International Journal of Biological Macromolecules 50 (2012) 236–244

Contents lists available at SciVerse ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

# Presence of alternating glucosaminoglucan in the sheath of *Thiothrix nivea*

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ARTICLE INFO

*Article history:*
Received 29 August 2011
Received in revised form 6 October 2011
Accepted 31 October 2011
Available online 6 November 2011

*Keywords:*
Glucosaminoglucan
Structural analysis
Sheath
*Thiothrix nivea*

ABSTRACT

A sheath-forming sulfa oxidizer, *Thiothrix nivea*, was mixotrophically cultured in a medium supplemented with acetic acid and sodium disulfide. Its sheath, a microtube-like extracellular supermolecule, was prepared by selectively removing the cells with lysozyme, sodium dodecyl sulfate, and sodium hydroxide. The sheath was not visibly affected by hydrazine treatment, suggesting that it is not a proteinous supermolecule. From the acid hydrolysate of the sheath, glucose and glucosamine were detected in an approximate molar ratio of 1:1. Three other saccharic compounds were detected and recovered by HPLC as fluorescent derivatives prepared by reaction with 4-aminobenzoic acid ethyl ester. Nuclear magnetic resonance (NMR) analysis suggested that one of the derivatives was derived from an unidentified deoxypentose. NMR analysis for the other 2 derivatives showed that they were derived from β-1,4-linked disaccharides and tetrasaccharides, which were composed of glucose and glucosamine. The sheath was readily broken down by weak HCl treatment, releasing an unidentified deoxypentose and polymer. Chemical analysis showed the presence of β-1,4-linked D-Glc*p* and D-GlcN*p* in the polymer. NMR analysis revealed that the polymer had a repeating unit of →4)-D-Glc*p*-(β1→4)-D-GlcN*p*-(β1→. The solid-state 1D-$^{13}$C NMR spectrum of the polymer in *N*-acetylated form supported this result. The molecular weight of the polymer was estimated to be $8.2 \times 10^4$ by size exclusion chromatography. Based on these results, the sheath of *T. nivea* is hypothesized to be assembled from alternately β-1,4-linked glucosaminoglucan grafted with unidentified deoxypentose.



## 1. Introduction

*Thiothrix nivea* is the type species of the genus *Thiothrix*, which is the filamentous sulfur-oxidizing taxon of γ-*Proteobacteria* and is common in polluted aquatic environments such as activated sludge [1–4]. Some members of the genus *Thiothrix* constitutively form an extracellular microtube, or sheath, that surrounds a line of cells [5,6]. *T. nivea* mixotrophically oxidizes reduced sulfur compounds and is capable of forming the sheath [1,7,8]. Since sheath formers in the class γ-*Proteobacteria* are only recognized in the genus *Thiothrix* [9] and *T. nivea* is the best-studied species of the genus *Thiothrix*, we considered its sheath to be the representative sheath of this class. Another sheath-forming taxon in the phylum *Proteobacteria* is the class β-*Proteobacteria* [9,10]. Besides the phylum *Proteobacteria,* sheath-forming eubacterial strains are found in the phyla *Bacteroides* [9,11] and *Cyanobacteria* [12,13]. All of the known sheath-forming eubacteria are inhabitants of aquatic environments. The sheaths provide cells with physical protection against bacteriophage infection and protozoan predation. Another benefit of sheaths might be that they can stimulate attachment to solid surfaces. Although the ecological functions of sheaths are well recognized, the significance of sheaths as functional extracellular supermolecules has not been adequately appreciated. Common bacterial extracellular polymers do not assemble in an orderly manner but form either a random meshwork or a mucous layer around cells. In contrast, sheath-forming polymers are orderly assembled and form tubular structures. This contributes to the uniqueness and functionality of sheath-forming polymers. In recent years, marked progress was made in characterizing the sheath-forming polymers of β-*Proteobacteria*, namely, the genera *Sphaerotilus* and *Leptothrix* [14,15]. Members of these genera are widely distributed heterotrophic iron oxidizers and comprise the *Sphaerotilus–Leptothrix* group of bacteria as a basis of their taxonomical relatedness [10,16,17]. Because their sheath-forming polymers have similar chemical structures, they are collectively named thiopeptidoglycan, which is a subcategory of peptidoglycans [15]. Thiopeptidoglycan is a peculiar glycoconjugate that has a straight-chained amphoteric heteropolysaccharide core grafted with a dipeptide composed of glycine and L-cysteine. The most remarkable feature of thiopeptidoglycan is the extreme

