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Note

Isolation and characterization of an amino sugar-rich glycopeptide from the surface layer glycoprotein of *Thermoanaerobacterium thermosaccharolyticum* E207-71

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The characterization of surface layers (S-layers) [1] of different clostridia has indicated the presence of covalently linked carbohydrate residues [2,3]. The glycan content comprises up to 20% of the S-layer material and is considerably higher than that of aerobic bacilli [4,5]. There exist great variations, not only in the glycan structures of S-layers of different species of clostridia, but also in the glycans of different strains of the same species [3].

Thermoanaerobacterium thermosaccharolyticum (formerly *Clostridium thermosaccharolyticum* [6]) strain E207-71 is an anaerobic, thermophilic eubacterium that was isolated from extraction juices of an Austrian beet sugar factory [7]. The bacteria were completely covered by a squarely arranged S-layer lattice consisting of glycoprotein molecules with a centre-to-centre spacing of approximately 12.3 nm when they were continuously grown in the fermenter with an average dilution rate of $D = 0.10\ h^{-1}$ [8]. Each morphological unit consisted of four identical subunits. SDS-polyacrylamide gel-electrophoresis (SDS–PAGE) of the purified S-layer glycoprotein showed distinct

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ladder-like bands in the molecular mass range between approximately 80 and 210 kDa (not shown), representing the glycosylated S-layer protomers. Glycopeptides were prepared from purified S-layer glycoproteins by exhaustive digestion with pronase according to standard procedures [9,10]. The purification of the glycosylated degradation products was achieved by a combination of gel-permeation chromatography on Bio-Gel P-4 and cation-exchange chromatography with Dowex 50W-X8, yielding a mixture of glycopeptides and peptides. Approximately 38% of the glycopeptides bear a glycan with a hexasaccharide repeating unit structure, and approximately 4.5% contain an amino sugar-rich compound. Upon chromatofocusing between pH values of 9.2 and 5.7 using the Polybuffer™ system (Pharmacia) three glycopeptide fractions, designated FI–FIII, were obtained (not shown), all of which contained an identical polysaccharide as major glycan component. This polysaccharide consists of branched hexasaccharide repeating units [8]. As demonstrated by reversed-phase-HPLC of the desalted glycopeptide pools after chromatofocusing, only fraction FI and FIII appeared to be homogeneous. Fraction FII, in addition to the major glycopeptide (referred to as **HS**), contained approximately 30% of an amino sugar-rich glycan (**PS**) consisting of 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-mannose and D-ribose in the ratio 1.5:1:1, as determined by high-performance anion-exchange chromatography using pulsed electrochemical detection on a Carbo-Pac PA-1 column (HPAEC/PED). The configuration of these glycoses was established by capillary GLC of their trimethylsilylated (−)-2-butyl glycosides [11]. Separation of the two overlapping glycopeptides into pools 1 and 2 could be achieved by gel filtration of FII on Bio-Gel P-100 (1 × 110 cm) (Fig. 1). Pool 1 (fractions 32–43) contained 42% of the total amount of FII comprising almost exclusively of **HS** (96%). Pool 2 (fractions 44–58) represented 56% of the total amount of FII and contained the **PS** component to an extent of 78%. The elution profile was recorded at 280 nm for the detection of tyrosine residues, and at 214 nm for the monitoring of peptides. Absence of absorption at 280 nm, corresponding to the amino sugar containing component, made it clearly distinguishable from the contaminating hexasaccharide bearing glycopeptide and indicated that there was no detectable amount of tyrosine present in the examined glycopeptide fraction [8]. This was confirmed by N-terminal sequencing of the respective peptide fragment. The molecular weight of the amino sugar-rich glycopeptide **PS** was significantly lower than that of the major heteroglycan **HS** (Fig. 1). Pool 2 was rechromatographed on a Bio-Gel P-100 column, with only minor portions of **HS** being removed, yielding **PS** with a purity of 80%. Further enrichment of the **PS** compound was achieved by semipreparative C_{18} reversed-phase HPLC. It eluted with 6.3% acetonitrile as a broad peak containing 13% of a contaminating **HS** material (not shown). The **PS**-containing fractions were collected and analyzed further. The amino acid sequence of the HPLC pool from this material was determined as X–Asp–Pro by N-terminal sequencing, where X represents presumably the glycosylation site which was blocked in the Edman degradation. The carbohydrate content of the investigated material was approximately 93%, and it exclusively contained the above-mentioned S-layer glycopeptide. The chemical composition of this amino sugar-rich component **PS** of fraction FII is summarized in Table 1.

