Structural Study of an Exocellular Polysaccharide of *Bacillus circulans*

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*Bacillus circulans*, a soil bacterium, produced an exocellular polysaccharide of high viscosity. On the basis of the results of methylation analysis, mild acid hydrolysis, and 1D and 2D $^1$H-NMR spectroscopy, it was concluded that the polysaccharide has a basic repeating unit composed of β-1-rhamnopyranose, α-1-D-mannopyranose, α-1-D-galactopyranose, and α-1-D-glucopyranuronic acid with the following structure:

\[ \beta-1-Rhap \]

\[ \rightarrow 3\alpha-1-D-GlcUAp1 \rightarrow 2\alpha-1-D-Manp1 \rightarrow 3\alpha-1-D-Galp1 \rightarrow \]

**Key words:** *Bacillus circulans*; exo-polysaccharide; $^1$H-NMR

Some bacterial polysaccharides have been used as raw materials in food and non-food industries, because the structure and biological properties of bacterial polysaccharides are very different from those of plants.\(^1\)

In our laboratory, we have isolated a soil bacterium, *Bacillus circulans*, which produced a highly viscous exopolysaccharide when it was grown on a medium containing glucose as a carbon source.\(^2\) Isolation and production of bacterial polysaccharides produced by *Bacillus* strains have been reported, and an isolation of a heteropolysaccharide produced by *Bacillus circulans* has been reported by Forsyth and Webley\(^3\) and Ishikawa *et al.*\(^4\), but the details of its chemical structure were not studied.

In this paper, we report the chemical structure of an exopolysaccharide produced by *Bacillus circulans*.

**Materials and Methods**

**General methods.** Gas liquid chromatography was done with a Shimadzu GC-14 apparatus with a flame-ionization detector. A glass capillary column (d=0.15 mm, l=50 m) coated with OV-17 (Tokyo Kasei Co., Ltd., Japan) was used for the analysis of trimethylsilylated methyl glycoside (program I) and OV-1 (GLScience, Co., Ltd., Japan) was used for the separation of partially methylated alditol acetates (program II) and trimethylsilylated (−)-2-butyglycoside (program I). The temperature programs used were: I, 150°C for 5 min, then 2°C/min to 200°C; and II, 150°C for 10 min, then 2.5°C/min to 200°C. GLC-MS spectra were obtained with a HITACHI M2000 mass spectrometer.

**NMR spectroscopy.** For $^1$H-NMR spectra, polysaccharide or oligosaccharide samples were repeatedly exchanged in D$_2$O with intermediate lyophilizations. $^1$H-NMR (500 MHz), 2D COSY, 2D TOCSY, and NOESY spectra\(^6\) (internal standard: 3-trimethylsilylpropionate) of a mild acid hydrolysis product, native polysaccharide, and de-O-acetylated polysaccharide were recorded using a Varian Unity-500 spectrometer with its standard pulse programs (GMQCOSY, NTTOCSY, and NOESY, respectively) for D$_2$O solution at 25°C.

**Organisms.** *Bacillus circulans* isolated from soil was used in this study. *B. circulans* was stored at 4°C in basal medium.

**Culture conditions of bacteria for polysaccharide production.** A basal medium for polysaccharide production by bacteria contained 1% glucose, 0.05% asparagine, 0.005% yeast extract, 0.005% K$_2$HPO$_4$, 0.005% MgSO$_4$·7H$_2$O, 0.005% CaCl$_2$·2H$_2$O, and 0.005% FeCl$_3$·6H$_2$O. The pH was adjusted to 7.0 before autoclaving at 120°C for 15 min. A jar-fermentor (MSI-30, B. E. Murakoshi Co., Ltd.) culture was done at 30°C for 48 h with 10 liters of the medium by inoculating 1 liter of pre-culture, which was cultured as described in our previous paper,\(^7\) with aeration at 1.5 liters per minute and agitation speed at 300 rpm.

**Isolation and purification of the polysaccharide.** Culture broth from the fermentor was diluted with an equal volume of water, and centrifuged at 7000 x g for 20 min to remove bacterial cells. One percent cetyltrimethylammonium bromide (CTAB) solution was added to the supernatant solution until no more precipitation occurred. The precipitates were collected by filtration, washed with distilled water, and dissolved in 10% sodium chloride solution by stirring. Two volumes of ethanol were added to the solution, and the resulting precipitate was collected by centrifugation at 2000 x g for 10 min, dissolved in distilled water and dialyzed overnight against distilled water at 4°C. The dialyzed solution was lyophilized, and the lyophilized preparation was used for analysis.

