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ISOLATION AND CHARACTERIZATION OF THE SHEATH FROM THE CYANOBACTERIUM (BLUE-GREEN ALGA) *GLOEOTHECE* PCC 6909.[1]

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ABSTRACT

The sheath of the unicellular ensheathed cyanobacterium *Gloeothece* strain PCC 6909, was isolated from a crude cell envelope fraction by means of a discontinuous sucrose gradient (60-40% sucrose, 3020 × g, 4 h). It had a fibrillar fine structure, a high density and is composed predominantly of carbohydrates (17% w/w) and amino compounds (20% w/w). Rhamnose, xylose, mannose, glucose, galactose, 2-0-Methyl pentose and an unknown sugar were the major carbohydrates. A characteristic high proportion of arginine, glutamic acid, alanine and leucine was observed. Uronic acids were entirely absent. Glucosamine was present in trace amounts. Lipids and phosphorous content amounts to 1.7% (w/w) and 0.45% (w/w) respectively.

The protein was not solubilized by treatment with Triton X-100 (2%, w/v; 20°C; 1 h) or sodium dodecyl sulfate (4 % w/v; 100°C, W15 min). Absence of muramic acid diaminopimelic acid and B hydroxy fatty acids in the sheath fraction showed that it was free from the typical components of gram-negative cell wall of cyanobacteria.

INTRODUCTION

The cyanobacterial cell wall structure is similar to that of gram-negative bacteria, only difference is the cyanobacterial peptidoglycan in many strains is much thicker than in gram-negative bacteria. Cyanobacteria, too, have an outer membrane consisting of proteins (Golekei, 1977), phospho-lipids (Schrader *et al.*, 1981) and lipopolysaccharides (Weckesser *et al.*, 1979; Schmidt *et al.*, 1980; Schrader *et al.*, 1981). Fruther like gram-negative bacteria, many cyanobacteria bear outside their outer membrane additional surface structures. These structures, however, are virtually uncharacterized and are in fact of terminological confusion (Stanier and Cohen-Bazire, 1977). These layers have been variously named: Cell wall layers, sheath, capsule and slime. Recently Drews and Weckesser (1982) named the structured external layers as sheath and

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the undefined, unstructured zones as slimes. These terms are independent whether one or more cells are surrounded by the external layers.

Some unicellular cyanobacteria such as *Glocobacter, Gloeothece,* and *Gloeocapsa* show external layers when collected from any type of habitat and in laboratory cultures. These layers are deposited initially adjacent to the outer membrane of a single cell. In *Gloeothece* the enclosed cells synthesize new layers. Thus aggregates of cells are formed in which singles, doubles and multiples of cells are wrapped by envelope layers. Since the outer most surface structures are exposed to the environment, they play an important role in the physiology and ecology of these organisms. The step for the exact characterization of these structures has not yet been taken in a majority of cyanobacteria. For the first time an attempt is made in the present work to isolate, prify and characterize the sheath materials of an ensheathed unicellular cyanoabacterium Gloeothece PCC 6909.

MATERIAL AND METHODS

Cultivation of Cyanobacteria :

Gloeothece PCC 6909 (from Pasteur Culture Collection, Paris, France) was used as the experimental material. Cells were grown photoautotrophically at 25°C in BG 11 medium (pH, 7.5) irradiated continuously with white fluorescent lamp (2000 lux). Mass cultures were prepared in a 12 liter fermenter (Jungkeit, Göttingen, W. Germany) and were gassed continuously by a steam of air (250 liters/h) and CO_2 (2.5 liters/h). The generation time of the alga was 40 h. Cells were harvested always between 18-20 days of growth, washed once with 20mM Tris-hydrochloride buffer (pH, 8.0) and stored at -20°C until use (this buffer was used throughghout the isolation procedures).

Isolation of sheath materials :

A number of procedures were tried viz; treatment with ultraturrax, ultrasonication and French Press but none of these procedures were successful to separate the shath from the cells of *Gloeothece* PCC 6909. However, the isolation was successful when a vibrogen shaker was used (E. Bühler, Tubingen, Germany). Cells were suspended in buffer at 4°C, mixed with glass beads (0.25 mm in diameter); cell to glass bead ratio 1: 2 (v/v), and shaken at full speed for 2 hours. After removal of glass beads by filteration, crude cell envelopes were separated by differential centrifugation and wahsed 10 times with Tris buffer. Discontinuous sucrose gradient (10 ml of 60% and 5 ml of 55, 50, 45 and 40% sucrose in Tris buffer) were loaded with 10 ml of crude cell envelope suspension and run in an SW 25.2 rotor (Beakman Instruments, Fullerton, California) at 5,000 rpm for 4 hours. Sheaths were recovered from the band at 60% sucrose and were further purified by using the same gradient (3 times) (Fig. 1). No band was observed at the 60% sucrose when the crude cell envelope fraction of *Gloeothece* PCC 6909 mutant (obtained from Pasteur Culture Collection, Paris, France, without the sheath around the cells), prepared by similar method as described and subjected to discontinuous sucrose gradient (Fig.-1). This proves the density of the sheath materials present in the cell envelope of the parent strain. Sucrose was removed by washing the sheath materials with ice-cold tris buffer at least 5 times. Light mic-