In order to determine the backbone structure of the partially purified **PS** (87% purity) from the S-layer glycoprotein of *T. thermosaccharolyticum* E207-71, it was subjected to

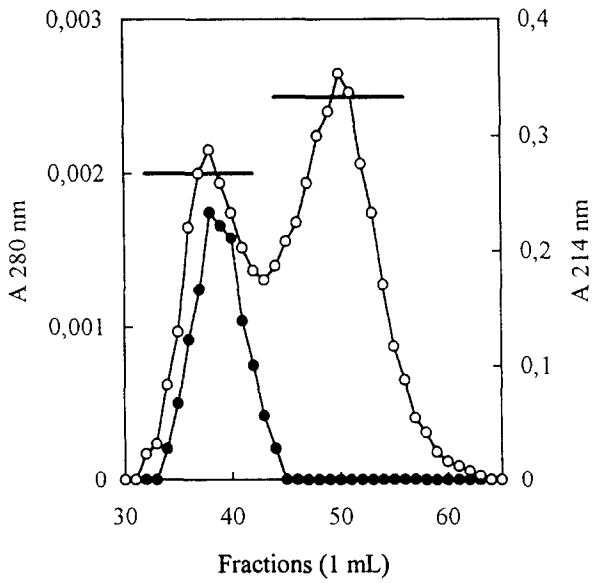


Fig. 1. Elution profile of FII after chromatofocusing on Bio-Gel P-100 (1×110 cm). An overlapping separation of the two glycopeptides contained in FII was achieved by gel-filtration chromatography with 0.1 mM NaCl as eluent [(●) A 280 nm; (○) A 214 nm]. Fractions 32–43 (pool 1) and 44–58 (pool 2) were collected (————).

periodate oxidation and Smith-type hydrolysis [8]. After reduction with $NaBH_4$, the products were separated on a Bio-Gel P-2 column (1 × 110 cm). The oxidized amino sugar-rich compound **PS** eluted in the void volume of the column, whereas the hexasaccharide **HS** was completely degraded by periodate (not shown). Carbohydrate analysis by HPAEC/PED showed 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-mannose in the ratio of 1.1 to 1.0.

Chemical and spectroscopical analyses were performed on both the native and the oxidized material. The characterization of the amino sugar-rich polymer included monosaccharide analysis, methylation analysis, and one- and two-dimensional nuclear magnetic resonance spectroscopy (1D and 2D NMR spectroscopy).

The 1D ^1H NMR spectrum of **PS** showed four resonances in the low-field region (5.5–4.4 ppm) in the relative ratio of 1:2:2:2, of which three were attributed to resonances from anomeric protons by direct correlation with corresponding ^{13}C resonances in a 2D heteronuclear ^{13}C–^1H correlation (HMQC) spectrum.

Table 1
Chemical characterization of the amino sugar-rich glycopeptide fraction FII

Sugar component	Molar ratio	pI value	Peptide sequence [a]
GlcNAc	1.5	7.65	X–Asp–Pro
ManNAc	1.0		
Ribose	1.0		

[a] X, unknown amino acid; Asp, aspartic acid; Pro, proline.

