Isolation and Chemical Composition of the Sheath of *Sphaerotilus natans*

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A sheathed bacterium, *Sphaerotilus natans*, was cultured with vigorous shaking in a medium containing peptone. Then the biomass was harvested and treated with lysosome, sodium dodecyl sulfate, and protease. With treatment, 1.6 mg of sheaths was obtained from 15 mg of biomass. For the preparation of sheaths of high purity, cultivation must be in the absence of glucose with sufficient aeration to prevent poly(3-hydroxybutyrate) accumulation. Carbohydrate (54.1%), protein (12.2%), and lipid (1-3%) were detected in the sheaths by colorimetric reactions and solvent extraction. Gas-liquid chromatography showed glucose and galactosamine to be present in the molar ratio of 1:4. The most abundant amino acids in the sheath protein were glycine (49.2 mol%) and cysteine (24.6 mol%). The sheaths were resistant to agents that reduce disulfide bonds (dithiothreitol and 2-mercaptoethanol) and to protease. However, sheaths were degraded completely by hydrazine, and a heteropolysaccharide composed of glucose and galactosamine (1:4) was released. The weight-average molecular weight of the polysaccharide was estimated to be $1.2 \times 10^5$ by gel filtration chromatography with a low-angle laser-light scattering photometer and a rotation index detector. A ladder of 1.5-kDa peptides separable by sodium dodecyl sulfate gel electrophoresis was obtained by partial hydrolysis of sheaths, suggesting the sheath protein has repeating units of 1.5 kDa.

Key words: sheath; *Sphaerotilus natans*; isolation; chemical structure

*Sphaerotilus natans* is a sheathed bacterium that constructs a tube-like sheath surrounding each cell. There are two genera of sheathed bacteria, *Sphaerotilus* and *Leptothrix*. Their morphological and physiological characteristics are similar, so the classification "*Sphaerotilus-Leptothrix group*" has been proposed. Their similarity has been confirmed by phylogenetic analysis based on 16S rRNA sequences.

The genus *Leptothrix* is a typical filamentous inhabitant of unpolluted streams rich in metal ions and can oxidized Mn$^{2+}$, but the genus *Sphaerotilus* is a filamentous bacterium often found in sewage and polluted streams. *Leptothrix* spp. have deposits of metal oxide in the sheath that may protect cells from toxic metal ions and harmful oxygen species. The sheath of *Sphaerotilus natans* seems to provide cells with physical protection from infection by a bacteriophage specific to this organism. Other benefits of the sheath in sheath-forming bacteria might be ready attachment to solid surfaces for advantageous positioning and the holding in of moisture to protect against drying.

The sheath of *Leptothrix discophora* is formed of a fibril matrix made of an anionic heteropolysaccharide and protein, connected by disulfide bonds. The polysaccharide is a 1:1 mixture of uronic acids and N-acetylated galactosamine, and the protein is rich in cysteine. A protein that oxidizes manganese-oxidizing is associated with the sheath of *L. discophora*. The sheath of *L. discophora* is resistant to a variety of enzymes, solvents, detergents, and other reagents, but not to agents that reduce disulfide bonds. The sheath of *Sphaerotilus natans* also is resistant to the effects of lysosome and detergents and is a complex of polysaccharide, protein, and lipid. Glucose and hexosamine are the sugar components and many kinds of amino acids are present. The chemical composition of the sheath of *Sphaerotilus natans* has been described only by Romano and Peloquin in 1963.

*S. natans* is only species in the genus *Sphaerotilus* and is a typical filamentous inhabitant of activated sludge. When abundant, sludge settles badly, in the phenomenon called bulking, probably because the water content of the sludge floc is greater when the biomass is large. The surface of the sheath of *S. natans* is covered with an acidic exopolysaccharide composed of fucose, galactose, glucose, and glucuronic acid. A surface with such a composition is presumably hydroscopic. The water content of sludge floc will increase as the bulk of these hydroscopic sheaths increases. This may be why excessive growth of *S. natans* results in poor settling of activated sludge. Bulking is an important problem at sewage treatment plants, but little is known about the composition and structure of the sheaths that seem to cause the problem. In this paper, we describe the isolation and chemical composition of sheaths and discuss the possible sheath structure.

