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Structural characterization of the exopolysaccharide PS-EDIV from *Sphingomonas pituitosa* strain DSM 13101

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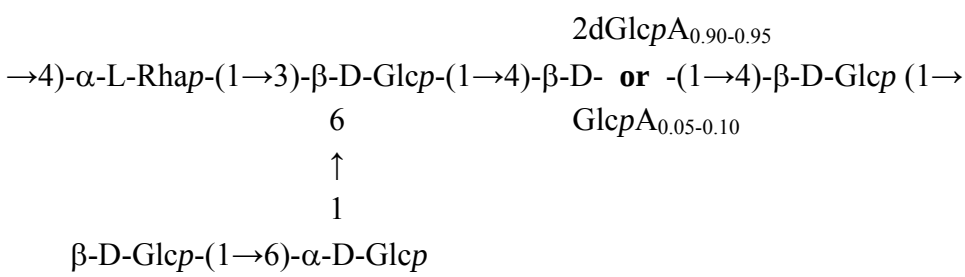
Abstract

Members of the bacterial genus *Sphingomonas* are known to produce highly viscous polysaccharides in solution. The exopolysaccharide PS-EDIV was produced by *Sphingomonas pituitosa* strain DSM 13101, purified using centrifugation, and precipitation and its structure was elucidated by 1D and 2D NMR techniques and chemical microderivatization combined with various mass spectrometric techniques. The following repeating unit of the polysaccharide could be identified:



In addition, the polysaccharide also contains acetyl and glyceryl groups whose exact positions were not determined. PS-EDIV is similar in structure to a known exopolysaccharide but differs in being the first bacterial polysaccharide in which two different glucuronic acids are combined. It caused a high viscosity of the culture broth after cultivation for 48 h, although a gelation was not observed.

Keywords:

Sphingomonas pituitosa, exopolysaccharide, structural characterization, sphingan, deoxyglucuronic acid

Introduction

In recent years many sphingomonads including species of the genus *Sphingomonas* were reported to be able to degrade a broad spectrum of xenobiotic and/or recalcitrant organic compounds (Balkwill et al. 2005) and to produce extracellular biopolymers with viscous properties. In general, most biopolymers are of great interest for a wide variety of applications, e.g., as aqueous rheological control agents in food, cosmetics and pharmaceuticals. In nature, polysaccharides often occur as capsules around bacterial cells to prevent dehydration or make carbon source inaccessible to other species.

Depending on their rheological properties they are used, for example, as thickening agents in skin creams and soups, where xanthan gums are the most popular within the range of microbial polysaccharides. The capsular EPS produced by sphingomonads are referred to as sphingans and include well-known members such as gellan gum and welan gum (Lobas et al. 1992, 1994, Martins et al. 1996, Hashimoto et al. 1998, Fialho et al. 1999).

These sphingans have similar but not identical structures and include gellan (Kang and Veeder 1982), S-88 (Kang and Veeder 1985), welan (Kwon et al. 1987) and rhamsan (Peik et al. 1983, Podolsak et al. 1996). They usually consist of a linear repeating tetrasaccharide containing rhamnose, glucose, mannose and glucuronic acid in differing proportions and frequency, and with distinct side chains, e.g., glycosidic bound glucose or rhamnose (Pollock 2002).

Sphingomonas pituitosa DSM 13101 was isolated and characterized by Denner et al. (2001). The exopolysaccharide (EPS) PS-EDIV, which is produced by this strain, reveals a high viscosity even at low concentrations. Moreover, this high viscosity is stable at high ionic concentrations, at various pH-values, and over a wide range of temperature as described by Denner et al. (2001). With its distinct thickening properties it could thus be a potential alternative to xanthan gum. The authors identified the components glucose, rhamnose and acetyl and glyceryl groups, but could not characterize an additional monosaccharide component of the repeating unit of the EPS. The aim of this study was therefore to completely elucidate the structure of the EPS PS-EDIV. Extensive investigations on the growth and production characterization and purification of the EPS will be described elsewhere.