Table 2
Chemical shifts of the ^{1}H resonances (in ppm) and coupling constants ($J_{1,2}$ in Hz) for the native glycan (**PS**) [a] and its Smith degradation product (**SH–PS**) [b]

Residue		Glycose unit	H-1	H-2	H-3	H-4	H-5	H-6	NAc
a	**PS**	α-D-Rib*f*-(1 →	5.377 (4.5)	4.15	4.05	4.13	3.75; 3.65		
b	**PS**	→ 3)-β-D-Man*p*NAc-(1 → 4 ↑	4.840 (< 2)	4.658	4.269	3.74	3.55	3.92; 3.81	2.047 [c]
c	**PS**	→ 3)-β-D-Man*p*NAc-(1 →	4.840 (< 2)	4.658	4.04	3.61	3.46	3.92; 3.81	2.064 [c]
	SH–PS		4.835 (< 2)	4.658	4.04	3.61	3.46	3.92; 3.80	2.059 [d]
d	**PS**	→ 4)-β-D-Glc*p*NAc-(1 →	4.585 (8)	3.73	3.71	3.71	3.51	3.87; 3.74	2.018 [c]
e	**PS**	→ 4)-β-D-Glc*p*NAc-(1 →	4.569 (8)	3.73	3.71	3.71	3.51	3.87; 3.74	2.021 [c]
	SH–PS		4.609 (8)	3.75	3.72	3.70	3.52	3.88; 3.73	2.031 [d]

[a] Chemical shifts were measured at 27 °C in D_2O with acetone as internal reference (2.225 ppm).
[b] Chemical shifts were measured at 70 °C in D_2O with acetone as internal reference (2.225 ppm).
[c,d] Assignments may be interchanged.

Assignments of the ^{1}H resonances in **PS** and **SH–PS** (Table 2) were made using 2D proton chemical shift correlated spectroscopy (COSY) and total correlated spectroscopy (TOCSY). The glycose residues in **PS** were labelled **a–e**, in order of descending chemical shifts of the corresponding anomeric protons in the ^{1}H NMR spectrum. Assignment of the ^{13}C resonances (Table 3) was carried out by direct correlation of the ^{1}H resonances with the ^{13}C resonances in a 2D heteronuclear ^{13}C–^{1}H correlation (HMQC) spectrum and a comparison with the chemical shifts of monosaccharides [12] (Fig. 2).

Table 3
Assignments of the ^{13}C resonances for the native glycan (PS) [a], its Smith degradation product (**SH–PS**) [b] and corresponding data for monosaccharides [c]

Residue		Glycose unit	C-1	C-2	C-3	C-4	C-5	C-6	NAc
a	**PS**	α-D-Rib*f*-(1 →	104.81	72.27	70.46	85.78	62.45		
	ref	α-D-Rib*f*-OMe	103.10	71.1	69.8	84.6	61.9		
b	**PS**	→ 3)-β-D-Man*p*NAc-(1 → 4 ↑	100.46	50.61	76.17	73.09	76.17	61.29	22.83; 175.32
c	**PS**	→ 3)-β-D-Man*p*NAc-(1 →	100.46	50.61	77.65	65.71	77.00	61.29	22.83; 175.32
	SH–PS		100.27	50.60	77.85	65.87	77.04	61.45	22.87; 175.13
	ref	β-D-Man*p*NAc-(1 →	93.91	54.94	73.00	67.65	77.25	61.54	22.98; 176.39
d		→ 4)-β-D-Glc*p*NAc-(1 →	98.09	55.91	79.83	73.09	75.15	60.86	23.16; 175.32
e	**PS**	→ 4)-β-D-Glc*p*NAc-(1 →	100.46	50.61	77.65	65.71	77.00	61.29	22.83; 175.32
	SH–PS		100.27	50.60	77.85	65.87	77.04	61.45	22.87; 175.13
	ref	β-D-Glc*p*NAc	93.91	54.94	73.00	67.65	77.25	61.54	22.98; 176.39

[a] Chemical shifts (in ppm) were measured at 27 °C in D_2O with acetone as internal reference (31.07 ppm).
[b] Chemical shifts (in ppm) were measured at 70 °C in D_2O with acetone as internal reference (31.07 ppm).
[c] See Refs. [12,13].

