Extraction and Chemical Characterization of Bacterial Cell Wall-Associated Polysaccharide from Activated Sludge
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Abstract
Polysaccharide (containing 4.7% protein) from bacterial cell walls was extracted from activated sludge and purified by gel filtration column chromatography. The apparent molecular weight was $1.3 \times 10^6$ by size exclusion chromatography. The constituent neutral sugars included rhamnose, fucose, arabinose, xylose, mannose, galactose, and glucose, in a molar ratio of 10:5:1:2:7:8:10; the ratio did not change after degradation with 1% acetic acid. Results of degradation using 1% acetic acid and SDS-PAGE showed that the polysaccharides were a complex of saccharide-lipid-protein in which most of the peptides were linked to the saccharide chain through the lipid portion. Oligosaccharides obtained by partial hydrolysis with 50 mM trifluoroacetate (TFA) were separated into three patterns, according to molecular weight and the molar ratio of constituent sugars. Results from methylation analysis of the partially hydrolyzed saccharides indicated that the saccharide chain was composed mainly of (1→4)-linked mannose and (1→2)-linked glucose.

Introduction
In the activated sludge process, which is widely used in urban and industrial wastewater treatment plants, dissolved organic or inorganic pollutants are removed from solution by adsorption to the microbial floc. Therefore, it is important that the activated sludge floc possesses good settlement properties for obtaining a clear effluent. Many investigators have proposed that extracellular polymeric substances (EPS), produced by floc-forming bacteria, play an important role in floc formation and the adsorption of pollutants. EPS, which is extremely viscous in solution, is composed principally of polysaccharide, although proteins and nucleic acids from cell lysis may be present. Wilén et al. showed that protein was the largest fraction and carbohydrate was a minor component. In contrast, no aromatic substances were indicated by NMR. Because EPS is a complex of polysaccharide, protein, and nucleic acid, and is negatively charged, positively charged organic pollutants and metal ions may be adsorbed by it. Brown and Lester reported that the quantity of bacterial EPS in activated sludge is controlled by the concentrations of nutrients in the broth, sludge age, and the oxidation of polymers by other bacterial species. The proteins and fulvic-acid like substances in EPS increased in the substrate utilization phase, but decreased in the endogenous phase.

The quantity and the characteristics of EPS vary widely by extraction method. Brown and Lester compared five different extraction methods and a combination of two of these methods, and stated that steaming treatment was the most effective extraction method for activated sludge and that sodium hydroxide (NaOH) treatment caused extensive cell disruption. In addition, they proposed sonication as a useful preliminary treatment in conjunction with another extraction method. Kiff and Rudd et al. also described that the combination of sonication and centrifugation is an effective method because of the high recovery of EPS and low cell lysis. Frølund et al. showed that extraction based on a cation exchange resin (Dowex, Fluka) had high extraction efficiency with little cell lysis and polymer disruption. In a previous paper, NaOH extraction was the most effective method for extraction of bioflocculants from activated sludge.
Although the mechanism of flocculation in activated sludge is poorly understood, several hypotheses have been proposed, such as cation bridging\textsuperscript{20} and hydrophobic interactions\textsuperscript{1,3,10}. The major feature in common among these hypotheses includes the physicochemical characteristics of EPS and interaction with bacterial surfaces. Therefore, characterizing the polysaccharides associated with the bacterial cell wall that interact with EPS is important for understanding the mechanism of flocculation. This report describes the partial structure and the chemical characteristics of polysaccharides extracted with 40% phenol from activated sludge.

Materials and Methods

Activated sludge. Samples of activated sludge (return sludge) were obtained from the Obihiro-south public sewage treatment plant in Obihiro, Japan. The samples were transferred to a container and returned to the laboratory within 30 min. The collected sludge was used in experiments immediately after sampling.

