlation observed in the HMQC spectrum. The assignments for the ¹³C-NMR spectrum are summarized in Table 2. The resonance at 104.7 ppm was assigned to a signal of C-1 of a glucose residue, and the other three anomeric resonances in the lower field were due to galactose residues. The chemical shifts of the anomeric carbons of galactose residues agreed with those reported in the literature indicating their furanoside form. The resonances of the C-2, C-3, C-4, C-5, and C-6 of each of the four residues were also assigned.

Anomeric resonances with less intensity other than the four assigned residues were observed at 106.05, 105.32, 104.76, and 104.15 ppm. These resonances are assumed to be derived from galactopyranoside residues, as judged by their chemical shifts. However, they could not be assigned since their correlation peaks were too weak.

Linkage assignment was done by analysis of the HMBC spectrum. The anomeric proton of residue Gal-
Table 2. $^{13}$C NMR Chemical Shifts for the SP-PG-L from *Arthrobacter* sp. in D$_2$O at 35°C

<table>
<thead>
<tr>
<th>Residue No.</th>
<th>Sugar type</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>β-Galf</td>
<td>110.14</td>
<td>88.34</td>
<td>84.41</td>
<td>84.77</td>
<td>79.46</td>
<td>68.56</td>
</tr>
<tr>
<td>B</td>
<td>β-Galf</td>
<td>108.84</td>
<td>90.15</td>
<td>78.70</td>
<td>86.10</td>
<td>72.44</td>
<td>71.84</td>
</tr>
<tr>
<td>C</td>
<td>β-Galf</td>
<td>110.74</td>
<td>82.11</td>
<td>86.10</td>
<td>85.17</td>
<td>72.38</td>
<td>71.86</td>
</tr>
<tr>
<td>D</td>
<td>β-GlcP</td>
<td>104.67</td>
<td>75.83</td>
<td>78.32</td>
<td>78.66</td>
<td>72.24</td>
<td>63.35</td>
</tr>
</tbody>
</table>

Chemical shifts are referenced to internal standard TSP.

C had a long-range correlation to C-6 of Galf-A, demonstrating the linkage to be a Galf-C(1→6)Galf-A. The anomic protons of residues Galf-A and Galf-B had long-range correlations to C-6. However, the C6 could not be assigned due to overlapping of the proton and carbon signals in this region. The GlcP residue also had a long-range connectivity between its H-1 and C-2 of residue Galf-A, indicating the linkage is a Glcp(1→2) Galf-A. These linkage assignments and the presence of a trisaccharide unit in the desulfated SP-PG-L, $^9$ →6)-β-d-Galf(1→6)[β-d-GlcP(1→2)]-β-d-Galf(1→, show that the repeating unit is composed of $^6$Gal-fC(1→6) Galf-A(1→ and Glcp linked to Galf-A through a (1→2) glycosidic linkage.

The positions of sulfation were identified by chemical shifts in the $^1$H and $^{13}$C NMR spectra. In comparison with the chemical shifts of the desulfated SP-PG-L, $^9$ large downfield shifts were observed on the H-3 (0.74 ppm), H-5 (0.87 ppm), C-3 (9.18 ppm) and C-5 (9.14 ppm) in Galf-A and the H-3 (0.58 ppm) and C-3 (10.2 ppm) in Galf-C. These shifts can be ascribed to sulfite group effects, and the values of the shifts corresponded well to those reported in the literature. Therefore, the sulfated positions of the SP-PG-L were identified at the C-3 and C-5 positions of Galf-A and at the C-3 of Galf-C. The downfield shifts of H-2 and C-2 in Galf-A are due to the glycosidic linkage. The finding of no difference in chemical shifts between the Glcp in the SP-PG-L and that in the desulfated SP-PG-L indicates that there is no sulfate group in the Glcp.

Comparison of chemical shifts among Galf-A, Galf-B, and Galf-C indicated that the C-3 and C-5 of Galf-B are not sulfated and that the C-2 of Galf-B is sulfated or glycosylated. Since the integrated intensity of the anomic proton of Galf-A is the largest, and nearly equal to the sum of those of Galf-B and Galf-C (approximately 1:1:2), Galf-B is sulfated on C-2. In the desulfated SP-PG-L, there was no residue corresponding to Galf-B. $^9$ This also suggests that the Galf-B converged with Galf-C in the process of desulfation.

These findings demonstrated the presence of the $^6$→6)-β-d-(3-SO$_3$)-Gal-f(1→6)[β-d-GlcP(1→2)]-β-d-(3,5-SO$_3$)-Gal-f(1→ in the SP-PG-L, and that β-d-(3-SO$_3$)-Galf might be substituted for β-d-(2-SO$_3$)-Galf.

