Lipopolysaccharide Isolated from *Mycobacterium tuberculosis* Strain Aoyama B

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Methods for efficient extraction and simple purification of the lipopolysaccharide specific for *Mycobacterium tuberculosis* were developed. Crude lipopolysaccharide was obtained from sterilized cells through mechanical disintegration, Triton X-100 extraction, ethanol precipitation, glucosidase digestion, and gel-filtration chromatography. The lipopolysaccharide was further purified by treatments with pyridine-methanol and chloroform-methanol to remove the contaminating glycolipids and phospholipids, and by digestion with the immobilized trypsin to remove the contaminating proteins. The purified lipopolysaccharide was composed of a polysaccharide consisting of D-mannose and D-arabinose, and fatty acids, mainly palmitic, tuberculostearic, and stearic acids, which were bound in ester linkages. The lipopolysaccharide had strong tumor regressing activity on the mouse fibrosarcoma.

Constituents of *Mycobacterium tuberculosis* have been studied by many investigators over the past decades, and a considerable number of reports1~7 and reviews8~12 have been published. However, reports on lipopolysaccharide were few.2,3,8

The authors have studied to elucidate the constituents of *M. tuberculosis* strain Aoyama B.

The structures of polysaccharides in a hot water extract of this bacterium have been reported.13 In our previous work, the lipopolysaccharide contained in the extract as a minor component was presumed to be the constituent largely related to the biological activities of the bacterium from the structural point of view, but further analyses of this substance showed that it contained phospholipids and glycolipids.

To obtain a large amount of the pure lipopolysaccharide mechanical disintegration and extraction with detergent was found the most efficient. Furthermore, a method for purifying the lipopolysaccharide from contaminating phospholipids was established using a combination of several organic solvents.

In this paper we report the structure and antitumor activities of this lipopolysaccharide (MLS).

**MATERIALS AND METHODS**

**Bacterial strain.** *Mycobacterium tuberculosis* strain Aoyama B (maintained by Zeria Pharmaceutical Co., Ltd.) was used.

**Bacterial cultivation.** The strain of *M. tuberculosis* was cultivated on the surface of Sauton's liquid medium at 37°C for 5 weeks, and cells were collected by filtration, washed with water, and suspended in 20 volumes of distilled water. The suspension was sterilized at 100°C for 2 hr.

**Extraction.** The sterilized cells were collected by filtration, washed with water, and suspended in 5 volumes of distilled water. The suspended cells were disintegrated with

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1 Part of this paper was presented at the 339th Meeting of the Kansai Branch of the Agricultural Chemical Society of Japan in Kyoto, May 18, 1985.
a French press (Ohtake Work Co., model 5615) at 1400 kg/cm². To the disintegrated cell suspension was added Triton X-100 (Nakarai Chemicals Ltd.) to the final concentration of 1.5%. The mixture was stirred at room temperature for 24 hr and centrifuged at 20,000 x g for 20 min. The supernatant was concentrated to 1/5th volume under reduced pressure. It was then dialyzed against running water, and 9 volumes of ethanol was added to the nondialyze. The mixture was stirred and separated by centrifugation at 5000 x g for 10 min. The precipitate collected was washed with ethanol and then with ethyl ether to obtain a crude lipopolysaccharide.

Analytical methods. Sugar was measured by the phenol-H₂SO₄ method with D-mannose as a standard. A modification by Boas of the Elson-Morgan method was used for the measurement of hexosamines. Protein was measured by the Coomassie brilliant blue colorimetric method with bovine serum albumin as a standard. Total phosphorus was measured by the Chen method.

Molecular weight estimation. High-pressure liquid chromatography (HPLC) was done to measure molecular weights with a Hitachi liquid chromatograph model 635; column, TSK G-3000PW (Toyo Soda Ltd.); eluent, 50 mM NaCl aqueous solution; flow rate, 1 ml/min; and detector, a differential refractometer (Japan Analytical Industry Ltd., model RI-2). The calibration curve for molecular weights was obtained by plotting the consecutive elution volumes found for the following reference substances against their known molecular weights on semilogarithmic graph paper; Pullulan P800, P200, P100, P50, P20, P10, and P5 (Showa Denko Ltd.) and D-maltotriose, D-maltose, and D-glucose (Nakarai Chemicals Ltd.). From the calibration curve, the molecular weight of each polysaccharide and lipopolysaccharide was estimated.

Fractionation by molecular sieve chromatography on Bio Gel columns. Lipopolysaccharide and polysaccharides constituents were fractionated by gel filtration on Bio Gel A-5m and P-30 (Bio Rad Laboratories). Elution was done with distilled water, followed by detection and assay as sugar by the phenol-H₂SO₄ method and by collection of the respective fractions.