Extraction of polysaccharide from activated sludge floc. Activated sludge was washed with 0.01 M Tris (hydroxymethyl) aminomethane-hydrochloride (Tris-HCl) buffer, pH 8.0, three times, and the lipid was removed with chloroform-methanol 1:2 and 2:1 (v/v). Dried, defatted floc (5 g) was suspended in distilled water (100 mL) and the same volume of 40% phenol was added. The mixture was heated at 75°C in a water bath for about 5 min until the temperature reached 68-70°C, and then the mixture was chilled with stirring in an ice box to 10°C. The mixture was centrifuged at 2,500 rpm for 40 min at 4°C to separate the aqueous and phenol phases. Then the same volume of distilled water was added to the phenol phase, and the entire procedure was repeated. The combined aqueous phases were dialyzed against distilled water at 4°C for 48 h. The dialyze was evaporated to 30 mL at 30-40°C and the same volume of 0.1 M Tris-HCl buffer, pH 7.7, containing 0.02 M MgCl\textsubscript{2}, 0.02% NaN\textsubscript{3}, and 0.005% chloramphenicol was added. The mixture was treated with ribonuclease (2 mg) and deoxyribonuclease (3 mg) at 37°C for 72 h. Then the reaction mixture was evaporated to 40 mL and stirred with the same volume of 80% phenol at 4°C for 40 min. After centrifugation at 2,500 rpm for 40 min at 4°C, the aqueous phase was dialyzed against distilled water at 4°C for 48 h. This crude polysaccharide was lyophilized and stored in a freezer.

Fractionation of crude polysaccharide. Crude polysaccharide (100 mg) was dissolved in 0.2 M ammonium acetate buffer, pH 6.9 (25 mL) and applied to a Sepharose 6B column (2.6 cm ID × 55 cm), previously equilibrated with the same buffer. Elution was performed with the same buffer at a flow rate of 9 mL/h and 6-mL fractions were collected. Aliquot fractions were analyzed for saccharide, phosphorus, nucleic acid, and protein. Appropriate fractions were combined, dialyzed against distilled water at 4°C for 48 h, lyophilized, and stored in a freezer. The void volume of the column was determined by the elution volume of blue dextran.

Purification of polysaccharide. The polysaccharide fraction (20 mg) from Sepharose 6B column chromatography was dissolved in 0.01 M Tris-HCl buffer, pH 8.0 (10 mL) and applied to a Bio-Gel P-100 column (2.6 cm ID × 30 cm), previously equilibrated with the same buffer. Elution was performed with the same buffer at a flow rate of 9 mL/h and 5-mL fractions were collected.
Aliquot fractions were analyzed for saccharide, nucleic acid, and protein. Combined fractions were dialyzed against distilled water at 4°C for 48 h, lyophilized, and stored in a freezer.

**Determination of molecular weight.** For the determination of polysaccharide molecular weight, high-performance liquid chromatography (HPLC) was conducted with a Toyo-Soda GPC system model CCPM, CO-8011 equipped with TSKgelG4 and 3SWxL column (each 7.8 mm ID × 300 cm × 2). Mobility phase, flow rate, pressure, and column temperature were 0.2 M phosphate buffer (pH 6.8), 1.0 mL/min, 48 kg/cm², and 40°C, respectively. The eluate was monitored with RI (RI-8012), and UV (SC-8010) detectors, and molecular weights of eluted polysaccharides were obtained from their retention times.

**Electrophoretic analysis.** Sodium dodecyl sulfate slab-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the method of Laemmli. A separating gel was formed with 12.5% acrylamide and 0.1% SDS, with a 2.5% acrylamide stacking gel. The sample solution (5 mg/0.525 mL) was mixed with 10% SDS (0.2 mL), 2-mercaptoethanol (0.05 mL), and 70% glycerol (0.1 mL). After the mixture was heated at 100°C for 5 min, 5 μL of 0.1% bromophenol blue was added, and a 10-μL portion was applied to the gel. The electrode buffer was 0.025 M Tris-HCl (pH 6.8) containing 0.192 M glycine and 0.1% SDS. Electrophoresis was performed at 10 mA until the tacking dye entered the separating gel, followed by 20 mA. After SDS-PAGE, components on the gel were detected by Ag-protein stain and Ag-LPS stain.

