POLYSACCHARIDES FROM *Spirulina platensis*

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A lipoglucan and a lipopolysaccharide have been isolated from an aqueous phenol extract of the cells of *Spirulina platensis*. The carbohydrate moiety of the lipopolysaccharide consists of residues of rhamnose, glucose, 2-keto-3-deoxymanno-octanic acid, and glucosamine. A 2,3-di-O-methylpentose and a 2-O-methyl-6-de-oxyhexose have been detected as minor components, and the presence of galactose, mannose, and xylose residues in trace amounts is possible. The lipid component of the biopolymers includes residues of glucosamine and of fatty acids: myristic, palmitic, and stearic. The carbohydrate chain of the lipoglucan is constructed of 1,4-bound glucose residues. The side chains are attached to the main chain by 1,6-glycosidic bonds. The polysaccharide component of the liposaccharide is constructed mainly of rhamnose residues linked by 1,3- and 1,2-bonds and of glucose residues linked by 1,4-bonds.

The blue-green alga *Spirulina platensis* belongs to the enormous class of Hormogoneae. This class combines the most highly organized forms of blue-green algae. They are found in stagnant and slowly running waters and in hot springs. Blue-green algae are cultivated successfully under laboratory conditions both in special fermenters and in flasks. We have previously given the general characteristics of carbohydrate-containing biopolymers from *S. platensis* [1], and in the present paper we consider the results of a chemical study of these biopolymers.

The total carbohydrate-containing fraction A was isolated from *S. platensis* by extraction with 45% aqueous phenol [2]. On ultracentrifugation, the total fraction A was separated into a lipopolysaccharide (LPS), which remained in the supernatant liquid, and a lipoglucan, which deposited in the precipitate. In a hydrolysate of this, glucose was detected as the main component of the carbonhydrate chain, together with glucosamine.

The structures of the glucan and of the LPS were established by the methods of partial hydrolysis, methylation [3], and periodate oxidation [4].

The lipoglucan was subjected to acid hydrolysis with 10% acetic acid, as a result of which a lipid was isolated in a yield of 1.5%. In a hydrolysate of the lipid, the following fatty acids were detected with the aid of gas-liquid chromatography (GLC): palmitic, $C_{16:0}$; myristic, $C_{14:0}$; and stearic, $C_{18:0}$, the palmitic acid predominating in the mixture.

These facts were confirmed by chromato-mass spectrometry [5]. In contrast to Gram-negative bacteria, no β-hydroxymyristic acid was found here. Glucose and glucosamine were identified by paper chromatography (PC) in the aqueous phase after the separation of the fatty acids, the glucosamine being detected and identified both with the aid of an amino acid analyzer and by electrophoresis in pyridine-acetate buffer.

After the elimination of the lipid component, the hydrolysate of the lipoglucan was subjected to gel filtration on Sephadex G-75 and G-100, which led to the isolation of a high-molecular-weight substance with $[\alpha]_D^{20}$ + 130° (c 0.4; water) in a hydrolysate in which only glucose was detected.

The lipoglucan and the glucan obtained from it were methylated exhaustively by Hakomori's method [3], and in hydrolysates of the permethylated compounds 2,3,6-tri-O-methyl-D-glucose was identified as the main component with the aid of chromato-mass spectrometry [6] and GLC, while the 2,3,4,6-tetra- and 2,3-di-O-methyl derivatives of D-glucose were

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detected in small and approximately equal amounts. The results obtained indicate that the lipoglucan contained carbohydrate chains constructed of 1,4-linked glucose residues and side chains attached to the main chain by 1,6-bonds.

The periodate oxidation of the glucan [4] consumed 1 mole of periodate per anhydro unit. In a hydrolysate of the polyalcohol formed, glycerol and erythritol were identified. The formation of erythritol confirmed the presence of 1,4-linked glucose residues. The glycerol was obtained through the oxidation of terminal monosaccharide residues.

The investigation of the LPS from *S. platensis* showed that it contained mainly residues of rhamnose, glucose, 2-keto-3-deoxymannooctanoic acid (KDO), and glucosamine. The presence of KDO in the LPS makes it similar to the LPS of Gram-negative bacteria. According to the results of GLC and the mass spectrometry of the acetates of the polyols [7], the LPS contained small amounts of a 2,3-di-O-methylpentose and a 2-O-methyl-6-deoxyhexose in addition to the monosaccharides mentioned while the presence of trace amounts of galactose, mannose, and cylose is possible. Similar O-methylated monosaccharides have been detected previously in the LPS from the blue-green alga *Synechocystis* [8] and *Synechococcus* [9].