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doi:10.1016/j.ijbiomac.2011.10.024

abundance of thiol groups arranged at regular intervals (every 5 sugar residues). This is one of the key contributing factors for ordered aggregation of thiopeptidoglycan molecules for formation of the microtube structure. To gain a deeper understanding of the polymers that are capable of ordered aggregation, sheath-forming polymers of other taxa should be studied in detail. However, sheath-forming polymers from other taxa, including those from the class γ-*Proteobacteria*, are still not well understood. Aiming at a deeper understanding of sheath-forming polymers of the phylum *Proteobacteria*, we attempted to isolate and investigate the sheath of *T. nivea*.

## 2. Materials and methods

### 2.1. Preparation of sheath

*T. nivea* ATCC35100 was statically cultured at 30 °C for 5 days in a 500-mL flask containing 200 mL of a modified DSMZ573 medium of the following composition (per liter water): 0.5 g sodium acetate, 0.3 g $Na_2S{\cdot}9H_2O$, 0.2 g $NH_4Cl$, 0.01 g $K_2HPO_4$, 0.01 g $MgSO_4{\cdot}7H_2O$, 20 mL $CaSO_4$ saturated solution, and 5 mL trace element solution (corresponding to DSMZ155 medium). The filaments of *T. nivea* from 2.8 L of culture were recovered by filtration with a glass filter (DP-70, Advantec) and subsequently washed with water. The washed filaments were lyophilized to measure the dry weight otherwise they were suspended in 30 mM Tris–HCl buffer (pH 8.0) containing 0.5 g/L ethylenediaminetetraacetic acid and then homogenized. About 5 mg of lysozyme was added to the suspension, followed by shaking at 37 °C for 3 h. The supernatant was removed by centrifugation, and the precipitate was suspended in 10 mL of 2 M NaOH solution. After the suspension was shaken at 37 °C for 42 h, 1 mL of 100 g/L sodium dodecyl sulfate (SDS) solution was added and shaken for an additional 3 h. The sheath was collected from the suspension and thoroughly washed with water by repeated centrifugation. To prepare *N*-acetylated sheath, the wet sheath originating from 2.8 L of culture was suspended in 15 mL of saturated $NaHCO_3$ solution. While stirring, acetic anhydride was added drop-wise at room temperature until the suspension became lower than pH 7. The *N*-acetylated sheath was recovered from the suspension and thoroughly washed with water by repeated centrifugation. When needed, the sheath and *N*-acetylated sheath were lyophilized.

### 2.2. Treatments of sheath with reagents and enzymes

The wet sheath (about 0.1 g) was put into a screw-capped micro tube containing 1 g of zirconia beads (0.5 mm in diameter) and 1 mL of water, followed by vigorous shaking (3000 rpm, 2 min) using a Mini-Beadbeater (Biospec Products). The suspension was recovered and centrifuged to collect the homogenized sheath as precipitate. The homogenized sheath was suspended in a solution (0.5 mL) of 0.1 M HCl, 0.1 M acetic acid, 6 M guanidine–HCl buffered with 200 mM Tris–HCl (pH 8.0), or 8.7 mM Tris(2-carboxyethyl)phosphine (TCEP) buffered with 200 mM Tris–HCl (pH 8.0), and then the suspension was incubated at 25 °C overnight. After incubation, the morphology of the sheath in the suspension was observed using phase-contrast microscopy. The effects of hydrolytic enzymes were tested as follows. The homogenized sheath (about 20 mg in wet weight) was suspended in 1.2 mL of 50 mM sodium–potassium phosphate buffer (pH 7.0) containing 0.4 mg of α-amylase, lipase, chitinase, or chitosanase. The effect of cellulase was tested in 1.2 mL of 50 mM sodium citrate buffer (pH 5.0) containing 0.4 mg cellulase. Suspensions with buffers free of the enzymes were also prepared. The suspensions were incubated at 37 °C for 2 days and subsequently subjected to phase-contrast microscopy. The effect of hydrazine was examined as follows. The wet sheath (about 0.1 g) was homogenized with zirconia beads in the manner described above. The suspension and zirconia beads were transferred into a screw-capped test tube and then lyophilized. Hydrazine anhydride (1 mL) was added under argon (Ar) atmosphere, and the suspension was heated at 100 °C for 8 h with occasional shaking. After hydrazine was removed under reduced pressure in the presence of $H_2SO_4$, the hydrazine-treated sheath was obtained as dried matter. The hydrazine-treated sheath was suspended in water and subjected to phase-contrast microscopy.