roscopy showed that the sheath materials were nearly free of cell materials (Fig. -2).

Purification of isolated sheath materials:

For the purification of the sheath materials from cell wall contaminants (outer membrane, peptidoglycan and cytoplasmic membrane), the isolated sheaths were treated with lysozyme (5 mg in 25 ml of ammonium acetate buffer, pH 6.8, 37°C, 12 h) followed by treatment with detergents 1. Triton X-100 (2% in 0.01 M Na_4 EDTA and 0.01 M $MgCl_2$, 20 min) in room temperature and 2. Sodium dodecyl sulfate (SDS) (4% in tris-HCl buffer, pH 7.8, 15 min, 100°C). The sheath materials after the detergent treatment washed 7 times in distilled water, dialysed for a week, collected by centrifugation and lyophilized. The peptidoglycan layer is resistant to treatment with hot SDS (4%, 100°C) where as both the outer membrane and cytoplasmic membrane are solubilized by this detergent. By contrast, the peptidoglycan layer is completly removed or partially removed by lysozyme treatment. Applying this principle, the sheath materials were obtained free from the cell wall materials.

Electronmicroscopy :

The whole cells and the sheath fractions were fixed in 1% Osmium tetraoxide for 6 hours and embeded in Epon. Thin sections were mounted on formvar coated copper grids and stained with uranyl acetate followed by lead citrate. The electronmicroscopical preparations of isolated sheath materials and whole cells were carried out by negative staining with phosphotungstic acid adjusted to pH 7.2. Thes pecimens were examined in a Philip EM 400 and a Siemens Elmiskop IA at 80 K V.

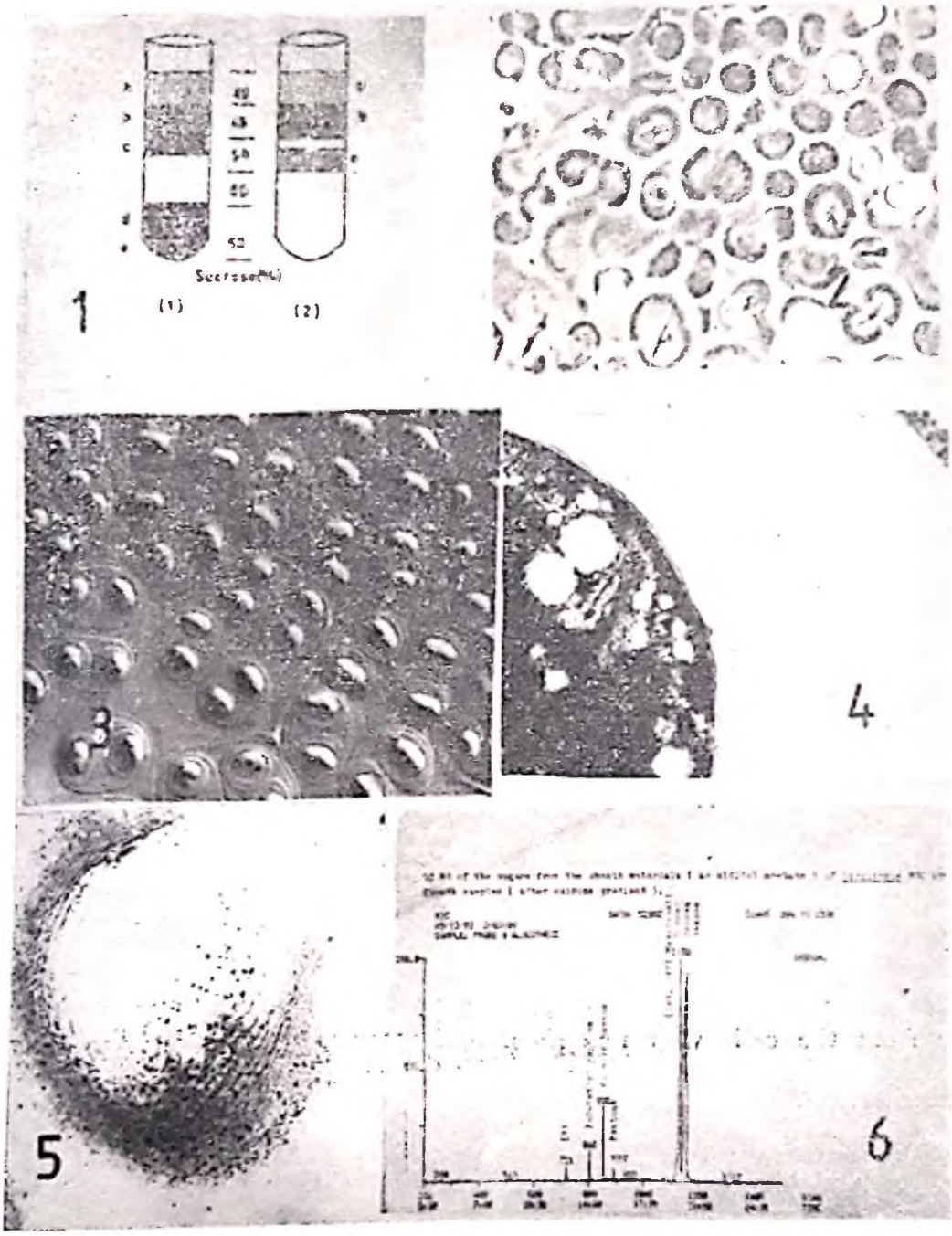
Chemical analyses :