Materials and methods

Bacterial strain and growth conditions

The bacterial strain used in this work was described by Denner et al. and is deposited at the DSMZ as DSM 13101 (Denner et al. 2001). It was cultivated

on a defined mineral salt medium containing (%, w/v) $NaNO_3$, 3.0; K_2HPO_4, 3.32 and NaH_2PO_4, 0.67 as pH-buffer; KCl, 0.5; $MgSO_4 \times H_2O$, 0.281; $FeCl_3 \times 6H_2O$, 0.025, and sucrose, 50.0. Exponentially growing precultures for submerged cultivations were used for inoculation at a volume ratio of 1:10. For cultivation a 7-L batch glass reactor from Applikon (Applikon® BIOTEK GmbH & Co. Vertriebs KG) with three-stage Intermig impellers was used (operating volume 5 L; temperature 30 °C; stirrer speed 600 min^{-1}; aeration 0.5 L/Lmin). The pH-value was set initially at 7.0 and did not change significantly during the cultivation.

Extraction and purification of PS-EDIV

For separation of the biomass from the polysaccharide the culture broth was centrifuged at 15,000 min^{-1} (26,000g) and room temperature for 30 min. The supernatant containing the polymer was decanted and precipitated by addition of isopropyl alcohol (80 %, v/v). The precipitated polysaccharide was redissolved in distilled water, reprecipitated and centrifuged. For further purification the polymer solution was dialyzed with a dialysis tube with permeability size of 10 kDa. After purification the polymer solution was lyophilized at -62 °C and a pressure of 0.009 mbar.

Gel permeation chromatography

Extracted and purified polymer was redissolved at a concentration of 1 mg/mL in deionized water with 0.2 % (w/v) sodium azide according to Cowie (1997). A PLaquagel-OH Guard 8 µm preliminary column (50 × 7.5 mm) and a PLaquagel-OH Mixed 8 µm column (300 × 7.5 mm) from Polymer Laboratories were used. The polymer was detected with a light dispersion detector (DAWN® DSP Laser Photometer from Wyatt Technology Corporation) and a refractive index detector (RI-101 from Shodex). The process was run at 40 °C with a flow rate of 0.5 mL/min and injection volume of 250 µL of which 100 µL were actually present in the sample loop, corresponding to 100 µg polysaccharide. Rayleigh scattering provides an absolute method to determine molecular weight when the differential index of refraction is known. In this work, 100 % mass recovery and AUX were assumed to be known, so the differential index of refraction dn/dc was calculated to 0.146 and the molecular weight of sphingan can be determined.

Carbohydrate compositional analysis

After methanolysis and trimethylsilylation, monosaccharide constituents of the polysaccharide were analyzed as the corresponding methyl glycosides on a Finnigan GCQ GC/MS (Thermo Finnigan, San Jose, CA) system with a 30 m DB5 capillary column according to Chaplin (1982).

Deacylation and lyophilization of the polysaccharide

Native polysaccharide material was dissolved in 0.1 M NaOH and maintained at 50 °C for 2 h. After neutralization, the deacylated product was dialyzed

against distilled water and the solvent removed at a reduced pressure of 0.009 mbar and at a temperature of -62 °C.

Partial hydrolysis of the polysaccharide

The deacylated polysaccharide was hydrolyzed at 100 °C for 2 h with 1 M acetic acid and the solvent was evaporated at reduced pressure and room temperature. Residual acid was removed by repeated co-evaporation with water (Lobas et al. 1994).

Methylation analysis of the polysaccharide

The intact polysaccharide was permethylated as described by Anumula and Taylor (1992), followed by a reduction with $NaBD_4$ to convert uronic acids to 6-deuterium labeled hexose derivatives. After hydrolysis (4 N TFA, 110 °C, 2 h), reduction ($NaBH_4$), and acetylation, the resulting partially methylated alditol acetates were analyzed on a ThermoQuest GCQ ion trap GC/MS system (MS: EI mode, GC: 30 m DB 5 column).

MALDI-TOF MS/MS

Polysaccharide fragments obtained by partial hydrolysis were reduced, permethylated and analyzed in the positive ion mode on a Bruker ULTRAFLEX time-of-flight (TOF/TOF) instrument using the reflectron with a matrix of alpha-cyano-4-hydroxy-cinnamic acid. For MS-MS analyses, selected parent ions were subjected to laser-induced dissociation (LID) and the resulting fragment ions were separated by the second TOF stage (LIFT) of the instrument. Samples of 1 µL and an approximate concentration of 1–10 pmol/µL were mixed with equal amounts of matrix solution. This mixture was spotted onto a stainless steel target and dried at room temperature before analysis.

