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Exopolysaccharides of *Synechocystis aquatilis* are sulfated arabinofucans containing N-acetyl-fucosamine

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ABSTRACT

Cyanobacteria are known to be a rather diverse group of organisms regarding e.g. morphology, metabolism and composition of excreted exopolysaccharides (EPS). Considering the high number of known cyanobacterial species the EPS from only a small percentage are investigated in detail. This work examined EPS from the unicellular strains of *Synechocystis aquatilis* and *S. pevalekii* with various methods. The results emphasize the heterogeneity of cyanobacterial EPS. *S. pevalekii* secrets complex hetero-polysaccharides and acidic proteins as proteoglycan-complexes whereas the protein-free but highly sulfated EPS from *S. aquatilis* only consist of 4 dominant monosaccharides. Especially remarkable is the composition of these EPS: an arabinofucan with higher amounts of N-acetyl-fucosamine (FucNAc) and only minor quantities of glucose. Both EPS and the newly found component FucNAc in EPS from *S. aquatilis* extend the possible components of cyanobacterial EPS and the knowledge of heterogeneity of cyanobacterial metabolites.

1. Introduction

Some cyanobacteria have a long tradition as nourishment in South America, Asia and Africa (Gantar & Svirčev, 2008) and actually they become still more important as dietary supplement, food additive and functional food. Additionally, cyanobacterial exopolysaccharides (EPS) can be used for different industrial applications such as thickener, emulsifying agent or for removing heavy metal ions from solvents and soil (Pereira et al., 2009). Two important structural characteristics are essential for the functionality of EPS: ionic charge and partial hydrophobicity. The former is given by carboxylic groups of uronic acids, pyruvate or sulfate substituents (Pereira et al., 2009) or by acidic amino acids of associated proteins as examples in literature show (Flaibani, Olsen, & Painter, 1989; Marra, Palmeri, Ballio, Segre, & Slodki, 1990; Nakagawa, Takamura, & Yagi, 1987; Schrader, Drews, Golecki, & Weckesser, 1982). Parts of EPS molecules are hydrophobic due to desoxy sugars, acylation or attached protein moieties (Pereira et al., 2009). In three quarters of so far described polymers cyanobacterial EPS have a complex and diverse structure with six or more different monosaccharides. In total, 12 different monosaccharides have been reported as components of cyanobacterial EPS together with a broad range of different linkage types. Various hexoses and pentoses as ketoses and aldoses as well as desoxy, amino and methyl sugars and uronic acids were found. Often glucose is the main component. The structure of cyanobacterial EPS was investigated in detail, however, only in a few cases, although detailed knowledge of them is essential for understanding the chemical and physical properties of these polymers as well as their biological activities (De Philippis & Vincenzini, 1998).

EPS from *Nostoc commune* and *Synechocystis aquatilis* (*S. aquatilis*) had shown an interesting biological activity: inhibition of the classical pathway of the human complement system (Brüll, Huang, Thomas-Oates, & Smestad Paulsen, 2000; Volk, Venzke, Blaschek, & Alban, 2006). The human complement system is part of the innate immune system (Tudoran & Kirschfink, 2012). Defects in this complex system cause rare serious diseases, but it can also participate in the genesis of widespread diseases like asthma. Especially EPS from *S. aquatilis* showed an impressing inhibitory activity (Volk et al., 2006). Nevertheless, a detailed analysis of the structure of these EPS was still missing. Therefore the composition and fine structure of the EPS from *S. aquatilis* were investigated in detail. For comparison and for extending the knowledge of composition of cyanobacterial material the EPS from the closely related *S. pevalekii* was analyzed additionally.

Abbreviations: EPS, exopolysaccharides; FucNAc, N-acetyl-fucosamine; GLC, gas liquid chromatography; HV, hydrodynamic volume; IEC, ion exchange chromatography; MW, molecular weight; MWCO, molecular weight cut-off; *S.*, *Synechocystis*; SEC, size exclusion chromatography; TFA, trifluoroacetic acid.

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2. Materials and methods

2.1. Material

The cyanobacterial strains *S. aquatilis* Sauvageau SAG 90.79 (*S. aquatilis*) and *S. pevalekii* Ercegovic SAG 91.79 (*S. pevalekii*) from the "Culture Collection of Algae at Goettingen University (SAG)" were cultivated in 10-liter-wide-neck-flasks containing 8 l of mineral medium under constant aeration (1 ml/min) and constant illumination (~150 μmol/m^2 s^{-1}). *S. aquatilis* was cultivated for a period of time of up to 42 days, *S. pevalekii* of up to 63 days. The medium consisted of (mg/l): 500 KNO_3, 50 K_2HPO_4, 5.5 $Na_2EDTA{\cdot}2H_2O$, 4.0 $FeCl_3{\cdot}6H_2O$, 0.4 $MnCl_2{\cdot}4H_2O$, 0.1 $CoSO_4{\cdot}7H_2O$, 0.1 $ZnSO_4{\cdot}7H_2O$, 0.1 $Na_2MoO_4{\cdot}2H_2O$, 0.01 $CuSO_4{\cdot}5H_2O$ and 10% of "Instant Ocean" (Aquatic Systems Inc., Atlanta, USA). Axenic culture was checked routinely by microcopy. Cells were separated by centrifugation (RZB ~15,000) and extracellular proteins were removed by heat denaturation (86 °C, 15 min) and centrifugation. EPS were precipitated with ethanol (final concentration 80%), dialyzed (MWCO 12–14 kDa) against demineralized water and lyophilized.

