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Lipid and lipopolysaccharide constituents of cyanobacterium *Spirulina platensis* (Cyanophyceae, Nostocales)

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ABSTRACT: Nature and quantity of lipids and lipopolysaccharides of *Spirulina platensis* were examined. Organic cell weight consisted of 50 % protein, 8.8 % carbohydrate and 16.6 % lipids. Carotenoid-to-chlorophyll ratio was 0.3. Lipids consisted of a diverse assortment of pigments, hydrocarbons, glycolipids, and phosphatidyl glycerol. Fatty acid contents were predominantly even-numbered chains with a relatively high proportion of polyunsaturated fatty acids. Lipopolysaccharide consisted of unsaturated fatty acids, 3-hydroxy myristate and the carbohydrates, hexoses, heptose, octulosonic acid and glucosamine. Assays of the lipopolysaccharide preparation by the Limulus amoebocyte lysate test and by the lethal toxicity test in mice demonstrated that the lipopolysaccharide of *S. platensis* is toxic but its toxicity is in the order of 10 % of the lipopolysaccharide fraction of *Salmonella abortus*.

INTRODUCTION

Spirulina platensis is a filamentous cyanobacterium that grows in salty and fresh water lakes and ponds. It contains more than 50 % protein and is commercially accepted as a dietary protein source and as health food (Richmond et al. 1982, Vonshak et al. 1982, Yanagimoto & Saitoh 1982, Vonshak et al. 1983). Biologically, Cyanophytes are curious organisms since they are photosynthetic prokaryotes that contain the basic structure and chemical composition of the cell wall of Gram-negative bacteria while possessing a photosynthesis apparatus like that found in eukaryotes. They contain lipopolysaccharides (LPS) (Weise et al. 1970, Weckesser et al. 1974, Stanier & Cohen-Bazire 1977, Weckesser et al. 1979, Keleti et al. 1981) that are characteristic of most Gram-negative bacteria and well recognized as an endotoxic and antigenic component of the cell wall. The lipid composition is relatively simple, containing principally mono- and di-galactosyl diacylglycerides, phosphatidyl diacylglycerol and sulfoquinovosyl diacylglycerol (Appleby et al. 1971, Sato et al. 1979, Sato & Murata 1982). Only a few of the Cyanophytes are recognized as producers of toxins (Gentile 1971, Carmichael & Gorham 1980) that cause animal poisonings.

The present report describes the lipid and LPS properties of *Spirulina platensis.*

MATERIALS AND METHODS

Spirulina platensis Lb 1475/4a was cultivated as previously described (Vonshak et al. 1982), harvested by filtration and spray-dried. Cell suspensions were extracted for lipids by the modified method of Bligh and Dyer (1959) (Kates et al. 1964). Nonpolar and polar lipids were separated by silicic acid column-chromatography (Tornabene et al. 1969) with hexane, benzene, chloroform, acetone and methanol. LPS was extracted from dried cells by the phenol-water method (Westphal et al. 1952). LPS was isolated by isopycnic density gradient ultracentrifugation. Lipid components were deacylated by mild alkaline methanolysis (Tornabene & Ogg 1971). Sample was hydrolyzed with 2 N HCl at 100 °C for 2 h for neutral sugars; 4 N HCl for 6 h at 100 °C for amino sugars; and 0.025 N sulfuric acid for 1 h at 100 °C for ketodeoxyoctulosonic acid (KDO). Lipids were analysed by thin-layer chromatography in diethyl ether-benzene-ethanol-acetic acid (45:50:2:0.2) as first solvent and hexane-diethyl ether (96:4) as second solvent or chloroform-acetone-me-

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thanol-acetic acid-water (50:20:10:10:5). Lipids were detected by exposure to I_2 vapor, acid charring, phosphate spray (Vaskovsky & Svetashev 1972), ninhydrin, α-napthol for glycolipids (Kates 1971), Dragendorff stain for quartenary amines (Kates 1971), or H_2SO_4-acetic acid (1:1) for sterols (Kates 1971). The deacylated water-soluble products were separated on cellulose-TLC plates (Short et al. 1969). Samples were made visible by the o-tolidine staining method overstained with an acidic ammonium molybdate solution (Kates 1971), or by ninhydrin. Total carbohydrates were determined by the phenol-sulfuric acid method (Kochert 1973b). Nucleic acids were estimated spectrophotometrically after hydrolysis with 0.1 N NaOH. Total protein was determined after hydrolysis in NaOH at 100 °C at 1 h (Kochert 1973a). Samples were assayed for 3-deoxy sugars (Cynkin & Ashwell 1960), KDO (Warren 1959), glucosamine (Boas 1953), heptose (Wright & Rebers 1972), and phosphorus (Bartlett 1959). Total fatty acid of LPS were determined gravimetrically (Raziuddin 1976). Chlorophyll and carotenoids were assayed as described by Jensen (1973). Fatty acids were converted to the methyl ester form in 2.5 % anhydrous methanolic-HCl (Kates 1971). Aliquots of fatty acids were hydrogenated with H_2 and 10 % Pd on activated charcoal. Free sugars were converted to alditol acetates (Albersheim et al. 1967). Derivatized components were analysed on a Varian 3700 gas-liquid chromatograph (GLC) equipped with dual flame ionization detectors and a Varian Vista 401 data systems. The following columns were used: 30 × 0.252 mm fused quartz capillary column, with 0.25 μM of OV 351 or 0.25 μM of DB-5 at 8 psi of He and 4 °C min^{-1} from 125 to 220 °C and held isothermally; 2 m × 0.31 cm glass column packed with 10 % SP2330 on 100/120 Gas Chrom W AW at 27 psi of He and 4 °C min^{-1} from 100 to 250 °C and held isothermally.