Electrospray MS/MS

The reduced ($NaBD_4$) and permethylated oligosaccharides were analyzed by ESI-MS/MS. The samples (~3 µL dissolved in MeOH/water 2:1 with 1 mM NaCl added) were placed in a gold-coated nanospray glass capillary (Protana, Odense, Denmark) and the tip of the capillary was placed orthogonally in front of the entrance hole of a quadrupole time-of-flight (Q-TOF 2) mass spectrometer (Micromass, Manchester, UK) equipped with a nanospray ion source. A voltage of ~1000 V was applied. For collision induced dissociation (CID) experiments, parent ions were selectively transmitted from the quadrupole mass analyzer into the collision cell. Argon was used as the collision gas and the kinetic energy was set to 60-90 eV for fragmentation of the sodium adducts of the oligosaccharides. The resulting daughter ions were then separated by an orthogonal TOF mass analyzer.

1D and 2D ^1H NMR spectroscopy

Prior to all NMR analyses, the polysaccharide (approximately 1.5 mg) was repeatedly lyophilized against D_2O (Fluka, > 99.95 atom% D) at pD 7 and ambient temperature. ^1H NMR spectra of the intact polysaccharide were recorded at 330 K on a Bruker AVANCE DMX 600 NMR spectrometer incorporating a gradient unit. A 1.3 s presaturation pulse was employed prior to all homonuclear proton pulse sequences in order to suppress the signal of the residual HOD resonance. For comparision purposes the ^1H chemical shifts are referenced to the signal of the rhamnose methyl group at 1.33 ppm in D_2O, with an accuracy of better than 0.02 ppm. 2D ^1H correlated spectroscopy (COSY), total correlated spectroscopy (TOCSY), and nuclear Overhauser enhancement and exchange spectroscopy (NOESY) were performed on the same instrument with mixing times of 110 and 500 ms, respectively, for the two latter techniques. All spectra were recorded using standard Bruker software.