2.2. Monosaccharide composition

After hydrolysis of ~5 mg EPS with 2.0 molar trifluoroacetic acid (TFA) for 1 h at 121 °C the monosaccharides were derivatized by reduction and acetylation as described by Blakeney, Harris, Henry, and Stone (1983). The resulting alditol acetates were analyzed by gas liquid chromatography (GLC) with flame ionization detection and by GLC-mass spectrometry (GLC-MS) with interpretation of MS-spectra using comparison with reference components (Goellner, Ichinose, Kaneko, Blaschek, & Classen, 2011a).

2.3. Determination of uronic acids and polysaccharide substituents

The content of uronic acids was determined as described by Blumenkrantz and Asboe-Hansen (1973). Acylation of polysaccharides was analyzed using the method of McComb and McCready (1957). Substitution of sugars with pyruvate groups was quantified according to Sloneker and Orentas (1962).

2.4. Elemental analysis, degree of sulfation and amino acid composition

Carbon, hydrogen, nitrogen and sulfur were quantified with a HEKAtech CHNS Analysator (Co. HEKAtech, Wegberg, Germany) by calibration with sulfanilamide using ~2 mg of EPS sample. The content of sulfate groups was calculated from the sulfur concentration using a conversion factor of 2.474. The amount of protein in EPS was calculated by multiplying the nitrogen content, reduced by the nitrogen content from amino sugars, with a conversion factor of 6.25 for *S. aquatilis* (acc. to Kjeldahl, 1883) or of 8.24 for *S. pevalekii*, based on the respective amino acid composition, determined as described by Goellner, Blaschek, and Classen (2010).

2.5. IR-spectroscopy

FT-IR spectra were recorded with a Perkin Elmer instrument (Spectrum 100 FT-IR-Spectrometer; Universal ATR Sampling Accessory).

2.6. Conductometric titration

Acidic functional groups of EPS were converted into their protonated form with Amberlite IR 120 (Co. Rohm/Haas, Philadelphia, USA) and titrated with 0.1 molar sodium hydroxide using conductometrical detection with a Seven Easy Instrument (Co. Mettler Toledo, Giessen, Germany) as described by Casu and Gennaro (1975).

2.7. Ion exchange chromatography (IEC) and size exclusion chromatography (SEC)

EPS were fractionated by IEC with Sepharose Q or DEAE Sepharose (Pharmacia Inc., 8 × 1.5 cm column). For elution different concentrations of NaCl (0–2 molar) or 5 molar urea were used stepwise. Fractions were analyzed for carbohydrate content (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956) and protein content (Starcher, 2001).

The molecular weight and hydrodynamic volume were determined by SEC on three different PL aquagel-OH columns in series (PL aquagel-OH mixed, MW range 0.1–1.000 kDa; PL aquagel-OH 40, MW range 10–200 kDa; PL aquagel-OH 20, MW range 0.1–20 kDa; in total good separation between 1 and 1.000 kDa; Polymer Laboratories, Darmstadt, Germany) as described by Goellner, Utermoehlen, Kramer, and Classen (2011b). For calibration pullulan reference compounds (5.9–788 kDa, PL Polysaccharide Standards, Varian Inc., Germany) were used.

2.8. Desulfation

Desulfation of EPS-pyridinium salts from *S. aquatilis* was performed according to Kolender and Matulewicz (2004) with 320 μl chlorotrimethylsilane per 10 mg EPS for 5.25 h at 100 °C. The desulfated EPS were dialyzed (MWCO 12–14 kDa) against demineralized water and lyophilized.

2.9. Analysis of linkage type

Genuine EPS from *S. aquatilis* were converted into their pyridinium salt. Activation and methylation of hydroxyl groups of the polysaccharides from *S. aquatilis* (~1 mg in 500 μl dry dimethyl sulfoxide (DMSO)) were obtained using a sodium hydroxide-DMSO-slurry and stepwise addition of iodomethane: 0.5 ml slurry – 0.05 ml iodomethane – 0.5 ml slurry – 0.2 ml iodomethane. First three additions were followed by a 20 min incubation time each, last addition by a 50 min incubation time. After addition of 5 ml of demineralized water, nitrogen was bubbled through the solution for 15 min and the mixture was dialyzed (MWCO 1 kDa) against demineralized water and lyophilized. The whole process was performed three times.

EPS from *S. pevalekii* were methylated as described by Harris, Henry, Blakeney, and Stone (1984) with freshly prepared dimethylsulfinyl-carbanion (dimsyl-reagent; potassium hydride in DMSO: 80 mg/ml). Samples were dissolved in DMSO (1–10 mg/460 μl) and treated three times alternating with dimsyl-reagent (40, 120 and 400 μl) and iodomethane (10, 30 and 300 μl). Reaction times were 40, 20, 40, 20, 30 and 10 min. Finally, samples were washed with a chloroform/methanol/water (2:1:2) mixture.

Further steps of hydrolysis, reduction and acetylation for methylated EPS from both strains were carried out as described by Harris et al. (1984). Samples were hydrolyzed in 1 ml 2 molar TFA for 1 h at 121 °C and dried. Reduction took place with 2 ml 0.5 molar sodium bordeuteride in 2 molar ammonia at 60 °C for 1 h and stopped by addition of 0.5 ml acetone. Dried samples were acetylated in 0.2 ml glacial acetic acid, 1 ml ethyl acetate and 0.1 ml perchloric acid with 3 ml acetic acid for 5 min at room temperature. Reaction was stopped by addition of 10 ml demineralized water and 0.2 ml 1-methylimidazole. Reaction products were extracted with 2 ml