Sugar analysis. Lipopolysaccharide and polysaccharides were hydrolyzed with 2 N H₂SO₄ at 100°C in a sealed tube for 2 hr. The hydrolyzates, with xylitol added as a standard, were analyzed as alditol acetates in the usual manner on a Shimadzu GC-4CM gas chromatograph (GLC) coupled with a hydrogen flame ionization detector, and on a Hitachi-6M gas chromatography-mass spectrometer (GC-MS) fitted with a total ion monitor by the method of Kobatake et al. for identification. GLC column used in sugar analysis was Tabsorb (Regis Chemical Co.). Hexosamines were identified by GLC by the method mentioned. All other analytical conditions were as specified in the literature.

Methylation analysis. To obtain a methylated monosaccharide mixture, the polysaccharide specimens were completely methylated by the method of Hakomori, hydrolyzed with 90% formic acid at 105°C for 6 hr, and for an additional 2 hr at 100°C after adding 5 volumes of water by the method of Lomax et al. and concentrated under reduced pressure at a temperature below 40°C. The hydrolyzates were analyzed as partially methylated alditol acetates in the usual manner on a Shimadzu GC-4CM and on a Hitachi-6M GC-MS equipment. The GLC column used was Tabsorb.

Fatty acid analysis. The dry lipopolysaccharide sample was methanolized with 4% (w/v) HCl solution in methanol at 75°C in a sealed tube for 16 hr with added pentadecanoic acid (guaranteed reagent, Nakarai Chemicals Ltd., used after recrystallization twice from acetone, mp 53°C) as an internal standard. The resultant mixture of fatty acid methyl esters was extracted 3 times with n-hexane and the hexane layers were combined. It was then washed with 2% KHCO₃ aqueous solution, dried over anhydrous sodium sulfate, and analyzed by GLC on a column of 10% Silicon gum SE-30/Chromosorb W. Individual fatty acid methyl esters were identified by GC-MS on the same column.

Analysis of fatty acid linkage. The dry lipopolysaccharide specimen was treated with methyl vinyl ether (Tokyo Kasei Kogyo Co., Ltd.) in dimethyl sulfoxide on a crushed ice bath for protection of the free hydroxyl groups with methoxyl groups. Fatty acid binding sites in lipopolysaccharides were then substituted with methyl groups by the method of Hakomori and hydrolyzed to eliminate methoxyl groups and simultaneously hydrolyze the polysaccharide. The resultant mixture of monosaccharides and partially methylated monosaccharides was analyzed in the form of alditol acetates by GLC on columns of 3% ECNSS-M/Gascrom Q (Wako Pure Chemicals Ltd.), Tabsorb and 3% Silar 10C/Chromosorb W (Gasukuro Kogyo Co.) to identify the location of fatty acid binding in the lipopolysaccharide.

Enzyme digestion. Glucosidase digestion: To eliminate contaminating α-glucan in the lipopolysaccharide it was incubated with α-amylase (Type IIA, Sigma Chemical Co.) and amyloglucosidase (from Aspergillus oryzae, Sigma Chemical Co.) in 0.1 M acetate buffer, pH 5.0, at 37°C overnight. For removal of the enzyme proteins, the reaction mixture was heated at 100°C for 15 min and the precipitate of the denaturated proteins was centrifuged out. Trypsin digestion: One g of trypsin (E. Merck, Darmstadt) was immobilized on 125 g of Sephalose 4B (Pharmacia Fine Chemicals Inc.) activated by 25 g of cyanogen bromide (Nakarai Chemicals Ltd.). For elim-
dration of contaminating proteins, the sample was incubated with the immobilized trypsin in 0.1 M Tris-HCl buffer, pH 7.4, with stirring at 37°C overnight and then the immobilized trypsin was centrifuged out (2000 x g for 10 min).

Measurement of antitumor activity. Eight week-old C57BL/6 female mice (Shizuoka Agric. Cooper. of Exptl. Animals) having body weights of 20.1 ± 1.1 g were used as test animals, and each mouse was inoculated subcutaneously in the upper belly region with 1 x 10⁵ cells of the methylcholanthrene-induced fibrosarcoma MC-1. On the 10th day after inoculation, the mice were divided into groups of 10 mice each, and the sample substance in 0.2 ml saline was administered intraperitoneally. The mice in the control group were given 0.2 ml of saline in the same manner. The sizes (length x width, mm²) of tumors were measured with a dial caliper. On the 23rd day after inoculation, all mice were killed and the tumors were cut out.