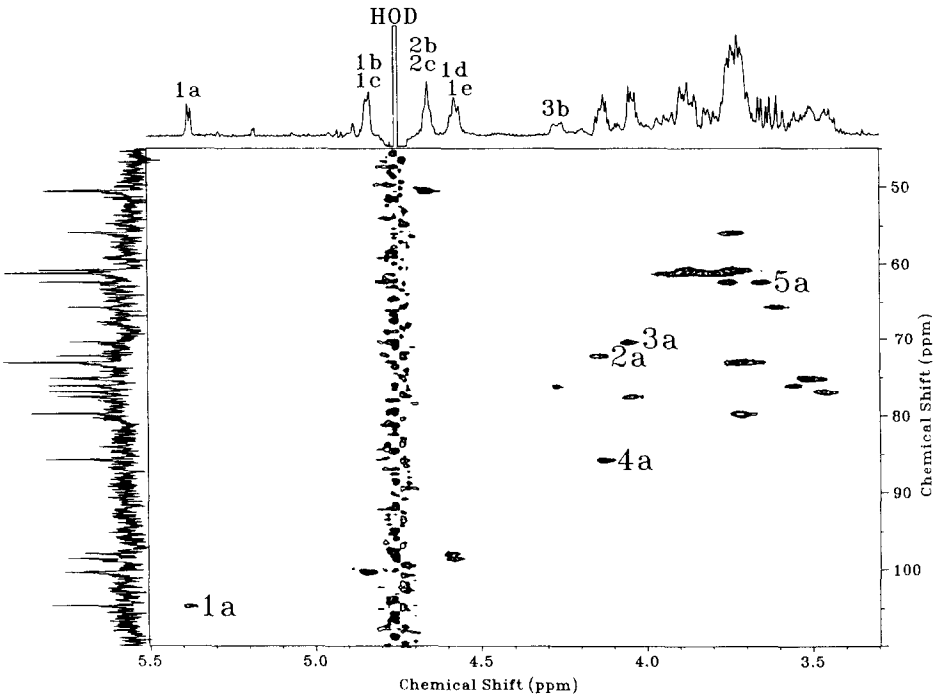


Fig. 2. Heteronuclear 2D $^{1}H-^{13}C$ chemical shift correlation of the anomeric and ring regions of the glycan from the minor glycopeptide fraction of the S-layer glycoprotein of *T. thermosaccharolyticum* E207-71. The cross-peaks for the α-D-ribofuranosyl residue (residue **a**) are indicated.

Large vicinal proton coupling constants for $J_{2,3}$, $J_{3,4}$ and $J_{4,5}$ (8 to 10 Hz) indicated the presence of two hexopyranosyl residues having the *gluco* configuration (residues **d** and **e**). Similarly, residues **b** and **c** were identified as having ^{1}H spin systems typical of *manno*pyranose ring systems on the basis of the observed small $J_{1,2}$ (< 2.0 Hz) and $J_{2,3}$ (4.5 Hz) and the large $J_{3,4}$ value (~ 10 Hz). Residue **a** was assigned to α-D-ribofuranose on the basis of comparison with the ^{13}C chemical shifts (Fig. 2) of the corresponding methyl α-D-furanoside [13] and the methylation analysis.

The sequence of the monosaccharides in the repeating unit was established from the 2D NOESY spectrum of **PS** (Fig. 3) and the methylation analysis. The NOEs produced upon saturation of each anomeric proton also provided information on the anomeric configuration of glycose residues. The β-linked glycopyranoses showed NOEs between H-1, H-3 and H-5 resonances within the same ring systems (residues **b**, **c**, **d** and **e**). Due to the presence of overlapping anomeric resonances more than one interresidue NOE was produced upon saturation of each anomeric proton (Fig. 3). The linkage position was determined from the methylation data.