**Preparation of carboxyl-reduced polysaccharide.** The carboxyl group of polysaccharide was reduced by the method of Taylor and Conrad.\(^8\) Polysaccharide (50 mg) in 100 ml of distilled water was adjusted to pH 4.7 with 0.1 N hydrochloric acid, followed by the addition of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (500 mg) and the stirred reaction mixture was kept at pH 4.7 for 3 h by addition of 0.02 N hydrochloric acid. After addition of sodium borohydride (500 mg) and one drop of n-octanol, the mixture was kept at room temperature with continuous stirring overnight and then dialyzed exhaustively against water. The reduced polysaccharide was obtained by lyophilization.

**Sugar analysis.** The native polysaccharide and carboxyl-reduced polysaccharide were hydrolyzed with 2 N CF$_2$COOH at 100°C for 12 h, and the acid was removed by repeated evaporations. GLC analysis of monosaccharides was done after conversion to their trimethylsilylated methyl glycosides. Glucuronic acid was analyzed by the carbazole method.\(^9\)

**Methylation analysis.** The native polysaccharide and carboxyl-reduced polysaccharide were completely methylated by Hakomori’s methylation.\(^8\) The permethylated polysaccharide was hydrolyzed by aq. 90% formic acid (at 100°C for 12 h). The solvent was evaporated under reduced pressure, and the residue was treated with 2 N CF$_2$COOH for 7 h at 100°C. The acid was removed by evaporation, and the resulting hydrolyzate was reduced with NaBH$_4$ at 25°C for 16 h. The excess NaBH$_4$ was removed by the

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addition of 10% acetic acid and the solution was evaporated to dryness under reduced pressure. After removal of borate by co-evaporation with methanol, the partially methylated sugars were acetylated and analyzed by GLC and GC-MS.

Mild acid hydrolysis. The polysaccharide was heated at 100°C in 0.25 N CF₃COOH for 6 h. The acid was removed by evaporation and the hydrolyzate was dissolved in water and put on a Dowex 1 × 8 (14 × 129 mm, CH₃COO⁻ form) column. The column was washed with water, and an acidic oligosaccharide was eluted with 0.1 N CH₃COOH, and the acid was removed by evaporation. The acidic oligosaccharide was lyophilized, then used for methylation analysis and ¹H-NMR analysis.

Results and Discussion

The crude exo-polysaccharide was isolated from the culture supernatant solution of Bacillus circulans by CTAB and purified by repeated ethanol precipitations. About 2 g of the purified polysaccharide was obtained from one liter of the culture supernatant solution. The purified polysaccharide had [α]D₂₀ + 188° (c, 0.25, H₂O) and its molecular weight was estimated to be about 1,200,000 by a pul lulan-calibrated gel permeation column (data not shown).

Hydrolysis of the polysaccharide followed by GLC analysis of trimethylsilyl ethers of methyl glycides and colorimetric measurement of the uronic acid showed the presence of rhamnose (Rha), mannose (Man), galactose (Gal), and glucuronic acid (GlcUA) in the molar ratio 2:2:3:3. GLC analysis of their trimethylsilylated (−) 2-butylic glycoses showed that Rha had the α-configuration and the other sugars had the α-configuration.

The native polysaccharide and carboxyl-reduced polysaccharide were methylated by Hakomori’s method and the derived partially methylated alditol acetates were analyzed by GLC-MS (Table I, PS and PSred). This methylation analysis showed that the polysaccharide is composed of terminal l-Rhap, 2-linked d-Manp, 3-linked d-Galp, and 3,4-linked d-GlcpUA residues. In Table I, smaller molar ratios of 2,3,4-tri-methyl Rha and 2,6-dimethyl Glc than those expected by sugar analysis were probably due to the high volatility of acetylated methyl ether of Rha and incomplete carboxyl reduction of GlcUA, respectively.

The 500 MHz ¹H-NMR spectrum of native polysaccharide in D₂O at 50°C (Fig. 1) contained methyl signals of l-Rhap at δ 1.2–1.3 and O-acetyl signals at δ 2.1–2.2 in the higher magnetic field, in addition, four major H-1 signals with small J₁,₂ values at δ 4.77, δ 5.10, δ 5.13, and δ 5.21 in the anomeric proton region. By tracing of cross peaks from methyl signal in the GMQCOSY spectrum (data not shown), referring with the TINTOCSY spectrum (data not shown), the ¹H-signal at δ 4.77 was assigned to H-1 of l-Rhap.

To identify the sequence and anomeric configurations of the monosaccharide residues in the repeating unit of the polysaccharide, it was hydrolyzed with mild acid with 0.25 N CF₃COOH for 6 h at 100°C, and the resulting oligosaccharides were fractionated by ion-exchange chromatography on Dowex 1 × 8 (CH₃COO⁻ form). The main oligosaccharide fraction was analyzed by methylation analysis and NMR spectroscopy.