**Degradation of polysaccharide with 1% acetic acid.** Polysaccharide (20 mg) was dissolved in 1% acetic acid (20 mL) and boiled for 2 h. Then, chloroform (20 mL) and methanol (22 mL) were added and mixed. The aqueous phase of this mixture was purified by Bio-Gel P-100 column chromatography as described above. The chloroform phase (lipid fraction) was evaporated to dryness, and chloroform (0.2 mL), methanol (0.3 mL), and 0.2 N NaOH in methanol (0.5 mL) were added. After deacetylation for 15 min at room temperature, chloroform (0.8 mL), methanol (0.2 mL), and distilled water (0.9 mL) were added to the reaction mixture and mixed. The aqueous phase was treated with Dowex 50WX8 and then evaporated. The components of these hydrophilic materials were analyzed by thin layer chromatography (TLC) with a solvent system of n-propanol-pyridine-H₂O (7: 4:2, v/v/v). The chloroform phase (free fatty acid fraction) was analyzed by gas liquid chromatography (GLC) using a Hitachi gas chromatograph model 163 fitted with a hydrogen flame ionization detector using a glass column (3 mm ID × 200 cm) packed with 10% DEGS at 180°C, as fatty acid methyl ester.

**Analysis of constituent neutral sugars in polysaccharide.** Gas-liquid chromatography (GLC) of constituent neutral sugar monomers was performed after conversion of the sugars into their corresponding alditol acetates, using a glass column (3 mm ID × 200 cm) packed with 3% ECNSS-M at 180°C in a gas-liquid chromatograph (Hitachi Model 163). The alditol acetates were prepared by the reduction of the sugars with sodium borohydride followed by acetylation with pyridine-acetic anhydride (1:1, v/v) at room temperature overnight in a sealed tube under N₂ gas. Complete hydrolysis of polysaccharide was accomplished with 0.5 N H₂SO₄ in 90% acetic acid at 80°C for 16 h, followed by an additional 5 h after addition of the same volume of distilled water.
Partial hydrolysis of polysaccharide. Polysaccharide (30 mg) was dissolved in 50 mM trifluoroacetic acid (TFA) (30 mL) and was boiled for 20 min. After evaporation to dryness with methanol and carbon tetrachloride, the partially hydrolyzed polysaccharide was dissolved in 3 mL of distilled water and washed with CHCl₃. The aqueous phase was applied to a Bio-Gel P-6 column (1.5 cm ID × 42 cm), previously equilibrated with distilled water, and 1-mL fractions were collected. The eluate was monitored with RI and UV detectors.

Analysis of partial structure of the saccharide chain. Partially hydrolyzed polysaccharide (8 mg) obtained by the treatment with 50 mM TFA was dissolved in dimethylsulfoxide (DMSO) (0.8 mL) and permethylated by the method of Yang and Hakomori.[19] The methylated polysaccharide was dissolved in tetrahydrofuran (1 mL), and 1.35% LiAlH₄ (suspended in 1.5 mL of diethylether) was added dropwise with stirring. The carboxyl group of uronic acid was reduced by stirring this mixture at room temperature for 18 h, followed by decomposition of the residual LiAlH₄ by addition of distilled water in an icebox. The carboxyl-reduced methylated polysaccharide was divided into two portions, and one was methylated again using the method of Hakomori as described above. The methylated polysaccharide was subjected to GLC and gas chromatography/mass spectrometry (GC/MS) analyses after conversion into corresponding alditol acetates as described above. The GC/MS was performed using a Shimadzu QP1000 instrument with a silica capillary column (0.22 mm ID × 25 m) coated with CBP-10 at 160-220°C (5°C/min). The ionization potential was 70 eV, and the ion source and molecular separator temperature were 250°C. The voltage of the detector was 1.08 kV, and the flow rate of the carrier gas (He₂) was 30 mL/min.

