Isolation and Chemical Characterization of the Sheath from the Cyanobacterium *Chroococcus minutus* SAG B.41.79

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(Received 3 February 1986; revised 28 April 1986)

The sheath of the unicellular cyanobacterium *Chroococcus minutus* SAG B.41.79 was isolated from a crude cell envelope fraction by discontinuous sucrose gradient centrifugation, and was further purified by treatment with lysozyme followed by Triton X-100 or sodium dodecyl sulphate (SDS) extraction. The absence of muramic and diaminopimelic acids and of β -hydroxy fatty acid showed the fraction to be free from cell wall components. The sheath had a fibrillar fine structure with the fibres parallel to the cell surface. The total neutral sugar content was 45.9% (w/w). The main sugars were glucose and 2-O-methyl-6-deoxyhexose. Additional O-methyl sugars, 2-O-methylhexose, 3-O-methylhexose and a 2-O-methyl sugar (not further identified), were present. Protein could not be completely removed from the sheath fraction by treatment with boiling SDS. The contents of fatty acids, phosphorus, uronic acids and glucosamine in the fraction were all less than 0.5% (w/w).

INTRODUCTION

Many cyanobacteria bear one or more additional layers of considerable thickness external to their outer membrane. These layers have been variously named as cell wall layers, sheath, capsule or slime (Stanier & Cohen-Bazire, 1977). Unicellular cyanobacteria such as *Gloeobacter*, *Gloeotheca* or *Gloeocapsa* (synonym *Chroococcus*) show light- and electron-microscopically visible sheath layers which enclose both individual cells and cell groups (Rippka *et al.*, 1979). Nothing is known about the chemical composition, physical properties or function of the sheath of unicellular cyanobacteria. Schrader *et al.* (1982*a*) have shown that the isolated sheath of *Chlorogloeopsis* PCC 6912, which has a rather complex developmental cycle, is composed of mainly neutral sugars and protein but lacks fatty acids. The present work describes the isolation and first chemical characterization of the sheath of the unicellular *Chroococcus minutus* SAG B.41.79.

METHODS

Organism and growth conditions. *Chroococcus minutus* (Kützing) Nägeli strain SAG B.41.79 was obtained from the Sammlung von Algenkulturen, Göttingen, FRG. Cells were grown photoautotrophically in BG-11 medium, pH 7.5 (Rippka *et al.*, 1979) at 25 °C, irradiated continuously with white light from a fluorescent lamp (1–4 klx). Mass cultures were prepared in a 12 l fermenter (Jungkeit, Göttingen, FRG) gassed continuously by a stream of air/CO₂ (99 : 1, v/v, at 250 l h⁻¹). The generation time was 38 h. Cells were harvested after 18–20 d growth, washed once with 20 mM-Tris/HCl buffer (pH 8.0) and stored at –20 °C until use. This buffer was used also throughout the sheath isolation procedure.

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Isolation of the sheath. Cells were suspended in buffer (as above), mixed with 0.25 mm diameter glass beads (cell to glass bead ratio 1 : 2, v/v) and DNAase (approx. 2 mg) and were broken at 4 °C in a Vibrogen shaker (Type V12, Edmund Bühler, Tübingen, FRG) at maximum speed for 2 h. After removal of the glass beads by filtration, crude sheath fractions were separated by centrifugation at 750 g, 4 °C, for 30 min. The supernatant was then centrifuged at 12 100 g, 4 °C, for 45 min, and the sediment was washed four times (same conditions). Discontinuous sucrose gradients (10 ml of 60 %, and 5 ml each of 55, 50, 45, and 40 %, w/w, sucrose in Tris/HCl buffer) were loaded with the sediment of the last centrifugation and run in a swing-out rotor model A4.13 (Hermle, Gosheim, FRG) at 16 300 g, 4 °C, for 4 h. Sheaths were recovered from the 60 % sucrose band and further purified once on the same type of gradient. The sheath fraction was finally washed four times at 12 100 g, 4 °C, for 45 min, in Tris/HCl buffer as above.