Methylation analysis of the oligosaccharide gave 2-linked d-Manp and 3-linked d-Galp in almost the same molar ratio. The 500 MHz ¹H-NMR spectrum of the oligosaccharide at 25°C (Fig. 2, projected along f₂ axis in GMQCOSY spectrum) contained four H-1 signals at δ 5.405 (A, unresolved), δ 5.277 (B, unresolved), δ 5.152 (C, unresolved), and δ 4.615 (D, J₁,₂ 8.0 Hz), where the ratio of A:(B+D):C was almost the same, indicative of a trisaccharide. Assignments of the non-anomeric protons of residue A, B, C, and D were made on the basis of cross-peaks observed in the GMQCOSY (Fig. 2) and TINTOCSY spectrum (data not shown) as far as H-3 or H-4. As a result, the chemical shifts of the sugar residues in the oligosaccharide are listed in Table II. From this ¹H-NMR chemical shift data and the result of methylation analysis, residues A, B, C, and D were identified as 2-linked α-d-Manp, 3-linked reducing terminal α-d-Galp, non-reducing terminal α-d-GlcpUA, and 3-linked reducing terminal β-D-Galp. These data described above permit the structure of the trisaccharide to be formulated as follows (1):

\[ \alpha-D-GlcUAp₁ → 2α-D-Manp₁ → 3α-D-Galp \]  

(1)

The NOESY spectrum of de-O-acetylated polysaccharide (data not shown) showed the inter-residual connective between Rha H-1 and GlcUA H-4 indicating the linkage between H-1 of Rhap and H-4 of GlcUA.

Overall, the methylation analysis and ¹H-NMR data permit the primary structure of the basic repeating units of the polysaccharide produced by Bacillus circulans to be formulated as follows (2):

\[ \beta-l-Rhap \]

\[ \downarrow \]

\[ \rightarrow 3α-D-GlcUAp₁ → 2α-D-Manp₁ → 3α-D-Galp₁ \]

(2)

Many papers concerning exocellular heteropolysaccharides produced by Bacillus strains have shown that they vary widely in their sugar compositions. For example, B. polymyxa produces heteropolysaccharide containing some or all of the following sugars: glucose, mannose, galactose, glucuronic acid and mannnuronic acid, and a polysaccharide containing glucose, N-acetyl-glucosamine, and N-acetyl galactosamine was isolated from the culture broth of B. cereus, and a polysaccharide containing glucose, galactose, fucose, and glucuronic acid is produced by B. subtilis. However, detailed structures of these polysaccharides have been left unclear. Concerning the exopolysaccharide of Bacillus circulans, Forsyth and Webley reported the production and isolation of an exocellularr

Table I. Methylation Analysis

<table>
<thead>
<tr>
<th>Methylated sugar</th>
<th>Molar ratio</th>
<th>PS¹</th>
<th>PSred²</th>
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<tbody>
<tr>
<td>2,3,4-Tri-O-Me-Rha¹</td>
<td>0.5</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>3,4,6-Tri-O-Me-Man</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>2,4,6-Tri-O-Gal</td>
<td>1.2</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>2,6-Di-O-Me-Glc</td>
<td>ND³</td>
<td>0.7</td>
<td></td>
</tr>
</tbody>
</table>

¹ Native polysaccharide.
² Carboxylic-reduced polysaccharide.
³ 1.5-O-Acetyl-2,3,4-O-methyl-rhamnitol, and so on.
⁴ ND, not detected.
Fig. 1. 500 MHz $^1$H-NMR Spectrum of Native Polysaccharide.
polysaccharide, and Ishikawa et al. have reported that a polysaccharide produced by *B. circulans* contained rhamnose, galactose, mannose, and glucuronic acid, but its chemical structure was not investigated. Although the sugar components of an exo-polysaccharide reported by Ishikawa et al. are the same as those in this study, the physiochemical properties of their polysaccharide are clearly different from those of our polysaccharide preparation. For example, their polysaccharide has a property of gel formation and is stable to heating, but our polysaccharide cannot form a gel and the viscosity of its solution is decreased by heating at 100°C. These facts suggest that the structural features of the polysaccharide investigated in this study are different from those of the one reported by Ishikawa et al.

![Fig. 2. COSY Contour Plot (3.0-5.7 ppm region) for the Trisaccharide Obtained by Mild Acid Hydrolysis.](image_url)

<table>
<thead>
<tr>
<th>Table II.</th>
<th>1H-NMR Chemical Shifts (δ) of the Oligosaccharide Obtained by Mild Acid Hydrolysis</th>
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</thead>
<tbody>
<tr>
<td>Residue</td>
<td>Chemical shifts (ppm)</td>
</tr>
<tr>
<td></td>
<td>H-1</td>
</tr>
<tr>
<td>A</td>
<td>5.405</td>
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<tr>
<td>B</td>
<td>5.277</td>
</tr>
<tr>
<td>C</td>
<td>5.152</td>
</tr>
<tr>
<td>D</td>
<td>4.615</td>
</tr>
</tbody>
</table>
Acknowledgment. We wish to thank Miss Tomomi Maekawa, Osaka City University, for recording 500 MHz NMR spectroscopy.

References