The lipid was separated from the polysaccharide component with the aid of mild hydrolysis. The amount of lipid was 1%. Fatty acids were identified in a hydrolysate of it with the aid of GLC: myristic, $C_{14:0}$; palmitic, $C_{16:0}$, and stearic, $C_{18:0}$, the palmitic acid predominating in the mixture.

No β-hydroxy acids were detected either in the glucan or in the LPS.

After separation of the lipid, the polysaccharide component (PS) was separated by chromatography on Sephadex into a high-molecular-weight fraction (PS-1) $[\alpha]_{Hg}^{20} - 48°$, constructed predominantly of rhamnose and glucose residues with the presence, also, of small amounts of galactose, mannose, cylose, 2-O-methyl-6-deoxyhexose, and 2,3-di-O-methylpentose residues. The main monosaccharide of the low-molecular-weight fraction was glucose (60%), with mannose, cylose, and 2-O-methyl-6-deoxyhexose residues in amounts of 6, 10, 11, and 12%, respectively.

Incubation of the LPS with α-amylase led to the formation of maltose, which may indicate the presence of 1,4-bound residues that may form part of the glucan present in the LPS.

The methylation of the LPS and of PS-1 by Hakomori's method [3] led to completely methylated compounds. In a hydrolysate of them, 2,4- and 3,4-di-O- and 4-mono-O-methylrhamnoses and 2,3-6-tri-O- and 2,3,4,6-tetra-O-methylglucose were identified by gas–liquid chromatography and chromato-mass spectrometry. The results obtained indicate that the rhamnose residues are included in the main chain by 1,3-bonds and, in addition, there is a small number of rhamnose residues included by 1,2-bonds. The presence of 4-O-methylrhamnose shows the branched nature of the carbohydrate chain. The presence of 2,3,6-tri-O-methylglucose confirmed that in the LPS, just as in the glucan, the glucose residues are 1,4-linked.

The results of methylation agree with those of Smith degradation. The periodate oxidation of PS-1 consumed 0.34 mole of periodate per anhydro unit, which shows the presence of 1,3-glycosidic bonds in the carbohydrate chain and/or branches.

In a hydrolysate of the polyalcohol formed, rhamnose was detected as the main monosaccharide (60%), the glucose residues being destroyed by the periodate. The results obtained show the existence of a 1,3-bond between rhamnose residues. The formation of erythritol in the process of Smith degradation indicates that the glucose residues were bound by 1,4-bonds.

The LPS isolated from *S. platensis* possesses a definite serological activity. Thus, the LPS activated by alkaline treatment [10] takes part in an interaction with concavalin A and gives a precipitation band. At the same time, no such interaction is observed with the phytohemagglutinin from *Phaseolus coccineus*.

The results obtained permit the conclusion that the LPS isolated from the blue-green alga *S. platensis* is similar to the LPS of Gram-negative bacteria.

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, an alkaline solution of silver nitrate, or a 0.2% solution of ninhydrin in acetone (for amino sugars).

Gas–liquid chromatography was performed on a Pye-Unicam 104 chromatograph (United Kingdom) with a flame-ionization detector in a column (0.4 × 150 cm) filled with Gas-Chrom (100-120 mesh) impregnated with 3% of QF-I.

The monosaccharides were analyzed in the forms of acetates of polyols [7], of aldononitriles [11], and of methyl glycosides [7] with a rise in the temperature from 125 to 220°C at the rate of 5 deg/min, the rates of flow of carrier gas (argon) and of air being 60 ml/min. Fatty acids were analyzed in the form of their methyl esters under the same conditions. Chromato-mass spectrometry was performed on a LKB-9000 instrument (Sweden) using the same column as in GLC. The methylated sugars were analyzed in the form of the peracetates of the corresponding methyl glycosides [7].

The total monosaccharide content was determined by the phenol/sulfuric acid method [12] and the amount of amino sugars by the Elson–Morgan method [13] after the preliminary hydrolysis of the biopolymers (4 mg) with 4 N hydrochloric acid (1 ml) at 100°C for 4 h, and the amount of proteins were determined by Lowry's method [14], that of nucleic acids by Spirin's method [15], and that of KDO by a method described previously [16]. IR spectra were recorded on a UR-20 spectrophotometer and PMR spectra on Bruker-Physik HX-90E instrument. Specific rotations were measured on a Perkin-Elmer 141 instrument.