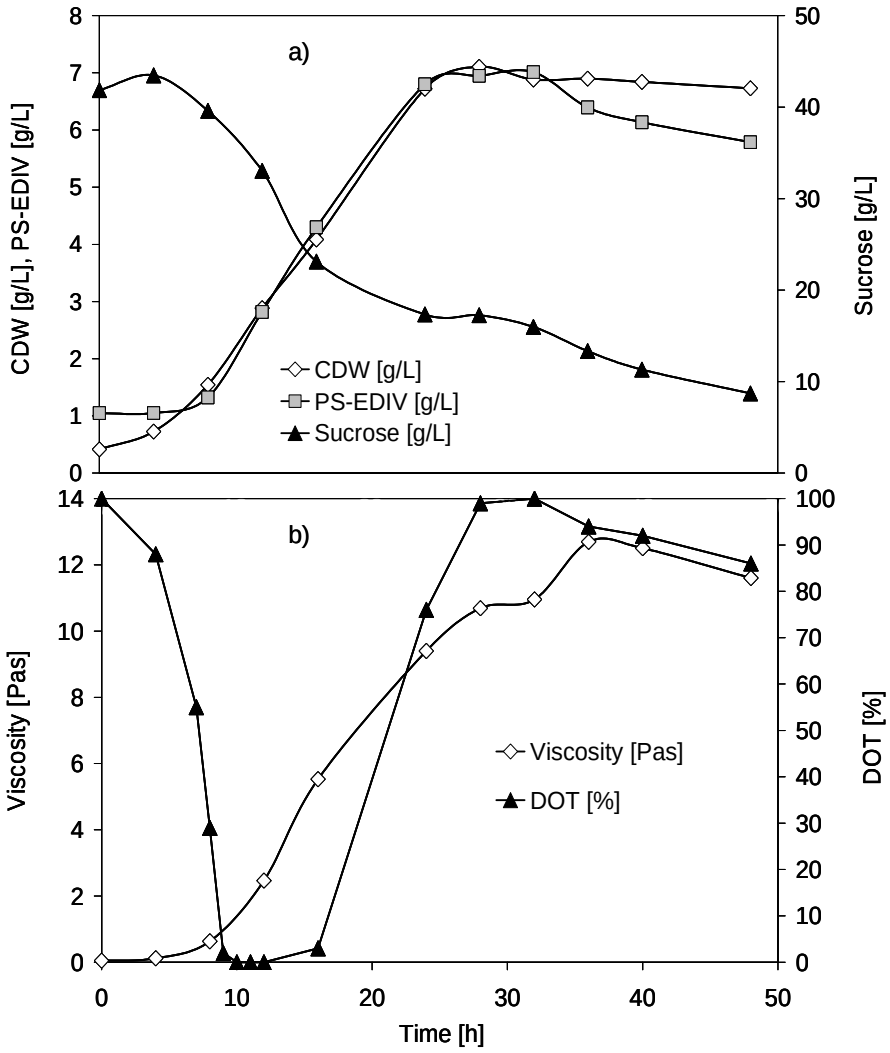


Fig. 1 a: Cell dry weight (CDW), substrate and polysaccharide (PS-EDIV) concentration during cultivation of *Sphingomonas pituitosa* DSM 13101 with sucrose,
b: Apparent viscosity at a shear rate of 1 s^{-1} and dissolved oxygen tension (DOT) during cultivation of *S. pituitosa* DSM 13101 with sucrose

Results

Cultivation of S. pituitosa

When cells of *S. pituitosa* DSM 13101 were incubated in a batch process on a 5 L-scale (600 min^{-1}, 0.5 L/Lmin) with 40 g/L sucrose, the production of the sphingan PS-EDIV proved to be mainly growth-related (Fig. 1a). After 24 h of cultivation, microbial growth stagnated, accompanied by an increased dissolved oxygen tension (Fig. 1b). In the end of cultivation, about 30 g/L of the carbon source were converted to approximately 7 g/L biomass and 7 g/L PS-EDIV. Further growth or production of the EPS was not observed although all required nutrients were present in the culture broth at sufficient concentrations. This discontinued growth might be explained by the high viscosity of the culture broth which increased rapidly during the cultivation process and, hence, caused mass transfer problems. At shear rates of 1 s^{-1} and PS-EDIV concentrations of 5.6 g/L viscosities of 5.8 Pas were observed. Such a high viscosity considerably impaired the oxygen supply to the cells. Detailed information in connection with fermentation process will be published briefly (Dreger et al. personal communication).

Gel permeation chromatography:

The average molecular mass of the PS-EDIV molecule was analyzed by gel permeation chromatography and was found to be approximately 1.2×10^6 Da correlated with the elution volume of 23.3 mL and 25.4 mL, respectively (Fig. 2).

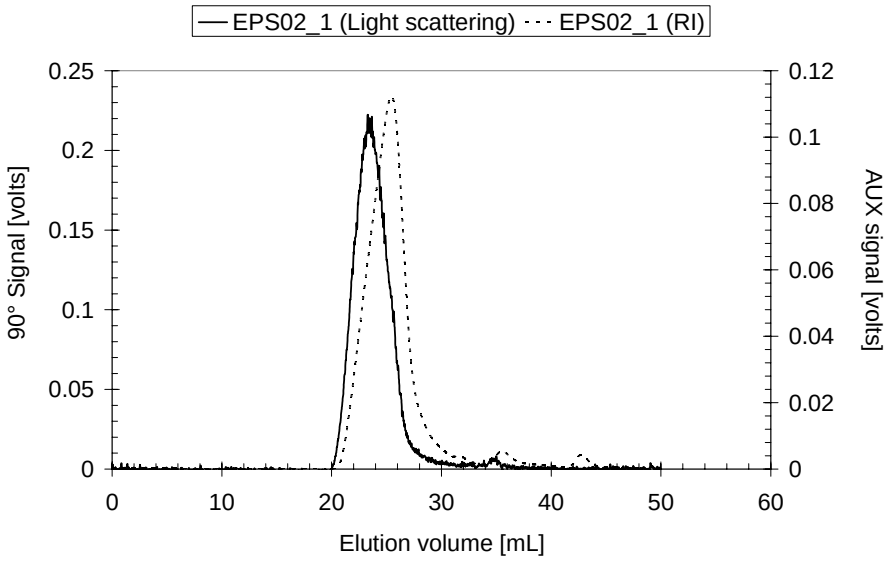


Fig. 2 Chromatogram of GPC with signals of PS-EDIV

Carbohydrate compositional analysis

Monosaccharide analysis of the polysaccharide material revealed that it contained rhamnose and glucose in a 1:4 ratio In addition, traces of glucuronic acid were found whereas deoxyglucuronic acid could not be detected as it decomposes under the conditions used (see below).