The *Limulus* amoebocyte lysate test was performed by mixing 0.1 ml of the lysate and 0.1 ml of sample and incubating at 37 °C. The mixtures were examined after 1 h and 24 h of incubation. A negative control of pyrogen-free saline and positive control of *Spirulina abortus equi* LPS were used. Mouse lethality tests of LPS were conducted using C57BL/6 male mice, 6 to 7 wk of age. Mice were injected intraperitoneally, and 6 mice were used for each concentration; deaths were recorded after 48 h.

RESULTS

Spray-dried *Spirulina platensis* contained 16.6 % lipid, 8.8 % carbohydrate and 50 % protein. In agreement with previous observations (Yanagimoto & Saitoh 1982), the carotenoid-to-chlorophyll ratio was about 0.3.

Most of the total lipids fractionated on a silicic acid column eluted with acetone and methanol. Combined hexane, benzene and chloroform eluates comprised only 5 % of the total lipids. The hexane fraction comprised only 0.2 % of the total lipids. Three components were identified in the hexane fraction by GLC; they were pentadecane (5.5 %), hexadecane (4.3 %), and heptadecane (90 %) as previously reported (Tornabene 1980). No unsaturated and branched hydrocarbons were detected. The benzene eluate representing 3.4 % of total lipids consisted of 5 components when analysed by TLC. Four of the components were yellow-colored pigments; the fourth component cochromatographed with a sterol ester standard. The component stained red with H_2SO_4 and acetic acid indicating a sterol component; however, no sterol was recovered after methanolic-HCl hydrolysis and analysis by the chromatographic procedures. The components were not identified. The chloroform eluate comprised only 1.4 % of the total lipids and appeared to consist of 4 components, 2 of which are yellow pigmented. Components in relatively low concentrations cochromatographed with a triglyceride, a free fatty acid, and a hydrocarbon standard.

The acetone and methanol eluates contained all of the polar lipids which represented 95 % of total lipids. In addition to the assortment of green, yellow, orange, and rose colored pigments fractionated by TLC, several components cochromatographed with authentic standards. On the basis of the R_f values, and staining behavior, they were tentatively identified as monogalactosyl diacylglyceride, phosphatidyl diacylglyceride, digalactosyl diacylglyceride and sulfoquinovosyl diacylglyceride. A major phosphate containing component and a glycolipid were also detected by differential staining procedures on TLC. The components were identified as phosphatidyl monoacylglycerol and digalactosyl monoacylglycerol after mild alkaline hydrolysis, cellulose-TLC and GLC.

The fatty acid composition is summarized in Table 1. Fatty acids are principally even-numbered carbon chains with a relatively large proportion of them being unsaturated. The existence of the polyunsaturated chains (Table 1) was substantiated by GLC of the hydrogenated components.

The LPS of *Spirulina platensis* comprised 1.6 % of the cellular dry weight. The LPS fraction was soluble in water and 1 % Triton X-100 but not in acetone, ethanol or pyridine. The chemical composition of the LPS is given in Table 2. It was free of nucleic acids but contained traces of proteins (0.6 %). The total carbohydrate (31.6 %) and fatty acids (14.3 %) represented 46 % of the LPS composition. Sugar analysis revealed

Table 1. *Spirulina platensis*. Relative retention and percentage composition data of fatty acids

Fatty acid	Ret. time (min)	Relative percent: Total lipids unhydro-genated	Total lipids hydro-genated	LPS unhydro-genated	LPS hydro-genated
14:0	19.0	0.8	1.6	–	–
14:1	19.6	0.6	–	–	–
16:0	24.1	26.4	38.9	9.5	19.8
16:1	24.8	4.6	–	10.4	–
16:2	26.5	1.0	–	–	–
16:3	29.2	2.1	–	–	–
17:0	26.9	0.7	0.7	3.1	3.0
18:0	30.0	0.4	55.5	1.5	64.1
18:1	30.9	22.8	–	1.7	–
18:2	32.6	9.8	–	5.0	–
3-OH-14:0	33.1	–	–	9.6	9.4
18:3	35.3	21.1	–	55.3	–
19:0	34.0	3.5	0.7	–	–
3-OH-16:0	45.3	–	–	2.1	2.0
Unidentified	(16–46)	6.2	–	1.8	–