Other analyses. Total saccharide was determined using the anthrone-H₂SO₄ method[20] with a glucose-galactose 1:1 standard. Hexosamine was determined by the method of Blix[21] with a glucosamine standard. Phosphorus was determined using the molybdenum-H₂SO₄ method[22]. Protein was determined according to the method of Lowry et al.[23] using a standard of bovine serum albumin. Nucleic acid and protein content in the gel filtration eluate were determined by measuring absorbance at 260 and 280 nm, respectively.

Chemicals. Sepharose 6B was obtained from Pharmacia Fine Chemicals, Uppsala, and Bio-Gel P-100 and Bio-Gel P-6 were the products of Bio Rad Laboratories. The Kieselgel 60 used for TLC analysis was obtained from E. Merck, Darmstadt. Deoxyribonuclease and ribonuclease were obtained from Sigma Chemical Co., St. Louis. DEGS and ECNSS-M were obtained from Gas-Chro Kogyo Co., Tokyo.

Results

Extraction and purification of polysaccharide from activated sludge

A total of 100 mg of crude polysaccharide was obtained from 5 g (dry weight) of defatted activated sludge floc by extraction with 40% phenol. Figure 1 shows the elution profile of crude polysaccharide obtained using a Sepharose 6B column. Gel filtration revealed a saccharide peak near the void volume, indicating a molecular weight greater than 10⁶ for the polysaccharide. In contrast, nucleic acid break-down products eluted at an elution volume between 200 and 300 mL,
producing broad tailing peaks. The portion corresponding to polysaccharide was further applied to a Bio-Gel P-100 column for purification. The elution profile from the Bio-Gel P-100 column shows that the polysaccharide was separated from the nucleic acid break-down products and was obtained as a single peak (Fig. 2). The procedure produced 35 mg of polysaccharide from 100 mg of crude polysaccharide.

**Molecular weight of purified polysaccharide**

As shown in Fig. 3, HPLC showed two peaks from polysaccharide obtained from activated sludge. The early peak (retention time, 11.47 min) was polysaccharide, and the later peak (retention time, 25.17 min) was contaminating nucleic acid. The molecular weight of the polysaccharide was calculated as $1.3 \times 10^6$ by comparison of the retention time with that of standard polysaccharides.

**Electrophoretic analysis**

Polysaccharide purified with Bio-Gel P-100 column chromatography was subjected to polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE). After electrophoresis, sacchar-
ides and proteins were visualized using LPS-Ag stain and protein-Ag stain, respectively. As shown in Fig. 4, saccharide bands were observed at lower molecular weights, while bands of proteins were observed in a broad range of molecular weights. The fast-migrating bands of proteins co-migrated with those of saccharides, which suggests these bands were complexes of saccharide and protein.

**Degradation of polysaccharide with 1% acetic acid**

**Hydrophilic fraction (degraded polysaccharide).**

Table 1 shows the effect of degradation with 1% acetic acid on the polysaccharide component. The GLC analysis identified seven neutral sugars (rhamnose, fucose, arabinose, xylose, mannose, galactose, and glucose) as constituent sugar monomers in polysaccharide; their molar ratio was not influenced by degradation. Hexosamine content was not changed by degradation, but protein content decreased from 47 to 30 µg (approximately a 36% decrease).

<table>
<thead>
<tr>
<th>Component</th>
<th>Before degradation</th>
<th>After degradation</th>
<th>molar ratio</th>
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<tbody>
<tr>
<td>Rhamnose</td>
<td>0.99</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>Fucose</td>
<td>0.58</td>
<td>0.52</td>
<td></td>
</tr>
<tr>
<td>Arabinose</td>
<td>0.14</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>Xylose</td>
<td>0.17</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>Mannose</td>
<td>0.72</td>
<td>0.67</td>
<td></td>
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<tr>
<td>Galactose</td>
<td>0.84</td>
<td>0.79</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>1.00</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Hexosamine</td>
<td>0.598</td>
<td>0.682</td>
<td>µmol/mg</td>
</tr>
<tr>
<td>Protein</td>
<td>47.0</td>
<td>30.0</td>
<td>µg/mg</td>
</tr>
</tbody>
</table>

Fig. 3. High performance liquid chromatogram of polysaccharide extracted from activated sludge.