1D and 2D NMR

After deacylation and dialysis a 1D ^{1}H NMR spectrum was recorded (Fig. 3) which was found to have very similar NMR data compared to those of the deacylated product from sphingan I-886 (Falk et al. 1996). In brief, the signals of six anomeric protons were observed at 5.18, 4.96, 4.81, 4.73, 4.57 and 4.50 ppm corresponding to those of sugar units E, D, F, C, B and A of sphingan I-886 reported at 5.17, 4.96, 4.81, 4.73, 4.58 and 4.50 ppm, respectively (Falk et al. 1996). Similarly, the high field signals at 2.36, 1.63, 1.33 ppm were also identical with H-2ax and H-2eq of sugar unit F and H-6 of E. A series of 2D spectra (COSY, TOCSY and NOESY) allowed the assignment of the remaining sugar protons in the overlapping region 4.2-3.3 ppm as well as unambiguous confirmation of the partial sugar sequence (C-E-B-F and A-D of sphingan I-886).

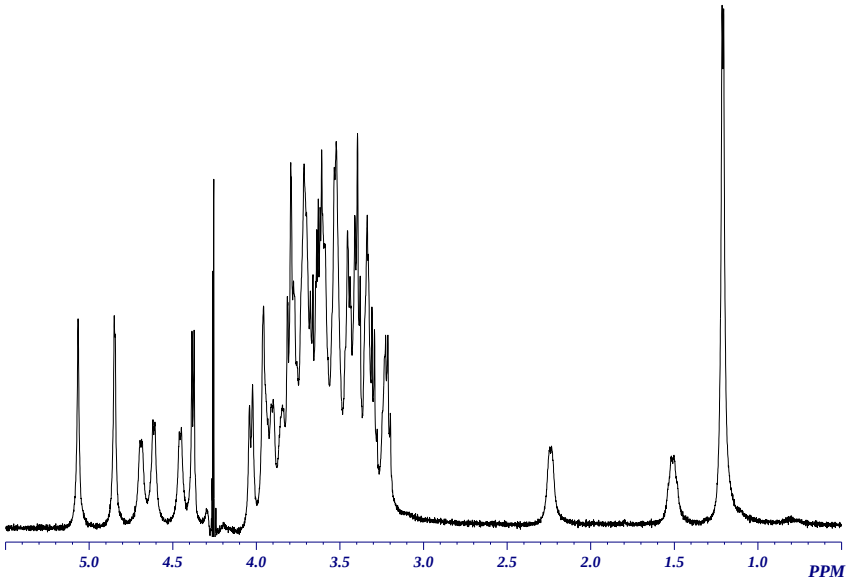


Fig. 3 ^{1}H NMR spectrum of deacylated sphingan PS-EDIV. Chemical shifts are with respect to the methyl group of the rhamnose moiety at 1.33 ppm. The spectrum was unambiguously assigned using a combination of 2D spectra. The chemical shifts were fully compatible with the deacylated product from sphingan I-886 (Falk et al. 1996), apart from the shift of H-5 from the 2-deoxyglucuronic acid moiety (F) which is assumed to be incorrectly reported in the original and overlaps with H-3 or H-4 at 3.83 or 3.64 ppm

However, despite the obvious similarity sphingan PS-EDIV is not identical with sphingan I-886 as is documented below from the methylation analysis of the intact polysaccharide and MALDI and ESI-MS/MS of the partially hydrolyzed polysaccharide fragments.

