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Note

Chemical structures of the secondary cell wall polymers (SCWPs) isolated from bovine mastitis *Streptococcus uberis*

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ABSTRACT

The cell envelope of Gram-positive bacteria is decorated with a variety of polysaccharides. In this study wall teichoic acid (WTA) and neutral polysaccharides were isolated from the cell envelope of bovine mastitis *Streptococcus uberis*. The polysaccharides were released by lysozyme treatment, and purified by hydrophobic interaction chromatography. Further separation was achieved utilizing anion-exchange chromatography which yielded two products, that is, a neutral polysaccharide with a high content of Rha and less Glc (rhamnan) and an anionic phosphate-rich one containing glycerol and Glc (WTA). The structures of these molecules were elucidated applying 1D and 2D nuclear magnetic resonance experiments as well as chemical analyses. In the rhamnan sample two independent molecules were identified, that is, a glucorhamnan with the structure →2)-α-L-Rha*p*-(1→3)-[α-D-Glc*p*-(1→2)-]α-L-Rha*p*-(1→, and a homopolymeric rhamnan →2)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→. The WTA comprised a polyphosphoglycerol chain substituted nonstoichiometrically with β-Glc*p*.

1. Introduction

Bovine mastitis, namely inflammation of the udder, is a result of intramammary infection by bacteria.[1] The course of the disease may be either sub-clinical or clinical depending on the factors in the host and the invading pathogen. Sub-clinical mastitis shows no obvious signs of disease, goes often unnoticed and untreated, resulting in long duration of the infection.[2] The clinical form of the disease is characterized by visible abnormalities in the milk (protein aggregates or clots) accompanied by pain and swelling in the affected gland and sometimes production of a secretion composed solely of aggregated protein in a serous fluid. In severe cases there may be systemic signs such as elevated temperature and loss of appetite which may lead to bacteremia, septicemia, and death of the animal.[1]

Over 135 infectious agents were reported to cause mastitis[3] but most infections are due to one of the three bacterial species *Streptococcus uberis*,[4] *Escherichia coli*,[4] and *Staphylococcus aureus*.[5]

The cell envelope of Gram-positive bacteria is decorated with a variety of polysaccharides. Based on their structural character, they can be classified into the three groups, (i) teichoic acids, (ii) teichuronic acids, and (iii) other polysaccharides (neutral or acidic) which do not belong to any of the other groups.[6]

Teichoic acids [TAs, lipoteichoic acid (LTA), and wall teichoic acid (WTA)] are phosphate-rich polyalditols which were shown to contribute to resistance to environmental stresses,[7,8] antimicrobial peptides,[9] cationic antibiotics, and lytic enzymes produced by the host.[10] Non classical polysaccharides were reported to play an important role in non-covalent attachment of the S-layer to the underlying peptidoglycan (PG).[11] The structure of the *S. uberis* LTA has been published recently.[12] A body of literature reveals a wide structural diversity of Gram-positive bacterial WTAs which are covalently bound to *O*-6 of muramic acid (MurNAc) of PG via a phosphodiester bond.[13] Their repeating unit often possesses a similar structure as that of LTA, comprising a polyglycerol- or polyribitolphosphate nonstoichiometrically substituted with alanine or glycosyl residues,[14,15] but may also show considerable differences. Structural analyses of non-classical SCWPs revealed that they are either charged[16] or neutral[17] heteropolysaccharides covalently bound to PG via a phosphodiester[18] or pyrophosphate linkage.[19] The present study deals with the structures of WTA and a neutral polysaccharide isolated from the cell envelope of one of the most important mastitis pathogens, *S. uberis*.

2. Results and discussion

The polysaccharides were released from disrupted bacterial cells of *S. uberis* 233 by lysozyme treatment, followed by incubation with RNase, DNase, and proteinase K. Hydrophobic interaction

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chromatography (HIC) was performed in order to remove possible remains of LTA. Separation of polysaccharides could be achieved by anion-exchange chromatography which yielded three fractions. Of these, the first and second contained the same material according to the anomeric regions of ^{1}H NMR spectra, a rhamnan, due to the high content of Rha residues. Since the quality of the 2D spectra recorded for fraction 1 was not sufficient for a successful assignment of the signals, further analyses were performed on fraction 2. The third fraction, phosphate-rich, was identified as WTA.