The methylated and hydrolyzed native glycan afforded a product that, after reduction ($NaBD_4$) and acetylation, gave 2,3,5-tri-*O*-methyl-D-ribose, 2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-glucose, 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-mannose and 2-deoxy-6-*O*-methyl-2-(*N*-methylacetamido)-D-mannose (0.6:1.0:0.3:0.2)

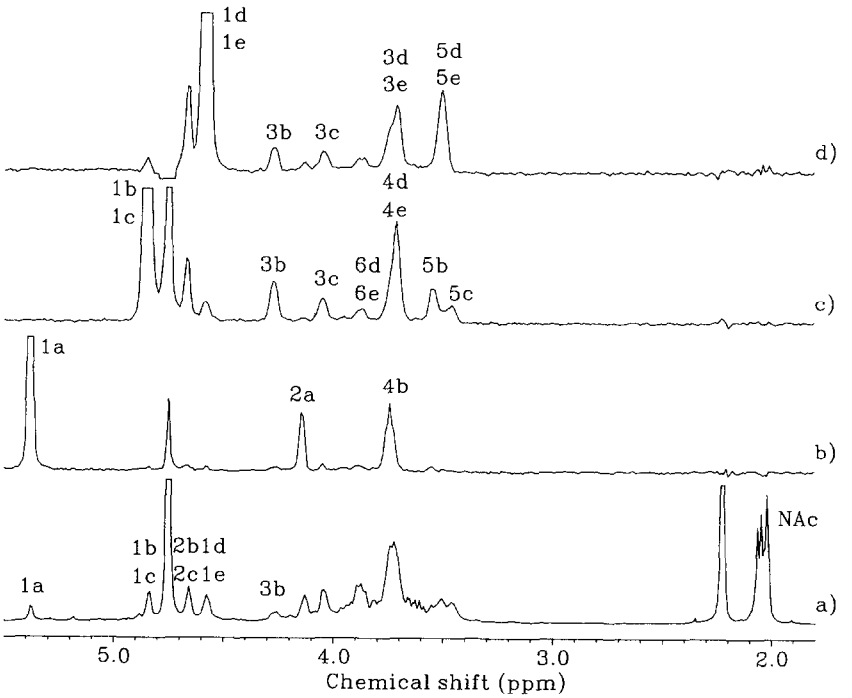
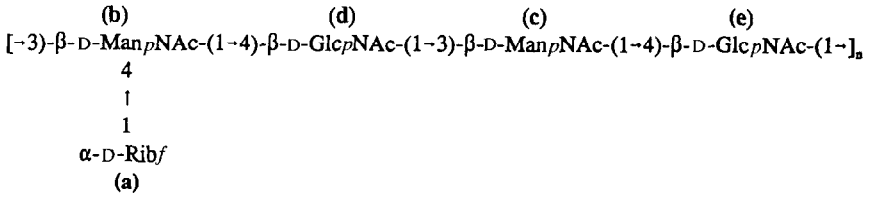


Fig. 3. Cross sections through the NOESY spectrum of the **PS** showing NOEs for the anomeric protons: (b) H-1**a**; (c) H-1**b**, H-1**c**; (d) H-1**d**, H-1**e**. The observed ^{1}H NMR spectrum of **PS** is shown in (a). HOD resonance is set at 4.76 ppm.

on GLC analysis, demonstrating that the native polymer consisted of 2-acetamido-2-deoxy-D-glucose and 2-acetamido-2-deoxy-D-mannose and that the branching occurred through position O-4 of the 2-acetamido-2-deoxy-D-mannose with D-ribofuranose being a terminal nonreducing sugar.

Methylation analysis of the oxidized polymer confirmed these results since only 2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-glucose and 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-mannose were obtained.