### 2.3. Sugar composition analysis

Neutral and basic monosaccharides were detected as their corresponding alditol acetates, using gas–liquid chromatography (GLC). The lyophilized sheath or the basic polysaccharide (2 mg) was hydrolyzed in 5 mL of 2 M trifluoroacetic acid (TFA) at 100 °C for 4 h, followed by evaporation. The sugars in the dried residue were derivatized to their corresponding alditol acetates by using the method previously described [18,19]. Separation and quantification of alditol acetates were performed using a GC-2010plus (Shimadzu) equipped with FID detector under the following conditions: column, TC-1 (0.25 mm × 15 m, GL Sciences); carrier gas, He; temperature program, from 150 to 250 °C at a rate of 2 °C/min. Uronic acids and neutral sugars in the hydrolysate were identified by gas–liquid chromatography/mass spectrometry (GLC/MS) as trimethylsilylated derivatives according to the method previously described [20]. GLC/MS conditions were as follows: equipment, JMS-600 (JEOL); column, InertCap 1MS (0.25 mm × 30 m, GL Sciences); carrier gas, He; temperature program, from 150 to 250 °C at a rate of 2 °C/min; ionization, EI (positive mode). The enantiomeric configurations of the sugars were identified by the formation of the (−)-2-butylglycosides, according to the method of Gerwig et al. [21,22]. Oligosaccharides in the hydrolysate were investigated using high-performance liquid chromatography (HPLC). Prior to analysis, the sugars were released from the sheath and subsequently converted to corresponding fluorescent derivatives by the following procedure. The lyophilized sheath (2 mg) was treated with 5 mL of 2 M trifluoroacetic acid (TFA) at 100 °C for 4 h or with 4 mL of 0.5 mM HCl at 80 °C for 3 h. After evaporation, the dried residue was dissolved in 50 μL of water, and then the sugars in the solution were derivatized with a fluorescent labeling reagent, 4-aminobenzoic acid ethyl ester (ABEE), using an ABEE labeling kit (Seikagaku Corporation) according to the manufacturer's instructions. The derivatives were subjected to HPLC under the following conditions: column, $5C_{18}$-AR-II (10 mm × 250 mm, Nacalai Tesque); temperature, 45 °C; mobile phase, 17.5% (v/v) acetonitrile containing 0.1% (v/v) TFA; flow rate, 2 mL/min; detection, fluorescence ($\lambda_{ex}$ 305 nm, $\lambda_{em}$ 360 nm) or absorbance at 305 nm. If necessary, the eluent containing the isolated derivatives were recovered and evaporated. Subsequently, they were hydrolyzed, derivatized with ABEE, and subjected to HPLC analysis by the procedure described above.

### 2.4. Preparation of fluorescent hydrolysate of sheath

For detailed structural analysis, 3 fluorescent derivatives (derivatives I–III) were isolated using HPLC. To prepare derivatives I and II, the lyophilized sheath was added to 2 M TFA solution at a concentration of 2.5 g/L and hydrolyzed at 100 °C for 4 h. After evaporation, hydrolysates were derivatized using the ABEE labeling kit according to the manufacturer's instructions, and the derivatives were subjected to HPLC under the following conditions: column, $5C_{18}$-AR-II (10 mm × 250 mm, Nacalai Tesque); temperature, 45 °C; mobile phase, 17.5% (v/v) acetonitrile containing 0.1% (v/v) TFA;