Methylation analysis

Methylation analysis of the native polysaccharide yielded the partially methylated alditol acetates listed in Table 1. In addition to the derivatives expected for the polysaccharide structure published by Falk et al. (1996), small amounts of the derivative typical for 4-substituted glucuronic acid were identified suggesting a particulate replacement of the 2-deoxyglucuronic acid by glucuronic acid (Falk et al. 1996). This assumption was confirmed by MS

and MS/MS analysis of oligosaccharides obtained by partial hydrolysis of the polysaccharide.

Table 1 NMR data for sphingan PS-EDIV

Peracetylated derivative of	Substituted in position	PS-EDIV
Rhamnitol		
2,3-Di-O-methyl-	4	++
Glucitol		
2,3,4,6-Tetra-O-methyl-	term.	+++
2,4,6-Tri-O-methyl- [a]	3	+
2,3,6-Tri-O-methyl-	4	++
2,3,4-Tri-O-methyl-	6	++
2,4-Di-O-methyl-	3; 6	++
2,3-Di-O-methyl-(6-d_2)- [b]	4	+

[a] The detection of this derivative can be explained by the partial absence/elimination during permethylation of the side chain of the polysaccharide.

[b] This derivative was obtained by an additional $NaBD_4$ reduction of the permethylated sample.

Matrix-assisted laser desorption/ionization-time-of-flight MS/MS

After partial hydrolysis, reduction and permethylation, the resulting oligosaccharides were analyzed by MALDI-TOF MS. Among others, intense signals at m/z 1264.6 $[Hex_3deoxyHexdeoxyHexAHex\text{-}ol\text{-}1D+Na]^+$, 2443.2 $[Hex_7deoxyHex_2deoxyHexA_2Hex\text{-}ol\text{-}1D+Na]^+$ and 3931.8 $[Hex_{11}deoxyHex_3$ $deoxyHexA_3Hex\text{-}ol\text{-}1D+Na]^+$ were detected suggesting a repeating unit consisting of 4 hexose residues, one deoxyhexose and one deoxy-hexuronic acid residue (Fig. 4a).

The sequence of the monosaccharide residues could be deduced from the daughter ion spectra, as shown in Fig. 5a confirming the NMR data.

Obviously, the linkage of the deoxyhexuronic acid to the hexose residue is the weakest and is preferentially cleaved during mild acid hydrolysis. Closer inspection of the MALDI spectrum, however, revealed the presence of a modified repeating unit which could be detected with an intensity of approximately 5-10 % per repeating unit (i.e., 10-20 % intensity in the case of the dimer depicted in Fig. 4b) with an increased mass of 30 Da corresponding to the addition of one oxygen atom in the deoxysugar and one additional methyl group obtained during the permethylation procedure.

MS/MS of this molecular ion clearly showed that the position of the modification corresponded to the deoxyhexuronic acid residue, suggesting the partial conversion of this residue to a hexuronic acid in PS-EDIV (Fig. 5b).

Additional peaks with mass reduced by 14 Da could occur due to undermethylation, and peaks with mass reduced by 205 Da could be due to β-elimination. Since all analyzed modified oligosaccharides (trimers and tetramers were analyzed by ESI-MS/MS; data not shown) had a deoxyhexuronic, but never a hexuronic acid residue at their reducing ends, the

linkage of hexuronic acid to its neighbouring hexose must be acid stable in contrast to the analogous linkage of its deoxyhexose derivative. Furthermore, the relative intensities of oligomers with zero, one or two glucuronic acids showed no clear repeating pattern suggesting a statistical replacement of every 10th-20th deoxyglucuronic by glucuronic acid.