Fig. 4. SDS-PAGE of polysaccharide extracted from activated sludge. A: LPS-Ag stain; B: protein-Ag stain.
Hydrophobic fraction.

The water-soluble materials freed by decylation of the hydrophobic fraction obtained by degradation with 1% acetic acid were subjected to TLC. Development was performed using chloroform-methanol-H₂O (65:25:4, v/v/v) for the hydrophobic fraction before decylation, and n-propanol-pyridine-H₂O (7:4:2, v/v/v) for the hydrophilic materials after decylation of this fraction. After development, amino groups were visualized with ninhydrine reagent. As shown in Fig. 5, several spots were detected in the aqueous phase, although they could not be identified. Because hexosamine is not detected by a colorimetric method in the hydrophilic materials, the lower Rₜ value spots in the materials seemed to be free amino acids (although determination of amino acid content was not performed). GLC analysis of fatty acid freed by decylation of the hydrophobic fraction showed that major constituent fatty acids were palmitic acid, stearic acid, and oleic acid, although myristic acid, palmitoleic acid, n-heptadecanoic acid, and linoleic acid also were identified in small amounts (Table 2).

Partial structure of polysaccharide.

Partial hydrolysis of polysaccharide.

Constituent sugar monomers in polysaccharide partially hydrolyzed with 50 mM TFA were compared after separation into seven fractions with a Bio-Gel P-6 chromatography column (Fig. 6). The fractions were separated into three patterns, according to the molar ratio of constituent neutral sugar monomers (i.e., pattern 1, fractions 1, 2, and 3; pattern 2, fractions 4, 5, and 6; and pattern 3, fraction 7). The molar ratios of constituent sugar monomers of fractions 1, 2, and 3 were similar to that of intact (before partial hydrolysis)

Table 2. Composition of fatty acids in polysaccharide extract from activated sludge.

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myristic acid (C14:0)</td>
<td>3.8</td>
</tr>
<tr>
<td>Palmitic acid (C16:0)</td>
<td>22.6</td>
</tr>
<tr>
<td>Palmitoleic acid (C16:1)</td>
<td>3.7</td>
</tr>
<tr>
<td>n-Heptadecanoic acid (C17:0)</td>
<td>1.5</td>
</tr>
<tr>
<td>Stearic acid (C18:0)</td>
<td>24.7</td>
</tr>
<tr>
<td>Oleic acid (C18:1)</td>
<td>31.5</td>
</tr>
<tr>
<td>Linoleic acid (C18:2)</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Fig. 5. TLC analysis of hydrophobic fraction obtained by degradation of polysaccharide with 1% acetic acid. A: hydrophobic fraction before decylation, developed with CHCl₃-MeOH-H₂O (65:25:4, v/v/v) and visualized with H₂SO₄; B: aqueous phase after decylation, developed with n-ProH-pyridine-H₂O (7:4:2, v/v/v) and visualized with ninhydrine reagent.

Fig. 6. Molar ratio of constituent sugars of partially hydrolyzed polysaccharide.
polysaccharide. Fractions 1 and 2 were combined and subjected to partial structural analysis of the saccharide chain.

**Structural characterization of partially hydrolyzed polysaccharide.**

Results of GLC and GC/MS analyses of carboxyl-reduced methylated polysaccharide and re-methylated material are summarized in Table 3. The (1→4)-linked mannose, (1→4)-linked glucose, (1→3)-linked galactose, (1→2)-linked glucose, and branched (1→4)-linked glucose existed in a molar ratio of 7:1:2.7:2. The nonreducing terminals were glucose (involving mannose) and galactose in a ratio of approximately 5:1. Re-methylation of carboxyl-reduced methylated polysaccharide resulted in an increase of the molar ratio of 2,3,6-tri-O-methyl-glucose from 1.00 to 1.95. In contrast, 2,3-di-O-methyl-glucose decreased from 3.13 to 1.31. This suggests that (1→4)-linked glucuronic acid exists in the chain at a ratio of about 5%. Deoxy sugars and pentoses could not be identified.