To remove cell wall contaminants, the sheath fractions were treated with hen eggwhite lysozyme (EC 3.2.1.17) (5 mg in 25 ml ammonium acetate buffer, pH 6.8, 37 °C, 12 h) followed by extraction with Triton X-100 (2 %, w/v, in 0.01 M-Na₂EDTA and 0.01 M-MgCl₂, room temperature, 20 min) or sodium dodecyl sulphate (SDS) (4 %, w/v, in Tris/HCl buffer, pH 7.8, 100 °C, 15 min). The purified sheath fractions were finally washed seven times with distilled water (12 000 g, 4 °C, for 45 min), dialysed, collected by centrifugation (12 000 g, 4 °C, for 45 min) and lyophilized.

Electron microscopy. Whole cells (freshly harvested) or isolated sheath fractions were fixed in 1 % (w/v) osmium tetroxide, dehydrated in alcohol and polymerized in Epon according to standard procedures (Kellenberger *et al.*, 1958; Golecki, 1977). Ultra-thin sections were mounted on Formvar-coated copper grids and stained with uranyl acetate (Watson, 1958) followed by lead citrate (Reynolds, 1963). Specimens were examined in a Philips EM 400 microscope at 80 kV.

Chemical analyses. Hydrolysis of carbohydrates and polypeptides and the determination of monomers have been described elsewhere (Schrader *et al.*, 1982b). The neutral sugars were separated by thin-layer chromatography (solvent: ethyl acetate/pyridine/water, 12:5:4, by vol.) and by gas-liquid chromatography as alditol acetate derivatives (Varian Aerograph, model 1400-1, ECNSS-M 2 mm × 1.5 m glass column, 3 % on Gas-Chrom Q, 100–200 mesh). Mass spectrometric analysis of sugar alditol acetates (reduced with NaB²H₄; Schmidt *et al.*, 1980) was done in a Finnigan MAT GC/MS automatic system, model 120. Alditol acetates of hydrolysates (0.1 M-HCl, 100 °C, 48 h) were separated on a fused silica capillary column SE-54 (25 m, i.d. 0.25 mm). Column temperature was from 120 °C to 230 °C in a 5 °C min⁻¹ programme; the injection temperature was 270 °C. Mass spectra were taken at 70 eV in the mass range 43–400 *m/e* in 1 s. Fatty acids were estimated as methyl esters by gas-liquid chromatography (EGSS-X column, 15 % on Gas-Chrom P, 100–200 mesh; or Castorwax column, 2.5 % on Chromosorb-G, 80–100 mesh). For detection of uronic acids (liberation in 0.5 M-H₂SO₄; 100 °C, 4 h) and amino sugars (liberation in 4 M-HCl, 110 °C, 16 h) high-voltage electrophoresis was performed (Kickhöfen & Warth, 1968). Paper electropherograms were stained with alkaline silver nitrate (Trevelyan *et al.*, 1950). Quantitative determination of amino sugars and amino acids was done in an automatic amino acid analyser (Durrum, model D-500). Organic phosphorus was determined according to Lowry *et al.* (1954).

RESULTS

Isolation of sheath

Various cell homogenization methods such as Ultra-turrax, ultrasonication, French press or Vibrogen shaker were applied for breaking *C. minutus* SAG B.41.79 cells. The best results were obtained using the Vibrogen shaker followed by differential centrifugation of the cell homogenate. A thick gelatinous sheath fraction, together with some whole cells and cell fragments, was obtained in the sediment after centrifugation at 3020 g for 30 min. This was further purified by centrifugation on a discontinuous sucrose gradient (see Methods). The sheath fraction was obtained from the gradient with a yield of about 28 % on a cell dry weight basis and it appeared to be almost free from unbroken cells and cell debris under the light microscope (Fig. 1). Residual contaminating cell wall components were removed by treatment with lysozyme (Fuhs, 1985; Jensen & Sicko, 1971) and detergents. Both the outer and cytoplasmic membranes from cyanobacteria can be solubilized with hot SDS (Golecki & Drews, 1974; Golecki, 1977). Triton X-100 removes the cytoplasmic membrane in Gram-negative bacteria (Schnaitman, 1971). By applying these methods, the sheath fraction of *C. minutus* was obtained free of diaminopimelic and muramic acids, indicating the absence of cell wall. The lack of pigments indicated the fraction to be free of membranes. The yields were 17.7 % (lysozyme + Triton X-100) and 12.4 % (lysozyme + SDS) on a cell dry weight basis.