Working Up of the Biomass of *S. platensis*. A culture of the blue-green alga *S. platensis* was grown under laboratory conditions in a medium of the following composition: $NaHCO_3$, 16.8 g; NaCl, 1.0 g; K_2SO_4, 1.0 g; $NaNO_3$, 2.6 g; K_2HPO_4, 0.5 g; $CaCl_2$, 0.04 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; and EDTA, 0.08 g, in 1 liter of settled mains water at an intensity of illumination of 5000 lux and a temperature of 30°C, and the algal cells were obtained after centrifugation of the culture mass at the end of the growth phase. The cells were fixed with ethanol and were then extracted with ethanol and acetone to obtain colorless extracts, followed by drying in the air.

Isolation of the Total Polysaccharide Fraction. The air-dry material (30 g) was extracted with 45% aqueous phenol (1200 ml) at 65°C for 30 min four times. The combined aqueous phases were dialyzed against water, concentrated in vacuum in a rotary evaporator, and precipitated with ethanol, and the precipitate was dissolved and lyophilized. The yield of combined fraction amounted to 4% of the weight of the air-dry cells. This fraction was centrifuged in an ultracentrifuge at 105,000 × g for 3 h. The resulting precipitate was separated off and dissolved in water, and the solution was dialyzed and lyophilized. This gave a lipoglucan with a yield of 0.9% (on the dry weight of the alga) containing 2.8% of nucleic acids, 5% of glucosamine, and 80% of carbohydrates. The supernatant was dialyzed against distilled water and lyophilized. This gave the lipopolysaccharide (LPS) with a yield of 3.1% (on the dry weight of the alga); it contained 53% of total carbohydrates, 6.6% of protein, 1.3% of amino sugars, 7.8% of nucleic acids, 0.6% of KDO, and 1% of lipid.

Complete Hydrolysis. The lipoglucan (3 mg) and LPS (4 mg) were heated in 1 N sulfuric acid (2 ml) in a sealed ampul 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 PC and GLC.

Partial Hydrolysis. The lipoglucan (73 mg) and the LPS (246 mg) were heated with 1% or 10% acetic acid (16 and 30 ml, respectively) at 100°C for 4 h. The lipid that deposited was separated off by centrifugation, washed with water and acetone, and dried in the air. The crude lipid so obtained was purified by extraction with chloroform–methanol (2:1, by volume). Yields were 1.46 and 2.5 mg, respectively. The supernatant solution was evaporated to small volume and was poured into ethanol (4 volumes). The precipitate that deposited was separated off by centrifugation and was dissolved in water, and the solution was dialyzed and lyophilized. This gave a degraded glucan (40 mg) or the total polysaccharide fraction (PS, 173 mg).

The degraded glucan and the PS were chromatographed on a column (1.8 × 45 cm) of Sephadex G-50 or G-100 with elution by pyridine–acetate buffer, pH 4.2. Separation was monitored by the phenol/sulfuric acid method [12]. The fraction issuing from the free volume of the column was dialyzed and freeze-dried. This gave a glucan with $[\alpha]_D^{20}$ + 130° (c 0.6; water) in a hydrolysate of which glucose was identified by PC. From the LPS was

obtained a high-molecular-weight fraction PS-1, yield 29 mg, $[\alpha]_{Hg}^{20}$ − 48°, and a low-molecular-weight fraction PS-2, yield 61 mg.

Smith Degradation. The glucan (10 mg) and the LPS (88 mg) were oxidized with a 0.06 M solution of sodium metaperiodate (34 ml) at room temperature for 48 h. The polyaldehyde so obtained was treated with sodium tetrahydroborate at 20°C for 16 h. The excess of tetrahydroborate was decomposed with acetic acid, and the residue was evaporated with methanol. The resulting polyalcohol was subjected to mild acid hydrolysis with 0.5 N hydrochloric acid (4 ml) at 20°C for 16 h. The hydrolysate was chromatographed of Sephadex G-15 (43 × 2 cm), and PS-1 was isolated.