Materials and Methods

Microorganism and culture conditions. The sheath-forming bacterium used was *Sphaerotilus natans* IFO 13543. In preliminary experiments, *Stokes medium*, often used in the cultivation of this bacterium, was used.

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(100 ml in 500-ml baffled flasks, incubated at 30°C for 2 days); glucose is present as the carbon source. An inoculum (100 μl) was obtained from a culture of this strain that had been kept at 20°C and allowed to settle for 1–7 days in 500-ml flasks containing 100 ml of medium containing 0.6% glucose, 0.3% proteose-peptone No. 3 (Difco), 0.05% CaCO₃, 0.02% yeast extract (Difco), and 0.02% MgSO₄·7H₂O. The medium for sheath preparation contained 0.2% proteose-peptone No. 3, 0.02% yeast extract, and 0.02% MgSO₄·7H₂O. Cultivation was in 500-ml baffled flasks containing 100 ml of medium with rotary shaking (150 rpm) at 30°C for 24 h.

Sheath isolation. Bacterial cells from 2 or 3 l of culture medium were harvested by centrifugation. The cell pellet was washed with distilled water, suspended with 90 ml of 30 mM Tris-HCl buffer (pH 8.0) containing 0.5 g/l EDTA disodium salt, and homogenized. To the suspension, 20 mg of lysozyme was added and the mixture was incubated at 37°C for 1–3 h. Then 10 ml of 10% SDS was added and the mixture was autoclaved at 110°C for 10 min. Partially purified sheaths appeared as a semitranslucent sheet floating on the surface. The sheaths were washed with distilled water and put into 45 ml of Tris-EDTA buffer containing 10 mg of protease (type XIV, Sigma). After incubation of the mixture at 37°C for 3 h, 5 ml of a 10% SDS solution was added and the mixture was autoclaved at 110°C for 10 min. Finally, purified sheath was obtained by washing with distilled water and lyophilized. When necessary, the sheaths were extracted with hot chloroform by a Soxlet apparatus for 6 h.

Hydrazinolysis. Isolated sheaths were dried over P₂O₅ under reduced pressure. The dried sheaths (10–20 mg) were suspended in 1 ml of anhydrous hydrazine and kept at 100°C for 8 h in an N₂ atmosphere. After the heat treatment, the solution was evaporated to dryness in a desiccator equipped with a sulfuric acid trap. Residual hydrazine was removed by evaporation of it together with 1 ml of toluene with a rotary evaporator at 40°C. This operation was repeated a total of four times. Then the residue was dissolved in 1 ml of 0.1 M HCl and centrifuged for removal of the small amount of insoluble materials. Five volumes of ethanol was added to the supernatant with shaking and the mixture was left at 4°C for 1 h. Polysaccharide released from the sheaths was recovered by centrifugation, dissolved in a small amount of 0.1 M HCl, and dialyzed against distilled water at 4°C. Finally, the dialyze was freeze-dried and stored in vacuo.

Sugar analysis. The gross sugar concentration of sheaths were examined by the phenol-sulfuric acid reaction (for neutral sugars),¹³ Elson-Morgan reaction (amino sugars),¹⁴ and carbazole-sulfuric acid reaction (acidic sugars).¹⁵ Before analysis, 1 mg of sheaths was hydrolyzed in 1 ml of 4 M trifluoroacetic acid at 100°C for 8 h.

Sugar components of sheaths were identified by TLC. The hydrolyzate was spotted onto a silica gel plate (Kieselgel 60, Merck) and developed with a solvent system of 2-propanol/aceton/0.1 M lactic acid (3:4:3). Before use, the silica gel plate was soaked in 0.5 M NaH₂PO₄ and dried at 105°C for 1 h. Sugars were detected by being sprayed with diphenylamine-aniline-phosphate reagent.¹⁶