**Discussion**

Many investigators have studied extracellular polymeric substances (EPS) produced by flocculating bacteria isolated from activated sludge, and the mechanisms of flocculation by these bacteria have been proposed[31-33]. However, the mechanism of flocculation by native activated sludge, which involves a complex mixed microbial system of bacteria, fungi, and protozoa, is incompletely understood. In general, the existence of filamentous microorganisms is regarded as a main factor for poor settling ability and can cause damage to wastewater treatment equipment. However, Urbain et al.[13] proposed the theory that they play a role as the backbone in the floc, because filamentous microorganisms were observed in all sludge samples and were not always associated with poor settling ability. Furthermore, Tsuge et al.[14] suggested that fungi in activated sludge could contribute to wastewater treatment under cold conditions.

In the present study, pentoses and deoxy sugars as well as aldohexoses were found in polysaccharide as the constituent neutral sugar monomers, results that agreed with the report by Brown and Lester[8]. However, the present results were different from those reported by Horan and Eccles[35], who found only glucose, galactose, mannose, glucuronic acid, and galacturonic acid as the sugar monomers. This difference may be due to the extraction method used. The extraction method with 40% phenol used in the present study is the one generally used to isolate polysaccharides.
ides associated with bacterial cell walls (lipoteichoic acid from Gram-positive bacteria and lipopolysaccharide from Gram-negative bacteria), suggesting that this polysaccharide is an endogenous compound rather than EPS. Although the major component of polysaccharide extracted with 40% phenol from activated sludge floc was thought to be lipopolysaccharide (most of the floc-forming bacteria was Gram-negative), glucosamine was not determined in the hydrophobic fraction obtained by degradation of the compound with 1% acetic acid. Therefore, further investigations are required to clarify the origin of the polysaccharide. However, the polysaccharide obtained in the present study was eluted as a single peak by gel filtration on a Bio-Gel P-100 column, which suggested that this purified polysaccharide was a mixture of several polysaccharides. Application of this polysaccharide to an ion exchange column resulted in extremely low recovery (data was not shown), suggesting that the polysaccharide adsorbed to the gel bed irreversibly. Because physicochemical characteristics (e.g., molecular weight and ionic properties) of polysaccharides produced by microorganisms in activated sludge are presumed to be similar, biochemical methods, such as affinity column chromatography, is needed for further purification.

The decrease in the protein content by degradation of polysaccharide with 1% acetic acid and the migration pattern of SDS-PAGE showed that the polysaccharides were a complex of saccharide-lipid-protein in which most of the peptides were linked to the saccharide chain through the lipid portion. Even though the gel filtration profile and the HPLC retention time indicated a molecular weight for the compound of approximately $1.3 \times 10^6$, the presence of a hydrophobic region means the polysaccharide may form a mixed micelle, resulting in an apparent macromolecule. The importance of hydrophobic regions on aggregation of bacteria has been reported. Goodwin and Forster also proposed that a lipid fraction (triglyceride mixture) had a great influence on the settlement properties. These observations suggest that the hydrophobic region formed by lipid in polymers may have strong effects on the interactions between EPS and bacterial cell wall-associated polysaccharide, resulted in the aggregation of bacterial cells.

Partial hydrolysis of the polysaccharide using diluted TFA was not satisfactory because sugar monomers rather than oligosaccharides were freed, even if under different hydrolysis conditions. Therefore, enzymatic hydrolysis will be needed to obtain oligosaccharide from this polysaccharide. The present report described the chemical characteristics of bacterial cell wall associated polysaccharide, which play a significant role in flocculation by attracting EPS. Further work is needed to determine the interaction of the polysaccharide with EPS and its contribution to the aggregation of bacteria.

References


29) Sakka, K., Endo, T., Watanabe, M., Okuda, S., and Takahashi, H., Deoxyribonuclease-susceptible floc forming