The neutral sugars were separated and identified by thin layer chromatography (solvent: ethyl acetate/pyridin/water; 12:5:4 v/v), and by gas-liquid chromatography as alditol acetate derivatives (varian aerograph, model 1445-1, ECNSS-M column, 3% on Gas Chrom R, 100-120 mesh). Mass spectrometric analysis of sugar alditol acetates were carried out in a Finnigan quadripole instrument (Finnigan Corp., Sunnyvale, California, USA, model 3200 E) coupled to a Finnigan data and graphic output system (model 6000). The partially methylated alditol acetates were separated on glass columns (0.3×152 cm) filled with ECNSS-M phase at a column temperature of 170°C. The spectra were taken at 70 eV in the mass range of 35-300 with an integration time of 7 ms per unit. Amino sugars and amino acids were separated and quantitatively analysed in an automatic amino acid analyser (Durrum model D-500). Fatty acids were studied as methyl esters by gas-liquid chromatography (EGSS-X column, 15% on Gas Chrom P, 100-120 mesh or Castorwax column, 2.5% on Chromosorb G, 80-100 mesh). For the detection of uronic acids, 2-Keto-deoxy octonate and amino sugars, high voltage paper electrophorosis was used. Paper electrograms of the materials were prepared and stained with alkaline silver nitrate solution. Organic phosphorous was determined according to Lowry *et al.*, (1954).

RESULTS AND DISCUSSION

Isolation and fine structure of the sheath:

Well defined sheaths lying one upon another enclose loosely both individual cells and cell groups of Gloeothece PCC



Figs. 1-6. Fig. 1. Behaviour of the cell homogenate of (1) *Gloeothece* PCC 6909 (homogenized in Mickle's discintigrator for 3 h at 0°C with 0.25 mm glass beads) and (2) *Gloeothece* PCC 6909 mutant (homogenized in Mickle's discintigrator for 15 min at 0°C with 0.25 mm glass beads) in sucrose gradient (40-60% g/g) at 3,020× g upto 4 h at 0°C. a. pigments b. membranes c. cell fragments d. sheaths e. intact cells. Fig. 2. Light microscopic photograph of isolated sheath material, shed from *Gloeothece* PCC 6909 by mechanical means. Fig. 3. Light microscopic photograph of *Gloeothece* PCC 6909 cells showing well defined sheaths. Fig. 4. Ultrathin sections of *Gloeothece* PCC 6909. The cell is surrounded by well defined sheath. Fig. 5. Ultrathin sections of the isolated sheath material, shed from *Gloeothece* PCC 6909 cells by mechanical means. Fig. 6. GCMS of the sugars from the sheath material (as alditol acetate) of *Gloeothece* PCC 6909. (Fig. 2×332 ; Fig. 3×425 ; Fig. 4×16920 ; Fig. 5×5000)

6909 has been observed by light microscope (Fig.-3). Their isolation and purification was facilitated by the relatively high density of the sheath materials. The sheath was shed from the cells by mechanical stress, separated from the cell homogenate by low speed centrifugation and purified by sucrose density gradient centrifugation. About 10% of the cell mass was obtained as the sheath fraction (Table-I). The lack or negligible content of meso-diaminopimelic acid, muramic acid and 6-O-methyl-D-Mannose, which are the components of cell wall of cyanobacteria (Schrader *et al.*, 1982) indicated that the sheath fraction was free of cell wall material.