Analysis of the Methylation Method. The glucan (6 mg), the PS-1 (6 mg) and the LPS (10 mg) were methylated with methyl iodide in the presence of the methylsulfinyl carbanion by Hakomori's method [3]. Completeness of methylation was determined from the absence of the absorption band of hydroxyl in the IR spectrum. The completely methylated compounds were hydrolyzed with 2 N hydrogen chloride in absolute methanol in a sealed ampul at 100°C for 12 h, and the methyl glycosides obtained were acetylated with acetic anhydride in pyridine and were analyzed with the aid of GLC and chromato-mass spectrometry.

Determination of the Fatty Acid Composition. The lipid (10 mg) was hydrolyzed with 4 N hydrochloric acid (2 ml) at 100°C in a sealed ampul for 7 h. The mixture was diluted with water and extracted with chloroform. The chloroform extract was dried over anhydrous sodium sulfate and was evaporated. The residue was treated with methanol in 2 N HCl (2 ml) at 100°C for 2 h. The fatty acid methyl esters so obtained were analyzed with the aid of GLC in column 1.

Determination of Serological Activity. The LPS (10 mg) was treated with 0.25 N NaOH at 56°C for 1 h. The activated LPS was deposited on a plate with 1% agar prepared in physiological solution. Samples of the phytohemagglutinin from *Phaseolus coccineus* and concanavalin A kindly provided by J. Kocourek (Czechoslovakia) were used as standards.

SUMMARY

1. A lipoglucan and a lipopolysaccharide have been isolated from the blue-green alga *S. platensis*.

2. It has been shown that the lipid component contains glucosamine and fatty acid residues.

3. The polysaccharide moiety of the LPS is constructed of L-rhamnose residues linked into a carbohydrate chain by β-1,3- and 1,2-bonds.

4. The polysaccharide moiety of the lipoglucan is branched; the carbohydrate chains are constructed of 1,4-linked D-glucose residues, and the side chains are attached by 1,6-bonds.

5. It has been shown that the LPS from the blue-green alga *S. platensis* is largely similar to the polysaccharides of Gram-negative bacteria, but differs from the latter by the absence of heptoses in the carbohydrate chain, by a low content of 2-keto-3-deoxymannooctanoic acid, and by the absence of β-hydroxymyristic acid.

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MODIFIED METHOD FOR THE STEREOSPECIFIC ANALYSIS OF TRIACYLGLYCEROLS

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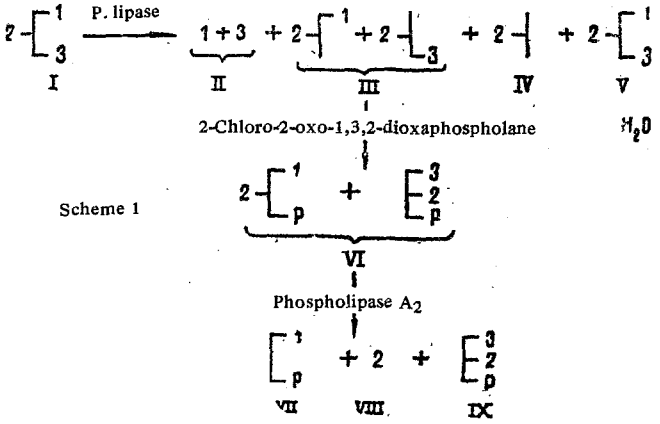
A modification of the well-known method of determining the structures of triacylglycerols that permits the time of analysis to be shortened is proposed. 2-Chloro-2-oxo-1,3,2-dioxaphospholane is used as phosphorylating agent in place of phenyl phosphorodichloridate. A description of the method is given and the results of the analysis of the triacylglycerides of cottonseed oil are presented.

The stereospecific analysis of triacylglycerols proposed by Brockerhoff [1] and consisting of three stages — the hydrolysis of the triacylglycerols with pancreatic lipase, the phosphorylation of the diacylglycerols with phenyl phosphorodichloridate, and the splitting with snake venom phospholipase A_2 of the phosphatidylphenols synthesized — permits the distribution of fatty acid acyl radicals between the sn-1, sn-2, and sn-3 positions of the triacylglycerols to be established.

Because of the length and laboriousness of the analysis (particularly the phosphorylation and phospholipiase hydrolysis stages), the method has not found wide application and is not used to determine the structures of triacylglycerols of modified fats.

To shorten the time of analysis we have proposed to use 2-chloro-2-oxo-1,3,2-dioxaphospholane as the phosphorylating agent in place of phenyl phosphorodichloridate (scheme 1).

Scheme 1



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