On the basis of the combined chemical evidence and 1D and 2D NMR analysis, the glycan structure for the minor glycopeptide fraction of the S-layer glycoprotein of *T. thermosaccharolyticum* E207-71 can be proposed as follows:



Interestingly, the S-layer glycoprotein of *T. thermosaccharolyticum* D120-70 also contains two different glycans [4]. An amino sugar-rich component of this S-layer glycoprotein has a composition rather similar to that of the E207-71 material, but galactose is substituted by ribofuranose in the latter (this study). Whether this reflects a common assembly principle among glycosylated S-layers of *T. thermosaccharolyticum* strains remains to be established.

1. Experimental

Analytical methods.—Colorimetric estimation of carbohydrates and amino acids and TLC were performed as previously described [4,10,14]. Quantitation of both the neutral and the amino sugars and amino acid analysis was done according to Ref. [4]. Deglycosylation of the S-layer glycoprotein by trifluoromethanesulfonic acid and *N*-terminal sequencing of the glycopeptides followed published procedures [15]. SDS–PAGE for molecular weight determination was carried out on 6–20% slab gels using the Multiphor system (Pharmacia). The staining procedures for carbohydrates and proteins are described in Refs. [16,17].

Monosaccharides and amino sugars were analyzed by GLC of their alditol acetates after hydrolysis of the sample with 4 M trifluoroacetic acid at 125 °C for 1 h. The release of ribose from the native glycan was achieved by mild acid hydrolysis with 0.05 M trifluoroacetic acid at 80 °C for 3 h.

Amino sugars were separated and quantified by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC/PAD) on a system consisting of a Dionex Bio-LC pump, CarboPac PA-1 column (4.6 × 250 mm) and a model PAD2 detector, using 15 mM sodium hydroxide with 150 mM sodium hydroxide wash between the injections. The absolute configuration of glycoses was established by capillary GLC of their trimethylsilylated (−)-2-butyl glycosides according to the method of Gerwig et al. [11].

Isolation of the glycopeptides.—Thermoanaerobacterium thermosaccharolyticum strain E207-71 was grown under anaerobic conditions at 60 °C in S-medium (10 g tryptone, 1 g yeast extract, 6 g glucose, 0.2 g sodium sulfite, 0.1 g sodium thiosulfate pentahydrate to 1 L water) in a 10 L fermenter (Biostat E, Braun, Melsungen, Germany). The pronase digest of the S-layer glycoprotein was separated into three major and one minor glycopeptide fractions as described [8]. Following chromatography and rechromatography on a Bio-Gel P-100 column (1 × 110 cm) equilibrated with 0.1 mM sodium chloride, the final purification of the minor glycan component was performed by reversed-phase HPLC on a semipreparative reversed-phase C_{18} column (Supersphere 100, 4 μm, 8 × 125 mm, Merck) using a linear gradient of 0–20% acetonitrile containing 0.1% trifluoroacetic acid [15].

Periodate oxidation, Smith-type hydrolysis, and methylation analysis.—A solution of the amino sugar glycopeptide in water was subjected to sodium metaperiodate treatment and Smith-type hydrolysis according to previously reported conditions [4]. Methylation analysis of both native and oxidized material was performed according to the Hakomori procedure [18].

Methylated polysaccharides were subjected to hydrolysis as described by Stellner et al. [19], and methylation analyses were made according to previously reported conditions [4].

NMR spectroscopy.—All NMR experiments were performed on a Bruker AMX 500 spectrometer using a 5 mm broad band-probe with the ^{1}H coil nearest to the sample. The spectra were recorded at 70 °C for an oxidized polymer and at 27 °C for the native glycan. Acetone was used as the internal standard with the CH_3 resonance at 31.07 ppm for ^{13}C spectra and 2.225 ppm for ^{1}H spectra. The experiments were carried out without sample spinning. Two-dimensional (2D) homonuclear proton correlation [correlated spectroscopy (COSY)] [20], total correlated spectroscopy (TOCSY) [21], nuclear Overhauser effect [2D NOE spectroscopy (NOESY)] [22], and heteronuclear $^{13}C-^{1}H$ chemical shift correlation (heteronuclear multiple quantum coherence [HMQC]) [23] experiments were carried out as previously described [8].

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