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Structural characterization of the acid-degraded secondary cell wall polymer of *Geobacillus stearothermophilus* PV72/p2

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Abstract—The secondary cell wall polymer (SCWP) from *Geobacillus stearothermophilus* PV72/p2, which is involved in the anchoring of the surface-layer protein to the bacterial cell wall layer, is composed of 2-amino-2-deoxy- and 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-mannose, and 2-acetamido-2-deoxy-D-mannuronic acid. The primary structure of the acid-degraded polysaccharide—liberated by HF-treatment from the cell wall—was determined by high-field NMR spectroscopy and mass spectrometry using N-acetylated and hydrolyzed polysaccharide derivatives as well as Smith-degradation. The polysaccharide was shown to consist of a tetrasaccharide repeating unit containing a pyruvic acid acetal at a side-chain 2-acetamido-2-deoxy-α-D-mannopyranosyl residue. Substoichiometric substitutions of the repeating unit were observed concerning the degree of N-acetylation of glucosamine residues and the presence of side-chain linked 2-acetamido-2-deoxy-β-D-glucopyranosyl units:

(**C**) (**D**) (**B**)
[-4)-β-D-Man*p*NAcA-(1-3)-β-D-Glc*p*N(Ac)$_{\sim 0.3}$-(1-6)-α-D-Glc*p*NAc-(1-]
3 4
| |
1 1
(β-D-Glc*p*NAc)$_{\sim 0.3}$ (**E**) (*S*)-Pyr-4,6-α-D-Man*p*NAc (**A**)

Keywords: *Geobacillus*; Secondary cell wall polymer; NMR spectroscopy; Mass spectrometry; Polysaccharide structure

1. Introduction

Crystalline bacterial cell surface layer (S-layer) proteins represent the outermost cell envelope component of many bacteria and archaea.[1–3] S-layers completely cover the cell surface during all stages of growth and division and they either exhibit oblique, square, or hexagonal lattice symmetry. Most S-layers are composed of single protein or glycoprotein species with molecular masses ranging from 40 to 200 kDa. The S-layer subunits are linked to each other by non-covalent forces. S-Layer proteins from gram-positive bacteria are bound to the rigid, peptidoglycan-containing layer via the so-called secondary cell wall polymers (SCWPs).[4–20] S-Layer proteins from *Bacillaceae* frequently carry an S-layer-homologous (SLH) domain on the N-terminal part.[21]

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SLH-domains typically consist of three modules of about 55 amino acids from which 10 to 15 residues are conserved. Several studies indicated that SLH-domain carrying proteins bind to pyruvylated SCWPs.[5,9–11,14,16,17] Detailed studies regarding the interaction between an SLH-domain and the corresponding SCWP were carried out with the S-layer protein SbsB and the corresponding SCWP of *Geobacillus stearothermophilus* PV72/p2.[15] Surface plasmon resonance (SPR) spectroscopic measurements indicated the existence of three differently strong binding sites with low ($K_d = 2.6 \times 10^{-5}$ M), medium ($K_d = 6.1 \times 10^{-8}$ M), and high ($K_d = 6.7 \times 10^{-11}$ M) affinities. This feature was explained by heterogeneities always associated with naturally occurring heteropolysaccharides; although regarding the high affinity binding site, avidity effects resulting from the binding of SLH-domain dimers could not be completely excluded.[15] The recognition mechanism between the SLH-domain of SbsB and the SCWP of *G. stearothermophilus* PV72/p2 was found to be highly specific, as the SLH-domain neither recognized the peptidoglycan, nor pyruvylated SCWPs of other organisms. Furthermore, not the *N*-acetyl groups from the amino sugars but the pyruvic acid residues played a crucial role in the binding process.[15,17]

In previous studies, the structures of the acid-degraded and the native SCWP of *Bacillus sphaericus* CCM 2177 were elucidated by NMR spectroscopy analysis.[10,22] Contrary to the results obtained with the S-layer protein SbsB, the three SLH-motifs of the S-layer protein SbpA were not sufficient for binding to the corresponding SCWP of *B. sphaericus* CCM 2177, and a 58-amino acid long SLH-like motif located just behind the third SLH-motif was required for reconstituting the functional, SCWP-binding domain.[9]

In the present study, the structure of the HF-treated SCWP of *G. stearothermophilus* PV72/p2 was analyzed with the tools of NMR spectroscopy and mass spectrometry in combination with N-acetylation, Smith-degradation, and hydrolytic removal of pyruvic acid acetal residues.