Fig. 1

Fig. 2

Fig. 1. Light micrograph of isolated sheath fraction of *C. minutus* (gradient purified, see text). Bar, 2.5 μ m.

Fig. 2. Ultrathin sections. (a) Part of a whole cell. Note the osmiophilic, fibrillar fine structure of the sheath, the fibres lying parallel to the cell surface. Bar, 200 nm. (b) Isolated sheath fraction purified by gradient centrifugation followed by lysozyme treatment and SDS extraction (see text). Bar, 1 μ m.

Table 1. *Chemical analysis of sheath fractions of C. minutus SAG B.41.79*

Constituent	Content (% of fraction dry wt) in sheath fraction:		
	From sucrose gradient	After lysozyme + Triton X-100 treatment*	After lysozyme + SDS treatment†
Unknown anhydro sugar	1.7	2.6	3.9
2-O-Methyl-6-deoxyhexose	6.2	9.7	11.9
2-O-Methyl sugar (not further identified)	trace	1.0	1.4
2-O-Methylhexose	3.3	5.2	6.7
3-O-Methylhexose	3.6	4.3	7.0
Glucose	7.8	10.8	15.0
Total amino acids	12.6	ND	5.8

ND, Not determined.

*Lysozyme: 5 mg in 25 ml ammonium acetate buffer pH 6.8; 37 °C, 12 h. Triton X-100: 2% (w/v) in 0.01 M-Na₂EDTA and 0.01 M-MgCl₂; room temperature, 20 min.

† Lysozyme: as above. SDS: 4% (w/v) in Tris/HCl, pH 7.8; 100 °C, 15 min.

Fine structure of the sheath

Ultrathin sections of cells of *C. minutus* revealed the sheath as an osmiophilic fibrous material with an orientation parallel to the cell surface (Fig. 2 a). The isolated sheath showed a similar fine structure stainable with osmium tetroxide in ultrathin sections (Fig. 2 b).

Chemical analyses

Neutral sugars were the major components of the sheath fraction (Table 1); their content increased from 22.6 % (gradient-purified) to 33.6 % (lysozyme + Triton X-100) and 45.9 % (lysozyme + SDS). All the fractions contained glucose and the four O-methyl sugars,

Table 2. Retention times and mass-spectrometric fragmentation pattern of *O*-methyl sugars (as alditol acetates) of the sheath of *C. minutus* SAG B.41.79

<i>O</i> -Methyl sugar	Retention time (relative to xylose)*	Primary fragments/ secondary fragments
Unknown anhydro sugar	0.66	74; other main fragments: 158, 129, 113, 87, 81, 69†
2- <i>O</i> -Methyl-6-deoxyhexose	0.88	118, 275/215, 201, 173, 129, 99
2- <i>O</i> -Methyl sugar (not further characterized)	1.09	245‡, 118/203, 171
2- <i>O</i> -Methylhexose	1.19	333, 118/273, 259, 171, 157, 139
3- <i>O</i> -Methylhexose	1.22	261, 190/201, 159, 141, 130

* Silica capillary column SE-54 (25 m; i.d. 0.25 mm; 120–230 °C, 5 °C min⁻¹).

† No distinction between primary and secondary fragments.

‡ Fragment 245 indicates the presence of two –CH₂ groups.

2-*O*-methyl-6-deoxyhexose, a 2-*O*-methyl sugar (not further identified), a 2-*O*-methylhexose and a 3-*O*-methylhexose, detected by gas-liquid chromatography. The *O*-methyl sugars were identified by their fragmentation patterns on mass spectrometric analysis after separation of their alditol acetate derivatives (Table 2; for a comparison see Jansson *et al.*, 1976). Uronic acids, glucosamine, fatty acids and phosphorus were detected only in trace amounts (each below 0.5 %) in the sheath fractions. The amino acid content was reduced from 12.6 % (gradient purified) to 5.8 % after lysozyme + SDS treatment (Table 1). A high proportion of aspartic acid, glutamic acid and leucine was observed in the sheath fractions (not shown).