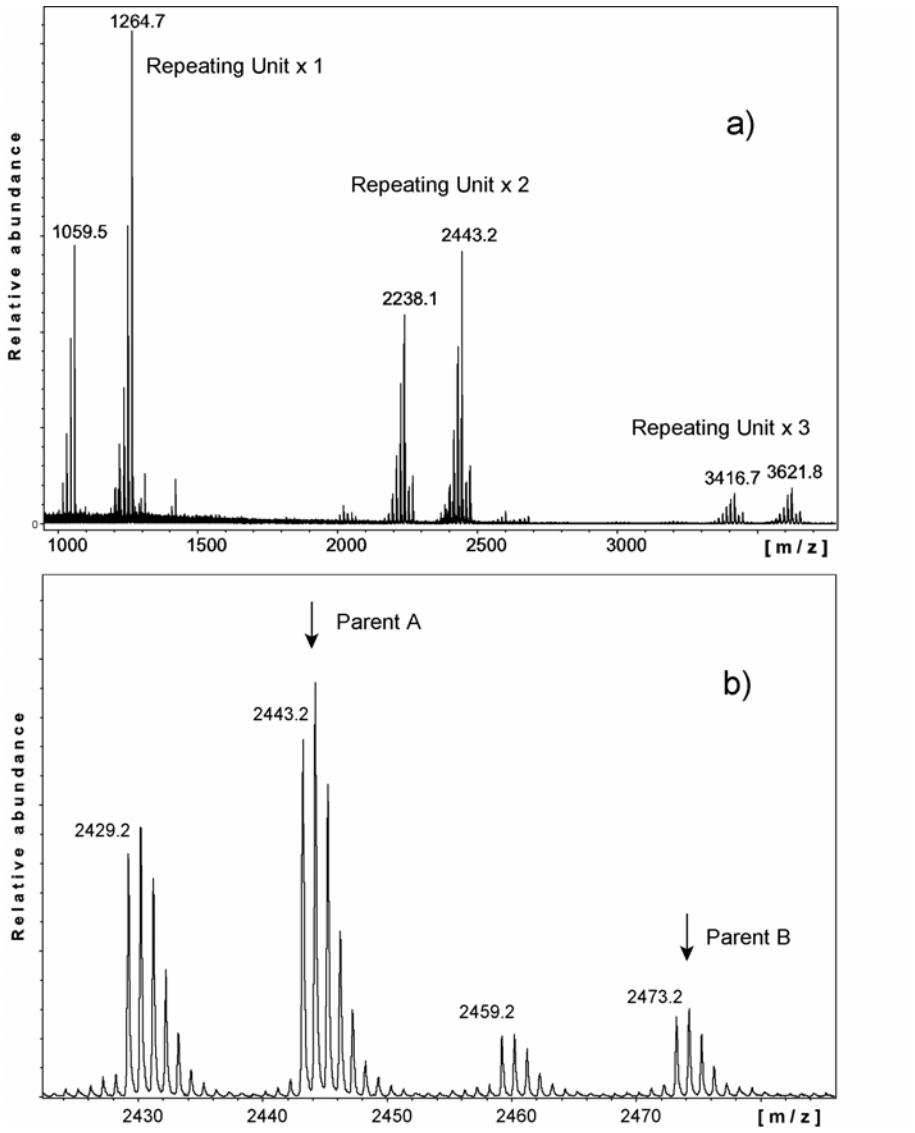


Fig. 4 a: MALDI spectrum of the monomer, dimer and trimer of the PS-EDIV, b: Section of the MALDI spectrum with the two dimer forms, (a) m/z = 2443 = 2x 2dGlcA, (b) m/z = 2473 = 1x 2dGlcA and 1x GlcA

Finally, the MS/MS experiments clearly showed, that both hexuronic acid forms are members of one polysaccharide chain and exclude the presence of two different polysaccharide forms having either deoxyhexuronic or hexuronic acid constituents.

Attempts to elucidate the structure of the native polysaccharide prior to deacylation failed. NMR as well as MS experiments gave clear indications of the presence of at least one acetyl group. However, the position of acetylation could not be determined due to a migration of the acetyl group during the NMR experiments.