2. Results and discussion

2.1. Chemical characterization and modification of the SCWP of *G. stearothermophilus* PV72/p2

Previous results on the linkage of SCWP to the peptidoglycan revealed the presence of pyrophosphate or phosphodiester bridges from 2-acetamido-2-deoxy-D-glucose to O-6 of muramic acid being susceptible to HF-induced cleavage.[28] Treatment of the SCWP of *G. stearothermophilus* PV72/p2 with HF at 4 °C afforded a polysaccharide material **1**, which was purified by GPC and eluted as a single peak. After hydrolysis of the GPC-purified

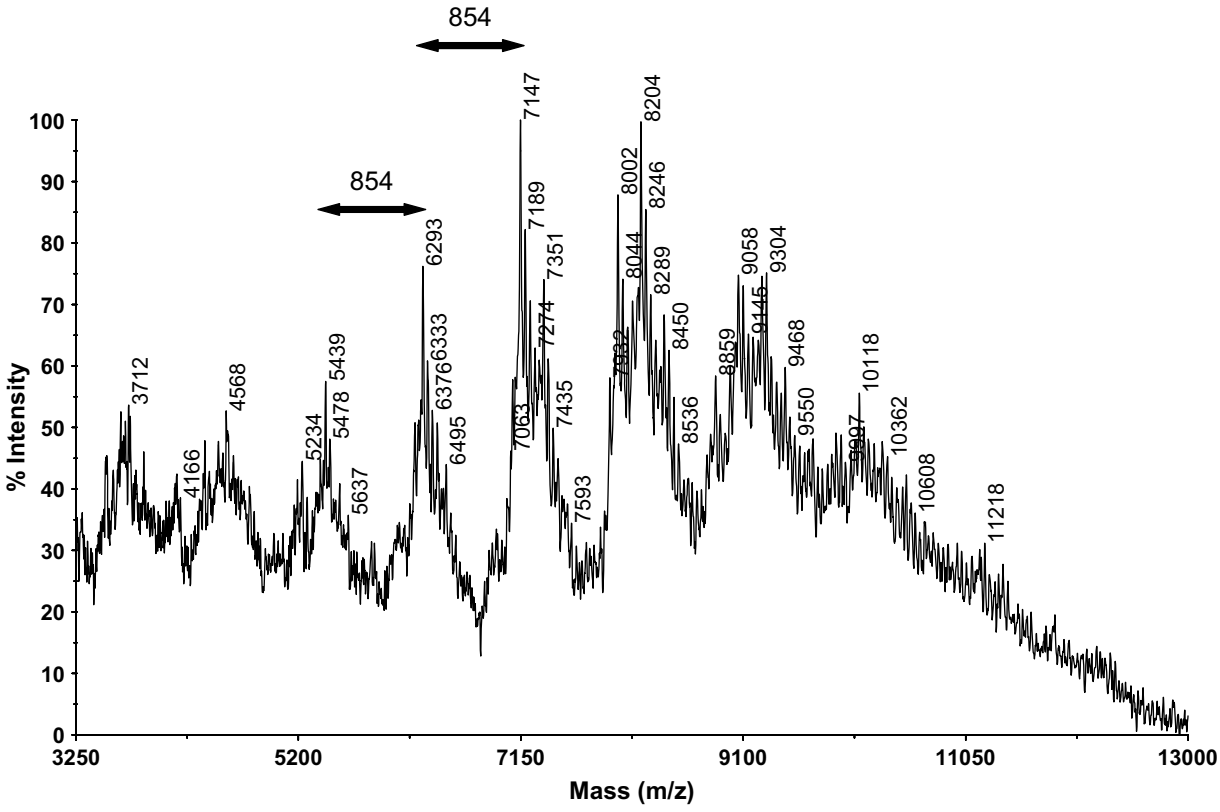

Figure 1. MALDI-TOF mass spectrum of the HF-treated secondary cell wall polymer **1** from *Geobacillus stearothermophilus* PV72/p2.