Amounts of sugars were measured by GLC by the method of Merkle and Poppe¹⁷ with some modifications mentioned here. Sheaths (1–2 mg) was suspended in 500 μl of methanolic HCl (5%, w/v) together with 20–60 μg of myo-inositol as an internal standard, and the mixture was kept at 80°C for 16 h. The mixture was cooled to room temperature and evaporated by being dried at 40°C under a stream of air. Methanol (250 μl) was added and evaporated, and this procedure was repeated for a total of four times. To the final residue, 200 μl of methanol, 40 μl of pyridine, and 40 μl of acetic anhydride were added, in this order, in an N₂ or Ar atmosphere. After the mixture was left at room temperature for 1–2 h, it was dried at 40°C under an air stream. To the residue, 200 μl of a commercially available silylating reagent (TMSI-C, GL Sciences) was added and the mixture was heated at 80°C for 20 min. The sample (1 μl) was injected into a gas chromatograph (GC-14B, Shimadzu) equipped with a TC-1701 capillary column (0.53 mm × 30 m, GL Sciences). The column temperature program was set for an initial temperature of 150°C, a final temperature of 250°C, and an increase at the rate of 4°C/min. Both the injection port and detector were set at 260°C. The mobile phase was helium. The component sugars produced multiple peaks were produced unlike myo-inositol, which produced a single peak. Amounts were calculated by comparison of the height of the most abundant peak of the component sugars to the height of the myo-inositol peak.

Lipid analysis. Freeze-dried sheaths (20–30 mg) were suspended in 19 ml of a mixture of chloroform, methanol, and distilled water (2:1:0.8). After vigorous shaking of the mixture, it was left for 3 h and then the chloroform phase was recovered. This phase was evaporated to dryness at 40°C under an air stream and then further dried under reduced pressure. The residue of free lipids that contaminated the sheaths was obtained and weighed. The sheaths, which were in the aqueous phase, were dried at 105°C and then hydrolyzed with 2 ml of 2 N HCl for 2.5 h at 100°C. Lipids released by the hydrolysis were extracted with a mixture of chloroform (10 ml), methanol (5 ml), and distilled water (2 ml). The chloroform phase was dried and weighed as the bound lipids.

The poly(3-hydroxybutyrate) (PHB) in the biomass was measured with the same gas chromatograph and column as for sugar analysis. The column temperature was set at 90°C, and the detection and injection ports were at 150°C. Sample preparation and measurement of PHB were done as described elsewhere.¹⁸,¹⁹

Protein analysis. Sheaths (10 mg) were hydrolyzed in 1 ml of 1 N NaOH at 100°C for 1 h in an N₂ atmosphere and the protein concentration was measured with a pro-
tein assay kit (based on either the Bradford method or the Lowry method; both, Bio-Rad). Bovine serum albumin was used as the protein standard. The amino acid composition of the sheaths was analyzed. In brief, sheaths (160 μg) were hydrolyzed in 50 μl of 6 N HCl containing 4% thioglycolic acid at 150°C for 3 h. Then the hydrolyzate was evaporated and dissolved in 375 μl of distilled water. The solution was examined with an amino acid analyzer (L-8500, Hitachi).

**Acetic acid analysis.** Sheath polysaccharide obtained by hydrolyzolysis (10 mg) was dissolved in 10 ml of a saturated solution of NaHCO₃. Acetic anhydride (100 μl) was added to the solution every 15 min for 10 times (total, 1 ml). After the solution was stirred for 30 min at room temperature, it filtered through a column (1 × 10 cm) packed with AG 50W-X8 resin (Bio-Rad) and dialyzed against distilled water. N-acetylated sheath polysaccharide was obtained by lyophilization of the dialyze. N-acetylated polysaccharide (2 mg) was dissolved in 500 μl of 2 N NaOH and deacetylated at 100°C for 2 h. The amount of acetic acid released was measured by HPLC of the hydrolyzate. The analytical conditions were as follows: column, Inertsil C8 (GL Sciences); eluent, 0.1 N NH₄HSO₄; flow rate, 1 ml/min; detection by absorbance at 210 nm; and injection size, 20 μl.