RESULTS AND DISCUSSION

Preparation of lipopolysaccharide

The 12 g of crude lipopolysaccharide extracted from 1 kg of the sterilized wet cells of M. tuberculosis strain Aoyama B as described in MATERIALS AND METHODS was purified in the following manner. Two g portions of the crude substance were put on a column of Bio Gel A-5 m (3.8 cm x 55 cm) equilibrated with 50 mM NaCl and eluted with the same solution to collect the milky white fraction excluded by the molecular sieve. This was dialyzed in running deionized water with a Visking tube overnight and the nondialyzed was treated with α-amylase and amyloglucosidase to digest the contaminating α-glucan. The solution was dialyzed again and lyophilized to yield 3.5 g of the lipopolysaccharide. To the solution of the lyophilized lipopolysaccharide in 70 ml of pyridine was added 210 ml of methanol, and the mixture was stirred and centrifuged at 3000 x g for 20 min. The precipitate was treated 2 more times with pyridine and methanol in the same manner. The supernatants were mainly the phosphatidyl inositol oligomannosides (PIMx). The precipitate was dissolved in the buffer and treated with the immobilized trypsin to give the purified lipopolysaccharide (MLS), yield 2.0 g (0.2% from wet cells). This yield was about 60 times those of phenol extraction and of hot water extraction. Figure 1 is a diagram of the preparation of MLS.

Properties of MLS

The purified MLS was white and wolly. The MLS was soluble in distilled water or saline solution to form a milky white solution but it was insoluble in organic solvents. The water solution of MLS did not have any precipitate on a high speed centrifugation at 20,000 x g and passed through a membrane filter of pore size of 0.45 μm. By molecular sieve HPLC, the MLS was eluted very sharply at V₀ on the chromatogram. The MLS is considered to have a hydrophobic micelle structure in water which behaves as if it were a very high-molecular weight substance. The analytical data are listed in Table I. The reason why the sugar content was low by the phenol-H₂SO₄ method seemed to be that fatty acids interfered with the color development.
Table I. Analytical Data of MLS

<table>
<thead>
<tr>
<th>Component</th>
<th>GLC wt%</th>
<th>CBB method wt%</th>
<th>Elson-Morgan method GLC</th>
<th>Chen method wt%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugars (PhOH-H2SO4, as Man)</td>
<td>89.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fatty acids (GLC)</td>
<td>64.0</td>
<td>6.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteins (CBB method)</td>
<td>1.7</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aminosugars (Elson-Morgan)</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphorus (Chen method)</td>
<td>0.1</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table II. Location of Fatty Acid Esters in MLS

<table>
<thead>
<tr>
<th>Methyl sugar</th>
<th>Molar %</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-O-Methyl-arabinose</td>
<td>1.8</td>
</tr>
<tr>
<td>2-O-Methyl-arabinose</td>
<td>0.5</td>
</tr>
<tr>
<td>Arabinose</td>
<td>26.7</td>
</tr>
<tr>
<td>6-O-Methyl-mannose</td>
<td>3.5</td>
</tr>
<tr>
<td>2-O-Methyl-mannose</td>
<td>1.1</td>
</tr>
<tr>
<td>Mannose</td>
<td>66.4</td>
</tr>
</tbody>
</table>

Constituent sugars of MLS

The MLS was hydrolyzed and analyzed as alditol acetates by GLC on Tabsorb (Fig. 2). From the data, the sugar component was arabinose and mannose (3:7). No hexosamines were detected by the Elson Morgan reaction and GLC.

Fatty acid constituents of MLS

The 1 mg of MLS was methanolized and analyzed as methyl esters by GLC. The analytical data are shown in Table I. The total fatty acid content was very much less than the value in the literature. The result shows that glycolipids and phospholipids which contained many fatty acids were removed. The main fatty acid methyl esters identified were palmitic, tuberculostearic, and stearic acids (56:34:10, w/w).

Fatty acid linkages of MLS

The 2.0 mg of MLS was treated with methyl vinyl ether, methylated, and then hydrolyzed. The relative content of monosaccharides and partially methylated monosaccharides detected by GLC as alditol acetates are shown in Table II. No peaks corresponding to dimethylated monosaccharides were observed. The finding would rule out the possibility that 2 or more fatty acids were bound to any single monosaccharide. The results suggested that fatty acids in MLS might be bound principally to the C-6 and C-2 positions of mannose and to the C-5 of arabinose, and in fewer instances to the C-2 of arabinose. Consequently, the content of monomethylated monosaccharides, that is, monosaccharides to which fatty acids were bound was estimated to be about 6.9%, being well in accord with the above-mentioned molecular percentage of fatty acids estimated by fatty acid analysis of MLS. Therefore, one out of 14 or 15 of monosaccharides was judged to be bound to a fatty acid.