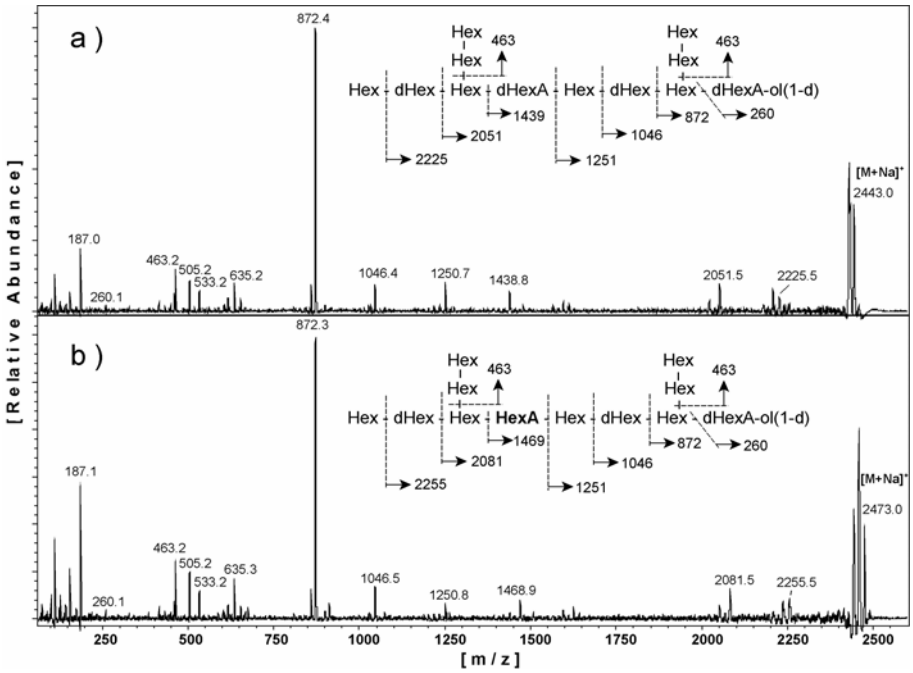


Fig. 5 MS-MS-Spectrum, a) Dimer with 2x 2dGlcA, b) Dimer with 1x 2dGlcA and 1x GlcA, fragmentation pattern is identical up to 1250, fragments always end with 2dGlcA, which is a predetermined breaking point

These results strongly suggested that the PS-EDIV is based on the following monomer structure as repeating unit:

$$\rightarrow 4)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-}\begin{matrix}2\text{dGlc}pA_{0.90\text{-}0.95}\\ \textbf{or}\\ \text{Glc}pA_{0.05\text{-}0.10}\end{matrix}\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\ (1\rightarrow$$

$$\begin{matrix}6\\ \uparrow\\ 1\end{matrix}$$

$$\beta\text{-D-Glc}p\text{-}(1\rightarrow 6)\text{-}\alpha\text{-D-Glc}p$$

Discussion

In this study, a detailed characterization of the exopolysaccharide PS-EDIV produced by *Sphingomonas pituitosa* DSM 13101 is presented. This is the first report of a bacterial polysaccharide containing deoxyglucuronic and glucuronic acids in the same molecule.

The molecular mass of the sphingan PS-EDIV found by Denner et al. was 3×10^6 Da (Denner et al. 2001). In contrast, the molecular mass determined in this study was only 1.2×10^6 Da, but these values are yet of the same magnitude. The difference is most probably due to differences in cultivation conditions. The exact dependence of the mean molecular weight of PS-EDIV on the cultivation conditions has to be elucidated in further studies.

The structure of PS-EDIV is quite similar to that of sphingan I-886 (structure with 100 % dGlcA), or rhamsan (S-194), which contains exclusively glucuronic acid at the third position in the repeating unit. However, a polysaccharide with both acidic residues has never been described before. The

substitution of glucuronic acid and deoxyglucuronic acid in the third position might occur through the same mechanism as that for the change of rhamnose and mannose in the first position in S-88 or S-198, where the substituents are distributed statistically at a ratio of 1:1 (Pollock, 2002), or in welan, where a similar substitution in the side chain is observed. Possibly, the substitution of the hydroxyl group in position 2 from GlcA to 2dGlcA might be analogous to the separation mechanism of the hydroxyl group in position 6 from mannose to rhamnose. Elucidation of such a mechanism may allow a controlled removal of the hydroxyl group for producing molecules with a defined ratio of GlcA and 2dGlcA. Alternatively, it might be possible to select strains which produce different ratios of these residues and - perhaps by modification of the biosynthesis - to create polysaccharides with interesting custom-made properties. As deoxyglucuronic acid is the most acid-labile residue of these carbohydrate polymers (see above), a controlled degradation of a polysaccharide matrix containing pharmacologically active substances, for instance, could be an interesting application. The interactive possibility for controlled degradation is the composition of a variable portion of 2dGlcA: An increased portion would lead to a faster degradation because more predetermined breaking points were available.

Acknowledgements

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