**Estimation of molecular weight of polysaccharide.** The molecular weight of polysaccharide released from sheaths by hydrolyzolysis was estimated by HPLC in combination with a laser-light-scattering photometer (LS-8000, Tosoh). The chromatograph was equipped with two columns of TSKgel GMPWXL (7.8 mm × 30 cm, Tosoh). The mobile phase was 0.2 M phosphate buffer with a flow rate of 0.5 ml/min. The column temperature was 40°C. The hydrolyzolysate was injected in the buffer to a concentration of 2.24 mg/ml and 200 μl of the solution was analyzed. A constant, $k$, was calculated with pullulan (1.00 × 10⁵) as the standard.

**Partial hydrolysis and electrophoresis.** Sheaths (4 mg) were soaked in 670 μl of 0.5 N NaOH (or 0.5 N HCl) and heated at 80°C for 1–18 h in an N₂ atmosphere. The suspension was neutralized by the addition of 670 μl of 0.5 N HCl (or 0.5 N NaOH) and dialyzed against distilled water with a dialysis tube with a molecular weight cutoff of 12,000–14,000 (Viskase). The dialyze was denatured and the sulfhydryl groups were alkylated by the method of Westermeier and then electrophoresed on a gel with 20% polyacrylamide. After the electrophoresis, proteins were stained with Coomassie brilliant blue R-250. Isolelectrofocusing was done with an Ampholine PAG plate (pH 3.5–9.5, Pharmacia). Dialyze prepared from 4 mg of sheaths was centrifuged and the supernatant was lyophilized. The dryness was dissolved in 50 μl of distilled water and 10–20 μl of the solution was put on a plate. Proteins were fixed and stained by the method of Westermeier.

**Results**

**Effects of culture conditions during sheath preparation**

In *S. natans* cells (Fig. 1a) grown in Stokes medium, and sheathes prepared from such cells, many granules were visible (Fig. 1b) in preliminary experiments. Of the total biomass, 19.0% was PHB, and sheathes were 38.5% PHB. Many globular materials remained in sheathes even after 6 h of extraction with hot chloroform done to remove PHB, and the sheath were 10% PHB.

When *S. natans* was cultured in a medium of proteose-peptone, yeast extract, and MgSO₄·7H₂O (Fig. 2a), the biomass was only 0.84% of PHB. Prepared sheaths contained almost no granules (Fig. 2b). Such sheaths were only 1–2% PHB even without chloroform extraction. In this medium, about 15 mg of total biomass was

![Fig. 1. S. natans Cells Grown on Stokes Medium (a) and Sheaths Prepared (b).](image1)

*S. natans* was cultured in Stokes medium at 30°C for 2 days with shaking. From the biomass, sheathes were prepared. Stain, Victoria blue.

![Fig. 2. S. natans Grown on Proteose-peptone to Prevent PHB Accumulation (a) and Sheaths Prepared (b).](image2)

*S. natans* was cultured in a medium mainly composed of proteose-peptone at 30°C for 24 h with vigorous shaking. From the biomass, sheathes were prepared. Stain, Victoria blue.
obtained from 100 ml of culture medium, and about 1.6 mg of sheaths was recovered, for a yield of 11% of the total biomass. Slower shaking or more medium, resulted in more PHB; for example, when the bacterium was grown in 300 ml of the medium for sheath preparation, which leached glucose, sheaths were 68% PHB.

**Composition of sheaths**

Some 0.7–2% of the weight of sheaths after extraction in a solvent mixture was free lipids. Bound lipids accounted for 0.3–1% of sheath weight and the total lipid content was therefore 1–3%. Extraction with the solvent mixture did not change the sheath structure seen by light microscopy.

Protease treatment also did not cause visible changes in sheath structure. Even after protease treatment, protein was detected in the sheaths at the concentrations of 12.2% by the Lowry method and 0.5% by the Bradford method. When protease was not used, the protein in sheaths was 27.0% by weight by the Lowry method. The protein in sheaths not treated with protease decreased to 12.0% (Lowry) when 2-mercaptoethanol or dithiothreitol were added, without any change in sheaths visible by light microscopy. The sheath structure was completely broken by the addition of hydrazine. We concluded that protein contributed to the sheath structure in combination with other components that protected it from protease attack.

The sheath hydrolyzate gave positive results in the phenol-sulfuric acid and Elson-Morgan reactions but negative results in the carbazole-sulfuric acid reaction, so the sheaths contained neutral sugars and amino sugars but not acidic sugars. Results of TLC showed glucose and galactosamine to be present. Some 29.7% and 24.4% of hydrolyzate of sheaths was glucose and galactosamine, respectively, as calculated from colorimetric reactions.