Analysis of saponificate of MLS

Two mg of MLS was saponified in 10 ml of 0.1 N NaOH at 100°C for 1 hr, neutralized, and dialyzed against running water, and the nondialyzate was concentrated to a volume of...
Lipopolysaccharide from *M. tuberculosis*

300 μl. A 30 μl sample was used in the HPLC for the molecular weight estimation under the conditions described in MATERIALS AND METHODS. The molecular sieve HPLC chromatogram is presented in Fig. 3. Peaks at average molecular weights of about 13,000 and 6000 were detected. The remainder of the saponified products were put on a Bio Gel P-30 column (1.6 × 50 cm) and eluted with distilled water. The peaks were checked by the phenol–H₂SO₄ method and the peaks at average molecular weights of 13,000 and 6,000 were fractionated, dialyzed, and lyophilized to give about 0.8 mg and 0.7 mg, respectively. Table III shows the GLC data of alditol acetates obtained by methylation analysis of each of the HPLC fractions. The first peak was arabinomannan and the second, mannan with a small amount of arabinose. All alditol acetates were identified by GC-MS. The structures of both polysaccharides were considered to be almost the same as those of arabinomannan and mannan described in the literature.¹³)

**Table III. The Molecular Ratio of Methyl Sugar Components from Saponified Products of MLS**

<table>
<thead>
<tr>
<th>Methyl sugar</th>
<th>Linkage</th>
<th>Molar ratio</th>
<th>Ara-Man¹ Arabinomannan</th>
<th>Man² Manan</th>
</tr>
</thead>
<tbody>
<tr>
<td>-O-Methyl-d-arabinose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,3,5-tri-</td>
<td>(Ara)₁→</td>
<td>0.6</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>2,3-di-</td>
<td>→5 (Ara)₁→</td>
<td>8.5</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>3,5-di-</td>
<td>→2 (Ara)₁→</td>
<td>1.0</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>2-</td>
<td>→5 (Ara)₁→</td>
<td>1.0</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>-O-Methyl-d-mannose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,3,4,6-tetra-</td>
<td>(Man)₂→</td>
<td>2.0</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>3,4,6-tri-</td>
<td>→2 (Man)₂→</td>
<td>1.0</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>2,3,4-tri-</td>
<td>→6 (Man)₂→</td>
<td>1.0</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>3,4-di-</td>
<td>→6 (Man)₂→</td>
<td>2.0</td>
<td>2.0</td>
<td></td>
</tr>
</tbody>
</table>

¹ *p*, pyranoside; ² *f*, furanoside.
³ Arabinomannan.
⁴ Mannan.

![Fig. 4. The Tentative Core Structures of Lipo-arabinomannan (a) and Lipomannan (b).](image)

R = fatty acyl or H−.
the backbone polysaccharides of MLS were arabinomannan and mannan. Therefore, MLS was assumed to be a mixture of lipoarabinomannan and lipomannan, although fractionation of them has not been successful. Figure 4 shows the tentative structures of core of MLS presumed from our results.

**Biological activities of MLS**

Various biological activities of the hot water extracts from *M. tuberculosis* have been already reported, but the biological activities of lipopolysaccharides from *Mycobacteria* have not been reported. An antitumor effect of MLS on the methylcholanthrene-induced fibrosarcoma MC-1 in C57BL/6 mice was shown in Fig. 5. The tumor growth was inhibited by one shot of MLS on the 10th day after inoculation, compared with that of the control group administered saline. Hemorrhagic necrosis began to occur in the tumors of mice on the day after administration in the groups administered 75.0 mg or 50.0 mg/kg of MLS, and the tumors had completely regressed on the 23rd day after inoculation. A similar tendency was observed in the other groups and the tumors had also regressed in 5 cases of 10 mice and 2 of 10 in the 25.0 mg/kg group and 7.5 mg/kg group, respectively.

Because these antitumor effects were obtained by the intraperitoneal administration of MLS, this lipopolysaccharide was indicated to have a systemic activity. MLS produced the complete regression of tumors with hemorrhagic necrosis, and so the lipopolysaccharide may produce a tumor necrosis factor. In addition, it was found that MLS had mitogen, interferon inducing, macrophage activating, adjuvant, and polyclonal B-cell activating activities; the detailed data on these will be presented elsewhere.

**REFERENCES**

Lipopolysaccharide from *M. tuberculosis*