DISCUSSION

The sheath of *C. minutus* SAG B.41.79 had a high density, which facilitated its separation from the cell homogenate. A similar property was observed with the sheath of *Chlorogloeopsis* PCC 6912, which on sucrose gradient centrifugation appeared to be even heavier (Schrader *et al.*, 1982*a*). The sheath of *C. minutus* showed a fibrillar fine structure similar to that of *Chlorogloeopsis* PCC 6912 (Schrader *et al.*, 1982*a*), *Gloeocapsa* NS4 (Cox *et al.*, 1981), *Gloeotheca* PCC 73107 (Vaara, 1982) and *Chroococcus* S24, N41 (Potts *et al.*, 1983), with the fibres lying parallel to the outer surface of the cell.

The isolated sheath fraction of *C. minutus* SAG B.41.79 mainly consisted of carbohydrates. The polysaccharides were not solubilized even after extraction with Triton X-100 or boiling SDS. Polysaccharides are common constituents of cyanobacterial cell envelopes (Dunn & Wolk, 1970; Drews & Weckesser, 1982; Schrader *et al.*, 1982*a, b*). Electron microscopic observations also suggested that the sheath of the coccoid unicellular ensheathed cyanobacterium *Gloeocapsa* NS4 (Cox *et al.*, 1981) was composed of acidic mucopolysaccharides, staining being observed only with uranyl acetate. Glucose was also reported as the main sugar in the polysaccharide moiety of the sheath of *Anabaena cylindrica* (Dunn & Wolk, 1970) and *Chlorogloeopsis* PCC 6912 (Schrader *et al.*, 1982*a*). However, galactose, mannose, xylose and arabinose, found in the sheath fractions of the latter species, were not detected in the sheath of *C. minutus* SAG B.41.79, which contained a number of *O*-methyl sugars, (2-*O*-methylhexose, 3-*O*-methylhexose, 2-*O*-methyl-6-deoxyhexose and an unknown 2-*O*-methyl sugar). The relative ratios of the sugars changed little even when drastic methods were used for purification of the sheath (Table 1). *O*-methyl sugars are characteristic constituents of *O*-antigens of a number of phototrophic bacteria and cyanobacteria (Weckesser *et al.*, 1979) but they were not found in the isolated sheath of *Chlorogloeopsis* PCC 6912 (Schrader *et al.*, 1982*a*) or *A. cylindrica* (Dunn & Wolk, 1970). Recently the isolated sheaths of two other coccoid unicellular cyanobacteria, *Gloeotheca* PCC 6909 and *Gloeotheca* PCC 6501, were found to contain a 2-*O*-methylpentose and 2-*O*-methylxylose, respectively (S. P. Adhikary & C. Broll, unpublished).

The sheath of *C. minutus* SAG B.41.79 contained negligible amounts of fatty acids, as found for sheath fractions of *A. cylindrica* (Dunn & Wolk, 1970) and *Chlorogloeopsis* PCC 6912 (Schrader *et al.*, 1982 *a*). These results give no indication of the presence of glycolipids in cyanobacterial sheaths. All the sheath fractions obtained from *C. minutus* SAG B.41.79 contained considerable amounts of protein(s), which could not be completely solubilized by extraction with hot SDS. This, together with the finding of protein(s) in sheath fractions of *A. cylindrica* (Dunn & Wolk, 1970) and *Chlorogloeopsis* PCC 6912 (Schrader *et al.*, 1982 *a*) indicates that protein(s) may be constituent(s) of the sheath of cyanobacteria; proof has not yet been obtained, however.

The authors thank R. Warth for amino acid and amino sugar analyses and H. Falk for light micrographs. The work was supported by the Deutsche Forschungsgemeinschaft.

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