LIPOPOLYSACCHARIDES FROM THE BLUE-GREEN ALGA *Microcystis aeruginosa*

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A lipopolysaccharide has been isolated from an aqueous phenol extract of the cells of *Microcystis aeruginosa*. Its lipids component includes residues of glucose and glucosamine and of fatty acids (myristic, palmitic, stearic, oleic), and phosphate groups. The polysaccharide component is constructed of D-mannose residues included in a linear carbohydrate chain by α-1,2- and α-1,3-bonds and D-glucose residues included by β-1,4- and β-1,6-bonds. The presence of a small number of branchings in the carbohydrate chain is possible.

We have previously described the isolation and have given the general characteristics of lipopolysaccharides (LPSs) from a number of fresh-water blue-green alga [1, 2]. It has been shown that the LPSs isolated are distinguished by a high serological activity and a low toxicity, and their study is of considerable fundamental and applied interest. The role of the LPSs of Gram-negative bacteria in the solution of very diverse theoretical and practical questions of immunity is widely known. The blue-green alga, occupying an intermediate position between the lower plants and microorganisms, have proved to be a basically new source of LPSs. Together with LPSs they contain ordinary plant polysaccharides. Thus, from the blue-green alga *Microcystis aeruginosa* new branched glucan constructed of α-1,4- and α-1,6-bound D-glucose residues have been isolated [3]. From the same alga we [2] have isolated an LPS by the phenol–water method [4]. In the present paper we consider the results of a chemical study of this biopolymer.

The crude LPS isolated was purified from free lipids by extraction with a mixture of chloroform and methanol, and the accompanying nucleic acids and glucan were separated by two ultracentrifugations. The purified LPS, obtained with a yield of 1.6%, was homogeneous according to the results of ultracentrifugation and gel filtration. Mild acid hydrolysis showed that the LPS included polysaccharide (about 80%) and lipid (8-10%) components. Complete acid hydrolysis showed that the carbohydrate moiety of the LPS consisted mainly of D-glucose and D-mannose residues in an approximate molar ratio of 1:2.7; only a relatively small amount (0.4%) of 2-keto-3-deoxyoctonic acid (KDO), one of the most characteristic components of the LPSs of the Gram-negative bacteria was detected. No pentoses or 3,6-dideoxyhexoses were found in the LPS studied, which distinguishes it from the LPSs of Gram-negative bacteria. In actual fact, the LPS from *M. aeruginosa* consists of a lipoglucomannan. The lipid component includes glucosamine, fatty acids, and phosphate groups. In a comparison with the composition of lipid A from the LPSs of Gram-negative bacteria, a certain similarity of the lipid component of the LPS of *M. aeruginosa* to lipid A becomes obvious.

In a hydrolysate of the lipid component in addition to glucosamine, the following fatty acids were identified: myristic ($C_{14:0}$), palmitic ($C_{16:0}$), stearic ($C_{18:0}$), and oleic ($C_{18:1}$). In its fatty acid composition, the lipid component of the LPS from *M. aeruginosa* is very similar to lipid A, but it lacks the β-hydroxymyristic acid that plays an important role in the manifestation of the properties of lipid A and of the LPSs of Gram-negative bacteria. This probably explains the low toxicity of the lipid component and of the LPS from *M. aeruginosa*.

The polysaccharide component (PS) was obtained by the mild partial hydrolysis of the LPS. It was separated by chromatography on Sephadex G-75 into two fractions: high-molecu-

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lar-weight (PS-1) and low-molecular-weight (PS-2). A similar pattern is observed in the case of the LPSs of Gram-negative bacteria, where the high-molecular-weight fraction forms the O-specific polysaccharide and the low-molecular-weight fraction the polysaccharide core [5].

PS-1 and PS-2 differ in their quantitative ratio of monosaccharide residues: In the first case, the ratio of glucose to mannose is approximately 1:2, and in the second case 2.5:1. In the LPS hydrolysate there was approximately twice as much PS-1 as PS-2.

When the LPS and PS-1 were methylated by Hakomori's method [6], completely methylated compounds were obtained. In a hydrolysate of them, 2,4,6- and 3,4,6,-tri-O-methylmannose and 2,3,4- and 2,3,6,-tri-O-methylglucose were identified as the main components by gas–liquid chromatography and chromato–mass spectrometry, together with trace amounts of 4,6-di-O-methylmannose and 2,6-di-O-methylglucose, and also permethylated glucose and mannose. The results obtained indicate that the LPS consists predominately of linear carbohydrate chains in which the mannose residues are included by 1,2- and 1,3-bonds and the glucose residues by 1,4- and 1,6-bonds. The presence of a small number of branchings is not excluded. These facts were confirmed by Smith degradation: On periodate oxidation, the LPS consumed 0.495 mole per anhydro unit, which shows the presence in the carbohydrate chain of 1,3-glycosidic bonds and/or branchings. Mannose residues were detected predominantly in a hydrolysate of the polyalcohol formed, while glucose residues were preserved only to a very small degree. It follows from this that an appreciable number of mannose reisudes is included in the carbohydrate chain by 1,3-bonds and the glucose residues remaining on periodate oxidation are the points of branching of the carbohydrate chain. In addition, erythritol and glycerol were formed in the hydrolysis of the polyalcohol. The presence of erythritol shows 1,4-bound glucose residues. Glycerol is obtained in the periodate oxidation of terminal monosaccharide residues and of residues included in a carbohydrate chain by 1,2- and 1,4-bonds.