Compositional analysis of the rhamnan identified Rha and Glc in an approx. molecular ratio of 4:1. Their absolute configurations were determined as L and D, respectively. Methylation analysis revealed the presence of 2-substituted, 3-substituted, and 2,3-disubstituted Rha, and terminal Glc by identifying 1,2,5-tri-*O*-acetyl-6-deoxy-3,4-di-*O*-methyl-[1-^{2}H]mannitol, 1,3,5-tri-*O*-acetyl-6-deoxy-2, 4-di-*O*-methyl-[1-^{2}H]mannitol, 1,2,3,5-tetra-*O*-acetyl-6-deoxy-4-*O*-methyl-[1-^{2}H]mannitol, and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-[1-^{2}H]glucitol. The WTA consisted of Gro, phosphate, and Glc. The absolute configuration of Glc was determined as D.

Both, rhamnan and WTA were investigated by 1D and 2D NMR experiments. The 1D ^{1}H NMR spectrum of the rhamnan revealed 5 major signals in the anomeric region [4 originating from Rha*p* (**A–C**, **E**) and 1 from Glc*p* (**D**) residues], several characteristic signals at δ 1.28–1.24 (methyl groups of 6-deoxyhexoses) and 2 signals at δ 2.06 and δ 2.02 (methyl group of *N*-acetyl function) (Fig. 1a). Since the latter signals were greatly diminished after further purification of the sample on TSK 40 (not shown) they were assigned as impurities. The anomeric configurations were determined on the basis of $^1J_{C\text{-}1,H\text{-}1}$ values ranging from 173 to 178 Hz (derived from a coupled heteronuclear single quantum coherence ^{1}H,^{13}C-HSQC spectrum) as α for all residues (**A–E**).

The anomeric region of the 1D ^{1}H NMR spectrum of WTA possessed two signals **A**′ and **B**′ (δ 5.16 and δ 5.09, respectively) of similar low intensity, and one more intense signal at δ 4.64 (**C**′, Fig. 1b). Additionally, several signals of ring protons and Gro at δ 4.22–3.31 and minor characteristic for the methyl group of *N*-acetyl functions at δ 2.08–2.02 were observed.

The signals from *N*-acetyl groups identified in both spectra belong neither to rhamnan nor to WTA. Presumably they originated from PG constituents, namely GlcNAc and MurNAc as the samples were treated with the lysozyme. However, the complete spin systems of GlcNAc and MurNAc could not be identified.

Detailed analyses applying ^{1}H,^{1}H correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), rotating frame Overhauser effect spectroscopy (ROESY), and ^{1}H,^{13}C heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC) experiments were performed in order to establish structures of both examined molecules.

The 2D spectra of the rhamnan identified several minor signals in the anomeric region and those originating from short phosphoglycerol chains which were not detected by the ^{1}H NMR experiment. After further purification of the sample by size exclusion chromatography on TSK-40 these signals could be assigned to impurities. Due to better quality of spectra, chemical shift assignments of strong signals corresponding to all peaks of ^{1}H spectrum were made on the non-purified sample (Table 2) and only those cross-peaks are depicted in Figure 2. In the HMBC experiment, connectivities between **A**, H-1/**C**, C-3; **B**, H-1/**E**, C-3; **C**, H-1/**A**, C-2; **E**, H-1/**B**, C-2 and **C**, H-2/**D**, C-1 (for **A-E**, see Table 1) were found which were confirmed by a ROESY experiment (*inter*-residual NOE contacts between **A** H-1/**C** H-3, **B** H-1/**E** H-3, **C** H-1/**A** H-2, **D** H-1/**C** H-2, and **E** H-1/**B** H-2). These data suggested the existence of two rhamnan polymers, first possessing the structure →2)-**B**-(1→3)-**E**-(1→, and the second →2)-**A**-(1→3)-[**D**-(1→2)-]**C**-(1→] (Fig. 3). No indication was obtained that both partial structures were linked (no cross connectivity between **E-**1 and **A-**2 was observed, and NOE contacts between **A** H-3/**D** H-4 and **A** H-6/**D** H-2 found in the ROESY spectrum were not observed between **B** and **D**).