Components analyzed by GLC on 0.25 µM of OV351. Retention times and percentages were determined with a Vista 401 data system. Identities were determined by comparison to authentic standards. Retention time of 2-OH-C_{14} was 32 min

Table 2. *Spirulina platensis*. Chemical composition of lipopolysaccharides

Components	Lipopolysaccharides % of the total
Glycerol[a]	7.4
Glucose[a]	7.5
Rhamnose[a]	17.1
Fucose[a]	3.3
Ribose[a]	8.1
Xylose[a]	4.5
3- or 4-0-Methyl hexose[a]	8.1
Mannose[a]	1.9
Galactose[a]	8.2
Inositol[a]	6.0
D-Glycero-D-mannoheptose[a]	1.6
D-Glycero-L-mannoheptose[a]	3.7
Unidentified[a]	22.6
Heptose[b]	1.4
3-Deoxy-D-manno-octulosonic acid[b]	1.2
D-Glucosamine[b]	2.1
Total carbohydrates[b]	31.6
Total fatty acids[b]	14.3
Phosphate[b]	0.6
Nucleic acids[b]	0
Protein[b]	0.6

[a] Determined by GLC on a 2 m × 0.31 cm glass column containing 10 % SP2330 on 100/120 Gas Chrom W AW. The unidentified represents the sum of 7 components
[b] Determined colorimetrically

the presence of 3-deoxy-D-manno-octulosonic acid, glucose, rhamnose, fucose, ribose, xylose, mannose, galactose, inositol, D-glycerol-D-mannoheptose, D-glycero-L-mannoheptose and 3- or 4-0 methyl hexose. Glycerol was also identified in the hydrolysate. Seven unidentified minor components were detected by GLC in the area where deoxyhexoses and pentoses elute. Glucosamine was the only amino sugar identified. The principal acids of the LPS were 16:0, 16:1 and 3-OH-14:0 (Table 1). Small amounts of 3-OH-C_{16} were also detected.

The results from the test of the *Limulus* lysate activities of the LPS of *Spirulina platensis* and *Salmonella abortus equi* are given in Table 3. Gelation of the *Limulus* lysate assay with LPS of *S. platensis* and *S. abortus equi* occurred with a concentration of 10^{-9} µg ml^{-1} and 10^{-12} µg ml^{-1}, respectively. These results demonstrated that the LPS of *S. platensis* was toxic. The toxic properties of LPS preparations were also measured by intraperitonal injections into mice. The lethal dose of LPS from *S. platensis* was 8.5 mg $animal^{-1}$ as compared to 0.8 mg $animal^{-1}$ for LPS of *S. abortus equi*. Similar toxicity has been observed in LPS preparations isolated from different commercial sources of *S. platensis*.

DISCUSSION

The lipid analysis of *Spirulina platensis* showed a relatively simple composition and one that has been previously described for blue-green bacteria (Appleby et al. 1971, Weckesser et al. 1974, Sato et al. 1979). One exception, however, is the occurrence of the lyso-forms of digalactosyl diacylglycerol and phosphatidyl diacylglycerol. The relatively large portion of C_{18} polyun-

Table 3. *Limulus* amoebocyte lysate activity of the Lipopolysaccharides of *Spirulina platensis* and *Salmonella abortus equi*[a]

Lipopolysaccharides	μg ml^{-1}							
	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}	10^{-13}
S. platensis	+ + +[c]	+ + +	+ +	±	–			
S. abortus equi[b]	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	±	–

[a] Activity of different concentrations of LPS, determined by incubating 0.1 ml of the test sample with an equal volume of *Limulus* amoebocyte lysate at 37 °C in a stoppered 10 × 75 mm glass test tube
[b] Lipopolysaccharide standard obtained from Sigma
[c] + + + Solid gel formed in less that 60 min; + + solid gel formed after 60 min; ± viscous or granular gel formed after 60 min; – no gel formed after 24 h

saturated fatty acid has been previously recognized in Cyanophytes and well reviewed (Stanier & Cohen-Bazire 1977), and their existence in Cyanophytes remains an exception to the general prokaryotic rule that polyunsaturated fatty acids do not occur in bacteria.

The fatty acid content of LPS was different from those in the free lipid fraction by the presence of OH-C14 and OH-C16 acids. These findings are similar to previous reports on LPS preparations from other Cyanobacteria (Weckesser et al. 1979). The sugar composition of the LPS preparation was fairly typical of many Gram-negative bacteria.

The toxicity of the LPS preparation was very significant but lower relative to the toxicity of LPS of *Salmonella abortus equi*. Toxicity in Cyanophytes has been shown to be dependent of many different environmental and physiological factors such as light, temperature, pH, nutrients, mixing, etc. (Carmichael & Gorham 1980). At this early stage it is not clear whether the LPS toxicity of *S. platensis* is environment dependent, but the presence of toxicity in various different commercial preparations of *S. platensis* points to a basic inherent level of toxicity in *S. platensis*.

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