Characterization of a Degraded Product Derived from 
*Serratia piscatorum* Polysaccharide by Ultrasonication

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Acidic polysaccharide, PLS F-II, was prepared from *Serratia piscatorum* polysaccharide, PLS N-I, by a sequence of ultrasonication and gel filtration and was examined for chemical composition and biological activity.

The purified PLS F-II preparation was shown to be homogeneous by ultracentrifugation, zone electrophoresis and column chromatography. The molecular weight was estimated to be about $2 \times 10^4$ by the Archibald method. PLS F-II was composed of L-rhamnose, D-galactose and D-galacturonic acid in the molar ratio of 2:1:1 and was partially acylated on the galacturonic acid residues.

PLS F-II was found to enhance the antibody formation in mice, although it showed no anti-inflammatory activity.

In previous papers, the authors reported that an acidic polysaccharide, PLS N-I, obtained from culture filtrate of *Serratia piscatorum* IFO 12527 was fractionated into two polysaccharide fractions, PLS F-I and PLS F-II, by gel filtration after treatment by ultrasonication. These two polysaccharide fractions differed from each other in their physicochemical, chemical and biological properties. It was especially noted that PLS F-II was a simple polysaccharide free from protein and lipid A-like moieties which were found in the native polysaccharide.

The present study examines the detailed chemical composition of PLS F-II and some of its biological properties.

**MATERIALS AND METHODS**

**PLS N-I preparation.** The native polysaccharide, PLS N-I, was prepared in the manner described previously.

**PLS F-II preparation.** The degraded polysaccharide, PLS F-II, was prepared from PLS N-I by gel filtration after treatment by ultrasonication as follows:

Eight hundred milligrams of PLS N-I was dissolved in 80 ml of water and sonicated for one hour at 10 kc in the cold. The sonicated polysaccharide was concentrated to 20 ml, applied onto a Sepharose 4B column (5×90 cm) and eluted with 1 M NaCl at a flow rate of 1 ml/min, the eluate being fractionated into 20 ml-portions as shown in Fig. 1. PLS F-II in the fractions from No. 56 to No. 78 was collected, dialyzed against running water, concentrated to about 20 ml and then rechromatographed as described above. The PLS F-II fraction thus obtained was dialyzed, passed through an Amberlite IR-120 (H+) column (1.3×30 cm), dialyzed against deionized water and lyophilized. Yield, 580 mg.

**Reduction of carboxyl groups.** Two hundred milligrams of PLS F-II was dissolved in 50 ml of water and mixed with 50 ml of ethylene oxide in the cold. The reaction mixture was left standing at 23°C for 5 days, dialyzed against running water and concentrated to 45 ml, to which 5 ml of glycerol was added. The resulting solution was admixed with 50 ml of 10% aqueous glycerol containing 1.5 g of NaBH₄ and left standing at 5°C for 2 days. The reaction mixture was treated with acetic acid to destroy the excess NaBH₄, dialyzed against running water and then lyophilized. Yield, 145 mg.

**Deacylation.** One hundred milligrams of PLS F-II was dissolved in 10 ml of 0.5 N NaOH and heated at 70°C for 60 min. The deacylated product was passed through an Amberlite IR-120 (H+) column (1.3×30 cm), dialyzed against deionized water
FIG. 1. Fractionation of PLS F-II on Sepharose 4B.

Eight hundred milligrams of sonication products of PLS N-I dissolved in 20 ml of 1 M NaCl was applied onto a column (5 x 90 cm) of Sepharose 4B and eluted with 1 M NaCl. The eluate was fractionated into 20 ml-portions which were then subjected to the sugar analysis by the phenol-sulfuric acid method. The PLS F-II fraction (No. 56~78) was rechromatographed and lyophilized. Yield, 82 mg.

Periodate oxidation and borohydride reduction. One hundred milligrams of PLS F-II or deacylated PLS F-II was dissolved in 50 ml of water, mixed with 50 ml of 0.1 M NaIO₄ and left standing at 5°C for 20 days in the dark. To the reaction mixture was added 10 ml of ethylene glycol to destroy the excess periodate in the solution and the resulting solution was dialyzed against running water. The dialyzed solution was supplemented with 100 mg of solid NaBH₄ and left overnight at room temperature. The resulting solution was treated with acetic acid to destroy the excess NaBH₄, dialyzed and lyophilized. The product was then subjected to sugar analysis.

Separation of the sugar components in PLS F-II. One hundred milligrams of PLS F-II was hydrolyzed with 5 ml of 1 N H₂SO₄ in a sealed glass tube at 100°C for 15 hr. After neutralization with BaCO₃, the hydrolysate was filtered. The filtrate and the washing were combined and passed through an Amberlite IR-120 (H⁺) column (1.3 x 20 cm). The effluent was then applied onto a Dowex 1 (OH⁻) column (1.3 x 20 cm), which was then washed with 50 ml of water and the passed solution and the washing were combined and evaporated to dryness to give the neutral sugar fraction. The Dowex 1 column was then eluted with 50 ml of 1 N NaOH and the eluate was passed through an Amberlite IR-120 (H⁺) column (1.3 x 20 cm) and evaporated to dryness to give the acidic sugar fraction.

Analytical methods. Total carbohydrate was determined by the phenol-sulfuric acid method; reducing sugars by the method of Somogy-Nelson; hexuronic acid by the carbazole-sulfuric acid method and hexosamine by the method of Elson-Morgan-Blix. Protein was determined by the method of Lowry et al. and fatty acid esters by the method of Skidmore and Entenman.