2D NMR experiments of the WTA allowed the assignment of all chemical shifts (Fig. 4, Table 2). The *intra*-residual NOE contacts observed in the ROESY spectrum between H-1 and H-2 of **A**′ and **B**′ (for **A**′**–G**′ see Table 2) and the chemical shifts of anomeric protons (δ 5.16 and 5.09) proved the α-configuration of the sugars. Their *gluco* configuration was confirmed by an *intra*-residual H-2/H-4 NOE connectivity. Additionally, the ROESY spectrum showed NOE contacts between **B**′ H-1/**A**′ H-2 and **A**′ H-1/**D**′ H-3 indicating the structure α-D-Glc*p*-(1→2)-α-D-Glc*p*-(1→3)-Gro. In our previous studies we identified this molecule to be a backbone of the lipid

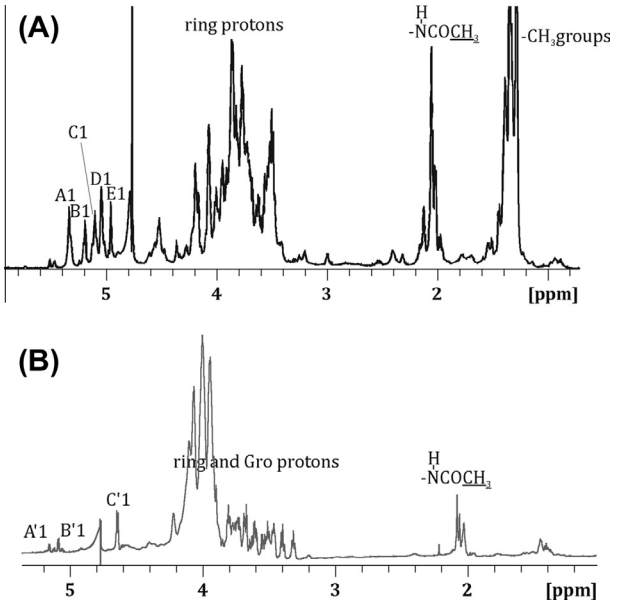

Figure 1. ^{1}H NMR spectra (700 MHz) of (a) rhamnan and (b) WTA isolated from *S. uberis* 233. The capital letters refer to the residues defined in Tables 1 and 2. Spectra were recorded in D_2O at 27 °C relative to external acetone (δ_H 2.225; δ_C 31.45).

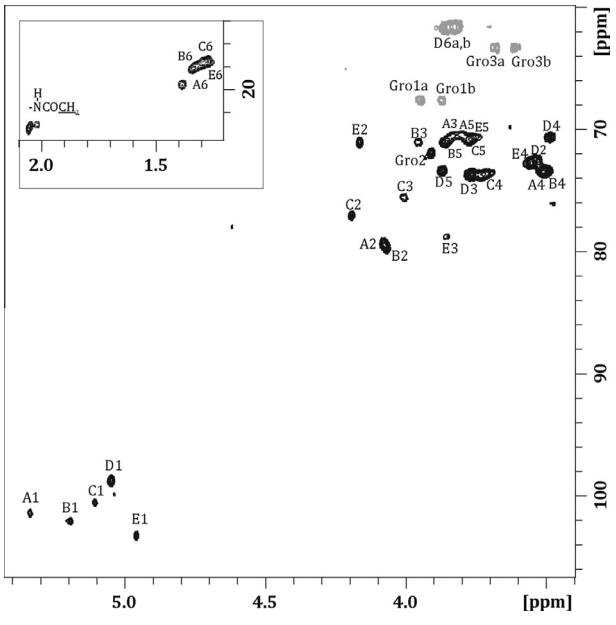

Fig. 2. Excerpt of ^{1}H,^{13}C HSQC-DEPT spectrum (700 MHz) of the rhamnan isolated from *S. uberis* 233. The spectrum was recorded in D_2O at 27 °C relative to external acetone (δ_H 2.225; δ_C 31.45).