**Sheath protein**

The amino acid composition of the sheaths is given in

![Fig. 3. SDS-PAGE of Partial Hydrolyzate of Sheath.](image)

Table I. In sheaths treated with protease, most abundant amino acids were glycine (49.2 mol%) and cysteine (24.6 mol%). Alanine, glutamine (or glutamic acid), and lysine were at amounts ranging from 4.7 mol% to 7.7 mol%. Other amino acids were detected in trace amounts (≤ 1.5 mol%) or not detected.

**SDS-PAGE of sheaths** not even partially hydrolysed gave no bands. Bands of proteins with molecular weights ranging from 18,700 to 30,500 was obtained by SDS-PAGE of sheaths which was partially hydrolyzed with 0.5 N NaOH for 6 h (Fig. 3). Acid hydrolysis with 0.5 N HCl for 1 h gave the same bands (data not shown). The mean intervals between adjacent protein band was a distance corresponding to 1.47 kDa. Prolonged hydrolysis for up to 18 h resulted in the loss of protein bands. The isoelectric point of protein released from sheaths by partial hydrolysis was near or at pH 5.8.

**Sheath polysaccharide**

The yield of polysaccharide released by hydrazinolysis was about 67.5% by weight of the sheaths. The results of elution of polysaccharides from a gel filtration column are shown in Fig. 4. A large peak was found by both detectors used. By comparison of the outputs of the photometer and the detector by the procedure of Takagi and Hizukuri, the weight-average molecular weight, $M_w$ of sheath polysaccharide at a particular retention time was found (Fig. 4). The range of molecular weight of the sheath polysaccharide was from $1.6 \times 10^4$ to $7 \times 10^4$, and the $M_w$ was $1.2 \times 10^5$.

The GLC chromatogram shown in Fig. 5 shows multiple peaks of glucose and N-acetylgalactosamine and no
Fig. 4. Elution patterns of sheath polysaccharide on gel filtration chromatography and calibration curve.

Elution patterns with the laser-light-scattering photometer and the rotation index detector are shown by the broken and solid lines, respectively. The time lag between the two detectors has been adjusted. Molecular weight was calculated by the method of to Takagi and Hizukuri and is plotted on a semilogarithmic scale.

Fig. 5. Gas-liquid Chromatography of Monosaccharides from Sheaths.

A 1.4-mg portion of sheaths was analyzed. As the internal standard, 60 μg of myo-inositol was used.

unidentified peak. The recoveries of glucose and N-acetylgalactosamine were calculated to be 7.4% and 37.4%, respectively, by comparison of the height of the main peak of each sample sugar with that of the peak of the internal standard (inositol). Therefore, the molar ratio of glucose and N-acetylgalactosamine was estimated to be 1:4. Similar chromatograms were obtained with sheath polysaccharide prepared by hydrazinolysis and the same molar ratio was found. In a check of the sugar composition, the acetyl groups in N-acetylated sheath polysaccharide were assayed by HPLC. Theoretically, 0.38 mg of acetic acid should be released from 2 mg of polysaccharide composed of glucose and N-acetylgalactosamine in the molar ratio of 1:4 by deacetylation. The amount of acetic acid actually released and detected from 2 mg of N-acetylated sheath polysaccharide was 0.31 mg, which is 82% of the theoretical value.

Discussion

One physiological characteristic used in the classification of S. natans is its ability to produce PHB. This organism accumulates more than 30% PHB during the growth phase in pure culture. Granules of PHB are observed even in filaments of S. natans inhabiting activated sludge. S. natans with a high PHB concentration survives longer when starved in phosphate buffer, but PHB is not completely exhausted. In preparing sheaths from S. natans, therefore, we must take PHB accumulation into consideration. Sheaths isolated from cells grown on proteose-peptone with aeration vigorous enough to prevent PHB accumulation contained a trace amount of lipid. We hypothesize that the sheath lipid detected by others is mainly contaminating PHB. In-sufficient aeration resulted in PHB contamination even when the microorganism was grown on proteose-peptone. This result shows that S. natans can convert amino acids into PHB when oxygen is limited. Once PHB granules were produced and enclosed in sheaths, PHB could not all be removed even by hot chloroform extraction. The only way to obtain sheaths of S. natans of high purity is to cultivate this organism under conditions preventing PHB accumulation. Sheath were not affected visibly under a microscope by solvent extraction, so we concluded that the sheath of S. natans did not contain lipids.