Separation and purification of oligosaccharide fragments

A chromatogram of the powder from the HF hydrolyzate (2 g) of the polysaccharide is shown in Fig. 4 and suggests that the HF-2 fraction is an oligosaccharide fraction. This fraction, HF-2, was collected and lyophilized to yield 1.3 g. The HF-2 fraction was pyridylaminated (HF-2-PA), and contained three major fractions in a chromatogram (Fig. 5). The fractions with the fastest retention time (the largest molecular weight), F-1, F-2, and F-3, were fractionated and lyophilized to obtain 15.7 mg, 12.3 mg, and 10.9 mg. These fractions were purified by ion-exchange chromatography and reverse-phase chromatography. The amounts of the resulting F-1, F-2 and F-3 were 4.6 mg, 3.0 mg, and 7.3 mg.
Structural characterization of the oligosaccharides

Structures of F-1 and F-2 were analyzed by NMR spectroscopy. F-3 was not analyzed, because it was clear from its $^1$H NMR spectrum that it was a disaccharide and a part of F-1 and F-2. The $^1$H NMR spectrum of the F-1 in D$_2$O (Fig. 6) included 22 signals in the hexose ring proton region (3.2–5.5 ppm) and four signals of the pyridylamino group at low field (6.8–7.9 ppm). These findings indicated that this oligosaccharide was composed of three hexoside residues. This was supported by the $^{13}$C NMR spectrum (Fig. 7), which had two anomic resonances (101.55, 106.63 ppm), and the TOCSY spectrum, which had three spin networks in addition to the pyridylamino group. Hereafter we refer to these three hexosides as residues A, B, and P, respectively. Tables 3 and 4 show the results of signal assignment.

The anomic $^1$H signal of residue A was observed at 4.68 ppm as a doublet ($J_{1,2}=7.8$ Hz), and the spin connectivity from H1 through H6 was observed well on the TOCSY spectrum. We therefore presumed that this residue was $\beta$-d-Glcp. The singlet at 5.31 ppm, which belongs to residue B, was assigned to an anomic $^1$H resonance of $\beta$-d-Galf based on the chemical shift of anomic $^{13}$C (106.65 ppm) and the small $J_{1,2}$ value (<2 Hz). Residue P was presumed to be a pyridylaminated hexoside (Hex), because it had no anomic resonance. Moreover, this $^1$H NMR study of the SP-PG-L showed it to be composed of three or more galac-

![Fig. 6. $^1$H NMR Spectrum of the F-1 in D$_2$O.](image)

![Fig. 7. $^{13}$C NMR Spectrum of the F-1 in D$_2$O.](image)
The difference in structure between F-1 and F-2 is only the position of sulfation. The C3 and C5 of glucosylated Galf of F-2 are sulfated, but no sulfation was observed in the Hex of F-2. In the structure of the SP-PG-L, there is variation in the sulfated position of the non-branched Galf which corresponds to the Hex of F-1 and F-2. This indicates that the Galf residue is sulfated at various positions or is not sulfated. In addition, it is possible that F-2 is desulfated in the process of HF degradation.

Therefore, these structures of the SP-PG-L and the fragments showed that one of the basic units of the SP-PG-L is the following, and that the β-glucosylated Galf residue is sulfated on C3 and C5. The other Galf residue is sulfated on C2 or C3, and there may be variation in the position of sulfation in the residue.

$$\beta$$-D-Glc p(1→6)(3,5-SO$_3$)-β-D-Galf(1→6)(3-SO$_3$)-Galg(phenolylaminated).

The structure of F-2 was analyzed as well as that of F-1. The assignments for 1H and 13C NMR spectra are shown in Tables 5 and 6. The structure was as follows:

$$\beta$$-D-Glc p(1→2)-(3,5-SO$_3$)-β-D-Galf(1→6)-(3-SO$_3$)-Galg(phenolylaminated).
converged to large ones that were easily detected (Fig. 8b). Therefore, it is too difficult to obtain pure oligosaccharide fragments derived from the disaccharide unit because of their small amounts.

Galactofuranar polysaccharides from cell walls containing Galf as a main chain component have been recently reported in \textit{Bifidobacterium},\textsuperscript{20} \textit{Penicillium},\textsuperscript{21} \textit{Eupenicillium},\textsuperscript{22} \textit{Neosartorya},\textsuperscript{23} and \textit{Cylindrocladium}.	extsuperscript{24} Their linkage types are different and the polysaccharide in this study is similar to the one from \textit{Cylindrocladium}, which has a repeating unit composed of (1→6)β-D-Galf as a main chain and branched β-D-GlcP through a (1→2) glycosidic linkage. However, the polysaccharide in the SP-PG-L is unique in the presence of sulfate groups and a lack of glucuronic acid, and is a novel polysaccharide.

References