To determine the configurations of its glycosidic bonds, PS-1 was first acetylated and was then subjected to oxidation with chromium trioxide. It has been shown previously [7] that in this case only monosaccharide residues bound by β-glycosidic bonds undergo oxidation. After the oxidation of the PS-1, only the mannose residues remained intact which indicates that they were included by α-glycosidic bonds. The glucose residues, consequently, are included by β-bonds. These results were also confirmed by PMR. The presence of signals at δ = 5-5.4 ppm shows α-bonds, and signals at 4.7-4.9 ppm are due to β-bonds.

EXPERIMENTAL

The monosaccharides were chromatographed on Filtrak-3 or -15 paper in the solvent systems butanol–pyridine–water (6:4:3) and ethyl acetate–pyridine–acetic acid–water (5:5:1:3). Indication of the spots was achieved with the aid of aniline hydrogen phthalate, and alkaline solution of silver nitrate, or a 0.2% solution of ninhydrin in acetone (for amino sugars).

Gas–liquid chromatography (GLC) was performed on a Pye-Unicam 104 chromatograph (United Kingdom) with flame-ionization detector with a column (0.4 × 150 cm) filled with Gas Chrom Q (100-120 mesh) impregnated with 3% of QF-I. The monosaccharides were analyzed in the form of the acetates of the corresponding polyols [8], and corresponding aldononitriles [9], and the methyl glycosides [8] with a rise in the temperature from 125 to 220°C at the rate five deg/min; the rate of flow of carrier gas (argon) and of air was 60 ml/min. Fatty acids were analyzed in the form of the methyl esters under the same conditions. Chromato–mass spectrometry [10] was carried out on an LKB-9000 instrument (Sweden) using the same column as in GLC. Methylated sugars were analysed in the form of the peracetates of the corresponding methyl glycosides [8].

The total monosaccharide content was determined by the phenol–sulfuric acid method [11] and the amino sugar content by the Elson–Morgan method [12] after hydrolysis of the biopolymers (4 mg) with 4 N hydrochloric acid (1 ml) at 100°C for 4 h, the amount of proteins was determined by Lowry's method [13], that of nucleic acids by Spirin's method [14], and that of KDO by the method of Burtseva et al. [15]. IR spectra were taken on a UR-20 spectrophotometer and PMR spectra on a Bruker-Physik HX-90E instrument.

Treatment of the Biomass of *M. aeruginosa*. A culture of the blue-green alga *M. aeruginosa* was grown on modified Fitzgerald medium [16] containing the following components (g) in 1 liter of mains water: $NaNO_3$ — 496; Na_2SiO_3 — 0.058; K_2HPO_4 — 0.039; $MgSO_4 \cdot 7H_2O$ — 0.075; Na_2CO_3 — 0.02; $CaCl_2$ — 0.036; ethylenediaminetetraacetic acid — 0.01; citric acid —

0.01%. Growth was carried out in flask at a temperature of 25-30°C with illumination of 5000 lx. The biomass obtained was separated by centrifugation, and the residue was extracted with ethanol and with acetone and was dried in the air.

Isolation of the LPS. Using Westphal's method [4], 20 g of the biomass of *M. aeruginosa* was extracted at 45% aqueous phenol. The crude LPS so obtained was treated with a mixture of chloroform and methanol (2:1 by volume) until the accompanying lipid impurities had been completely eliminated. The residue was dissolved in water and the solution was twice centrifuged at 105,000g for 3 h. The supernatant liquid was removed, and the precipitate was dissolved in water, dialyzed, and lyophilized. This gave purified LPS with a yield of 320 mg (1.6%), containing 1.2% of nucleic acids, 4.7% of protein, and 8-10% of lipid; the total carbohydrate content was 80% and the amount of amino sugars 7%.

Complete Hydrolysis. The LPS (5 mg) was heated with 1 N sulfuric acid (1 ml) in a sealed tube at 100°C for 4-5 h. The mixture was neutralized with barium carbonate, deionized with KU-2 cation-exchange resin (H^+), and evaporated. The monosaccharides were identified with the aid of paper chromatography and GLC.