We prepared sheaths from S. natans by lysozyme digestion, heating in the presence of SDS, and protease digestion. The most important difference from earlier work is the protease digestion. By treatment with protease, the protein content of sheath decreased from 27.0% to 12.2% without any change in sheath structures being visible under a microscope. Treatment with 2-mercaptoethanol had the same effect. Probably, proteins susceptible to protease are attached to sheaths by disulfide bonds and protease digestion can be replaced by the addition of 2-mercaptoethanol or other reagents that reduce disulfide bonds. A variety of amino acids were detected in sheaths by Romano and Peloquin. In contrast, sheaths prepared with protease digestion were rich in glycine and cysteine. The sum of the molar contents of glycine, cysteine, alanine, glutamic acid (or glutamine), and lysine was 92.6 mol%. The isoelectric point of sheath protein was pH 5.8, so glutamic acid was more likely to be present than glutamine. The sheath protein of S. natans seems to be mostly the five (or six) amino acids just listed. This unusual amino acid composition may explain why the protein content of the sheaths was different when the Lowry and the Bradford methods were used. Protease digestion is needed for sheath isolation.

Colorimetric reactions showed that sheaths of S. natans were composed of sugar and protein. The importance of peptide bonds in the structure was further confirmed when hydrazinolysis destroyed sheath structures, with release of polysaccharides. Recovery of the polysaccharide was 67.5%, with the assumption that sheaths were about 70% polysaccharide and about 30% protein and were constructed by cross-links between these polymers. The sheath of Leptothrix discophora is 24–35% polysaccharide and 25% protein, and less than 8% lipid. Leptothrix sheaths resist a variety of enzymes including protease. The structure of bacterial sheaths may resemble murein or pseudomurein because the sheathes are composed of polysaccharide and protein and are resistant to protease. Protease resistance suggests that the cross-linking peptides could be short enough to cause steric hindrance by the adjoining polysaccharide. Partial hydrolysis of sheaths of S. natans
gave a 1.5-kDa ladder of peptides separable by electrophoresis. Probably, sheath protein consists of a repeating unit of 1.5-kDa peptides, and the linkage between repeating units seems to be more unstable than ordinary peptide bonds. The repeating units might cross-link with the polysaccharide.

Murein and pseudomurein do not contain cysteine, but bacterial sheath proteins are rich in cysteine (6 mol% for *Leptothrix* and 24.6 mol% for *S. natans*). Emerson and Ghiorse reported that *Leptothrix* sheaths are easily broken down by agents that reduce disulfide bonds, showing the importance of such bonds in maintaining its structure. They found also that *Leptothrix* sheaths are constructed of fibril units assembled with disulfide bonds, and that the fibril units are composed of covalently linked polysaccharide and protein. Dispite the abundance of cysteine, *Sphaerotilus* sheathes were not affected by such reducing agents, suggesting disulfide bonds are not involved in sheath formation. *Sphaerotilus* sheathes seem to be constructed entirely of covalently linked polysaccharide and protein. One difference between *Leptothrix* and *Sphaerotilus* sheaths is, therefore, the presence of subunit structures. The difference may be reflected by *Leptothrix* sheathes looking rough under the electron microscope but *Sphaerotilus* sheaths looking smooth.

Polysaccharides of *Sphaerotilus* sheathes were composed of glucose and galactosamine in the molar ratio of 1:4. *Leptothrix* sheath polysaccharide is a 1:1 mixture of galactosamine and uronic acids, and galactosamine residues are N-acetylated in intact sheaths. Presumably, the galactosamine residues of *S. natans* sheathes also are N-acetylated, and N-acetylgalactosamine may be a common sugar component of bacterial sheathes.

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### References