Paper chromatography (PPC), gas chromatography (GLC), thin-layer chromatography (TLC) and gel filtration were all carried out by methods described previously. DEAE-cellulose column chromatography was performed by a gradient elution technique with 0~0.3 M tris buffer, pH 7.5. Electrophoresis was carried out as described previously. Sedimentation analysis was performed at a speed of 59,800 rpm with a Spinco Model E ultracentrifuge. The molecular weight was measured by the Archibald method.

Biological assay. Adjuvant effect on the antibody formation was assayed as follows: Five mice were intravenously injected with a polysaccharide sample together with washed sheep red blood cells (1 x 10⁹). The antibody response to mice was assessed by counting the number of plaque forming cells in the spleen according to the method of Jerne et al.

RESULTS AND DISCUSSION

Homogeneity and molecular weight of PLS F-II

The PLS F-II preparation showed a single peak in the gel filtration on Sepharose 2B, 4B and 6B, and also in the ion exchange chromatography on DEAE-cellulose. It moved as a single spot in the zone electrophoresis on a Whatman glass fiber paper and also showed a single boundary in the analytical ultracentrifugation (Fig. 2). These results indicate that this PLS F-II preparation is homogeneous.

The sedimentation coefficient, S₂₀,ₚ, was calculated as 1.6 S, and the average molecular weight was about 2 x 10⁴, as determined by the Archibald method.

Chemical composition of PLS F-II

As described previously, chemical analysis of PLS F-II indicated that this polysaccharide contained approximately 84% carbohydrate (as glucose) and 14% lipid (as palmitic acid ester) but neither protein nor hexosamine was present.
The hydrolysate of PLS F-II was fractionated into neutral and acidic sugars by ion exchange chromatography as described in the methodology section. The sugar components in the neutral and acidic sugar fractions were analyzed by PPC, TLC and GLC. The analytical data indicated that PLS F-II consisted of L-rhamnose, D-galactose and D-galacturonic acid. The results of quantitative analyses of sugars in the hydrolysate, before and after reduction of the carboxyl groups in PLS F-II, are shown in Table I. The carboxyl-reduced PLS F-II contained only a negligible amount of D-galacturonic acid, while the content of D-galactose increased almost twofold. Thus, the molar ratio of L-rhamnose, D-galactose and D-galacturonic acid was determined to be 2:1:1.

In the previous paper, PLS F-II was assumed to contain acyl groups, judged from its infrared spectrum. To confirm this, the NMR spectrum was measured. As shown in Fig. 3, the NMR spectrum revealed a signal around $\delta$2.04 ppm, indicating the presence of O-acetyl groups in PLS F-II. Furthermore, volatile fatty acid fraction obtained from the hydrolysate of PLS F-II was analyzed by gas chromatography, using the Tween 80-phosphoric acid column. The result showed that the main volatile acid was acetic acid and a small amount of n-butyric acid was also present. In another experiment, non-volatile long-chain fatty acids, found in the PLS N-I preparation, were not detected. The total O-acetyl content was determined by the alkaline titration method of Wolfrom and Thompson to be approximately 5%, indicating that the molar ratio of the acetyl group, L-rhamnose D-galactose and D-galacturonic acid corresponded to 1:2:1:1.

**Table I. Sugar Composition of PLS F-II**

<table>
<thead>
<tr>
<th>PLS preparation</th>
<th>Components (%)</th>
<th>Rhamnose</th>
<th>Galactose</th>
<th>Galacturonic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLS F-II</td>
<td></td>
<td>39.5</td>
<td>20.5</td>
<td>21.5</td>
</tr>
<tr>
<td>Carboxyl-reduced$^a$</td>
<td></td>
<td>37.0</td>
<td>35.5</td>
<td>4.5</td>
</tr>
<tr>
<td>PLS F-II</td>
<td></td>
<td>39.5</td>
<td>20.5</td>
<td>21.5</td>
</tr>
</tbody>
</table>

$^a$ PLS F-II in H+ form was treated with ethylene oxide in aqueous solution and then reduced with NaBH₄ at pH 8, as described in the methodology section.
The location of O-acyl groups was examined by periodate oxidation. Periodate oxidation of deacylated PLS F-II caused the complete destruction of galacturonic acid residues in its molecule, whereas galacturonic acid residues in native PLS F-II resisted the oxidation. Other sugar constituents, rhamnose and galactose, remained unchanged by deacylation of the polysaccharide.

Since the methylation analysis, which will be reported in the forthcoming paper, indicated that galacturonic acid residues are linked to the adjacent sugar unit by the 1→4 bond, the above results suggests that some of the galacturonic acid residues are most probably O-acylated at the C-2 and/or C-3 positions.

Adjuvant activity of PLS F-II

Although PLS F-II showed no anti-inflammatory activity as described previously, further biological examinations revealed that it enhanced antibody formation in mice, as shown in Table II. The plaque forming cells

<table>
<thead>
<tr>
<th>Dose of PLS (mg/kg, i.v.)</th>
<th>Number of PFC per spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PLS F-II</td>
</tr>
<tr>
<td>0.1</td>
<td>2,330</td>
</tr>
<tr>
<td>1.0</td>
<td>7,970</td>
</tr>
<tr>
<td>10.0</td>
<td>9,254</td>
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<tr>
<td>50.0</td>
<td>11,900</td>
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<tr>
<td>100.0</td>
<td>12,302</td>
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<tr>
<td>None</td>
<td>1,170</td>
</tr>
</tbody>
</table>

n.d.: not determined.

in the spleen of mice sensitized with sheep red blood cells increased approximately 10 times when more than 1 mg/kg of PLS F-II together with the antigen was intravenously administered to the animal. Table II also shows that deacylation of PLS F-II reduces its adjuvant effect to a great extent. It can be deduced from these results that the O-acyl groups attached to the galacturonic acid residues of PLS F-II may be essential for the activity.

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REFERENCES