Partial Hydrolysis. The LPS (77 mg) was heated with 1% acetic acid (30 mg) in a sealed tube at 100°C for 4 h. The lipid precipitate that had deposited was separated off by centrifuging, and it was washed with water and acetone and was dried in the air. Yield 6.1 mg. The supernatant solution was evaporated to small volume and was poured into ethanol (4 volumes). The precipitate that deposited was separated off by centrifugation, dissolved in water, dialyzed, and lyophilized. This gave the total polysaccharide fraction (PS) with a yield of 45 mg. The PS obtained was chromatographed in a column (1.8 × 48 cm) containing Sephadex G-75 with elution by pyridine–acetate buffer (4 ml of acetic acid, 10 ml of pyridine, and 986 ml of water; pH 5.4). Separation was monitored by the phenol–sulfuric acid method. The fractions corresponding to peaks on the elution curve were combined, dialyzed, and lyophilized. This gave the high-molecular-weight fraction PS-1 with a yield of 25 mg and the low-molecular-weight PS-2 with a yield of 10 mg. Glucose and mannose were found in hydrolysates of both fractions, with the aid of paper chromatography and GLC.

Methylation. The methylation of the LPS (10 mg) or PS-1 (10 mg) was carried out with methyl iodide in the presence of the methylsulfinyl carbon ion by Hakomori's method [6]. The completeness of methylation was determined from the absence of hydroxyl absorption bands in the IR spectrum. The fully methylated LPS and PS-1 were hydrolyzed with 2 N hydrogen chloride in absolute methanol in a sealed tube at 100°C for 12-14 h. The methyl glycosides obtained were acetylated with acetic anhydride in pyridine and were analyzed with the aid of GLC and chromato–mass spectrometry.

Smith Degradation. The LPS (10 mg) was dissolved in 2 ml of 0.03 M solution of metaperiodate and the mixture was left at room temperature in the dark, samples being taken every 3 h for determining the consumption of periodate. The maximum consumption of periodate amounted to 0.495 mole per anhydro unit. The polyaldehyde obtained was treated with sodium tetrahydroborate (50 mg) at 20°C for 16 h. The excess of tetrahydroborate was decomposed with acetic acid, and the residue was evaporated off with methanol. This gave a polyalcohol. The polyalcohol (5 mg) was subjected to complete hydrolysis with 1 N sulfuric acid as described above. The hydrolysate was analyzed by paper chromatography and GLC in comparison with authentic samples. Glucose (8%), mannose (70%), glycerol (19%), and erythritol (3%) were identified in the hydrolysate in the percentages shown.

Chromium Trioxide Oxiation [7]. A solution of 18 mg of PS-1 in 4 ml of formamide was acetylated with a mixture of acetic anhydride (1.3 ml) and pyridine (1.8 ml) at 20°C for 20 h. The reaction mixture was poured into cold water (50 ml) and was dialyzed and lyophilized. This gave the peracetate of PS-1 in a yield of 20 mg. The compound obtained (6 mg) was dissolved in acetic acid (1 ml) and was treated with chromium trioxide (50 mg) at 50°C for 2 h. The reaction mixture was poured into water (10 ml) and extracted with chloroform. The chloroform extracts were combined, dried over anhydrous sodium sulfate, and evaporated. This gave the oxidized peracetate of PS-1 with a yield of about 5 mg. It was deacetylated with barium methanolate and was hydrolyzed with 1 N sulfuric acid. The hydrolysate was analyzed by paper chromatography and GLC.

Determination of the Fatty Acid Composition. The LPS (10 mg) or the lipid (5 mg) was hydrolyzed with 4 N HCl (1 ml) at 100°C for 7 h in a sealed tube. The mixture was diluted with water and extracted with chloroform. The chloroform extract was dried over anhydrous

sodium sulfate and evaporated. The residue was treated with methanol (1 ml) in 2 N HCl (1 ml) in a sealed tube at 100°C for 2 h. The fatty acid methyl esters obtained were analyzed by GLC.

SUMMARY

1. A lipopolysaccharide has been isolated from the blue-green alga *Microcystic aeruginosa*. It has been shown that its lipid component includes residues of glucosamine and of a number of fatty acids and phosphate groups. The polysaccharide moiety is constructed mainly of D-mannose residues included in the carbohydrate chain by α-1,2- and α-1,3-bonds and of D-glucose included by β-1,4- and β-1,6-bonds. The presence of branchings in the carbohydrate chain is possible.

2. The LPS studied differs from the LPSs of Gram-negative bacteria by the absence of residues of 3,6-dideoxyhexoses and heptoses in the carbohydrate chain and by the absence of residues of β-hydroxymyristic acid in the lipid component.

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