TABLE I

YIELD OF THE SHEATH MATERIALS FROM *GLOEOTHECE* PCC 6909 IN DISCONTINUOUS SUCROSE GRADIENT (40-60%) AND IN DIFFERENT PURIFICATION STEPS. VALUES REPRESENTS % OF CELL MASS DRY WEIGHT.

1. Sheath after sucrose gradient	10.16
2. Sheath after lysozyme treatment	8.86
3. Sheath after lysozyme and Triton X-10 treatment	7.13
4. Sheath after lysozyme and SDS treatment	5.13

On treatment with the detergents: Triton X-100 and SDS., these cell wall contaminants were totally removed (Table II).

TABLE II

CHEMICAL COMPOSITION (% OF DRIED MATERIAL) OF THE ISOLATED SHEATH FRACTION OF *GLOEOTHECE* PCC 6909.

Constituent	sheath fraction after sucrose gradient	sheath fraction after Triton X-100 and lysozyme treatment	sheath fraction after SDS and lysozyme treatment
Sugar			
6-deoxy Rhamnose	2.1	3.8	4.2
Unknown sugar-X	0.9	2.1	2.4
2-O-Methyl Pentose	0.5	0.7	0.8
Xylose	0.4	0.3	0
Mannose	3.0	5.3	6.3
Galactose	4.2	4.8	7.5
Glucose	5.9	8.4	10.2
Total sugars	17.0	25.4	31.4
Total amino acids	18.9	12.2	8.4
Glucosamine	0.866	—	—
Glucosamine 6-PO_4	0.393	—	—
Muramic acid	0.613	—	—
Diaminopimelic acid	0.722	—	—
K. D. O.	—	—	—
Uronic acid	—	—	—
Phosphate	0.45	0.42	0.26
Total fatty acids	1.7	1.2	0.8

=absent

Ultrathin sections of the whole cell-envelope of *Gloeothece* PCC 6909 (Fig. -4) and the isolated sheath fractions showed a homogeneous fibrillar fine structure (Fig. 8). It was not clear, however, whether the sheath fraction represented the complete sheath. The loosening of sheath structure in the isolated sheath compared with the original state might be due to the mechanical treatment.

Chemical composition :

The sheath fraction obtained from the sucrose gradient was composed predominantly of 17% w/w carbohydrates. 6-deoxy Rhamnose, 2-0-Methyl pentose, xylose, mannose, galactose, glucose and an unknown sugar was present; galactose, glucose and mannose being the dominant sugars in the sheath materials. These sugars were detected by gasliquid chromatographic analysis of the neutral sugar hydrolysate and were identified by Mass spectrometric fragmentation (Fig. -6). Lipids and phosphorous were present in trace amounts (1.7% and 0.45% respectively, of dry weight). Presence of uronic acid and K D O in the sheath material was not detected even by high voltage electrophorosis. A considerable portion of the sheath consisted of protein. Amino acid residues accounted for 20% of the dry weight. A characteristic high proportion of arginine, glutamic acid, asparatic acid, alanine and leucine was observed (Table III).

The protein was not totally solubilized even by treatment with strong detergents like Triton X-100 and SDS (100°C, 15 min). However, the amount were considerably reduced with an increase in the % of carbohydrate content of the sheath material (Table II),.

The results presented here shows that the sheath fraction even after treatment with strong detergents to eliminate the cell wall contaminants was composed of high proportions of carbohydrates and protein. This proves that the sheath of the cyanobacterium *Gloeothece* PCC 6909 is of glycoprotein in nature. However, further work is needed on the characterization of isolated sheath fractions from various other cyanobacteria, to demonstrae the physical and chemical nature of the sheath of cyanobacteria in general.

TABLE III

AMINO ACID COMPOSITION OF THE SHEATH FRACTION (FROM SUCROSE GRADIENT) OF *GLOEOTHECE* PCC 6909

AMINO ACID	CONTENT (% DRY WEIGHT)
Lysine	0.522
Arginine	3.28
Treonine	0.538
Glutamic acid	1.743
Glycine	0.568
Valine	0.546
Isoleucine	0.507
Tyrosine	0.278
Histidine	0.020
Asparatic acid	4.48
Serine	0.511
Proline	0.384
Alanine	1.153
Methionine	0.169
Leucine	0.875
Phenylalanine	0.583
Total amino acids=	18.903 (%)

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