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Note

Structure of the O-specific polysaccharide of *Hafnia alvei* PCM 1196

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Abstract

An acidic O-specific polysaccharide was isolated from *Hafnia alvei* PCM 1196 lipopolysaccharide and studied by sugar and methylation analyses along with one- and two-dimensional ^{1}H and ^{13}C NMR spectroscopy, including NOESY and HMBC experiments. The following structure of the pentasaccharide repeating unit was established:

→4)-α-D-Gal*p*A-(1→3)-β-D-Glc*p*NAc-(1→2)-β-D-Gal*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Glc*p*NAc-(1→

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Hafnia alvei is an enterobacterial opportunistic pathogen, which is found in many nosocomial infections, including wounds, enteric, urinary and respiratory tract disorders. According to the serological classification,[1] this species includes 39 O-serotypes. The structures of over 20 O-specific polysaccharides (OPSs) of *H. alvei* lipopolysaccharides isolated from serologically different strains have been established so far.[2–4]

In this work we continue our studies on *H. alvei* O-antigens and report on the structure of a new acidic O-specific polysaccharide of the lipopolysaccharide of *H. alvei* strain PCM 1196.

The lipopolysaccharide was isolated in a 3.5% yield from dry bacterial mass by phenol–water extraction. Hydrolysis of the lipopolysaccharide with aqueous 1% acetic acid (1 h, 100 °C) followed by fractionation of the carbohydrate portion on Sephadex G-50 resulted in three well separated fractions: a high-molecular-mass O-specific polysaccharide (OPS, 7.5% of the lipopolysaccharide weight); a core oligosaccharide (17%); and low-molecular-mass products (Kdo and a small Kdo-con-

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taining oligosaccharide, 12%). The structures of the core and Kdo-containing trisaccharide from *H. alvei* PCM 1196 have been established earlier.[5,6]

Using enzymatic and colorimetric methods, it was found that the OPS contained D-glucose (16.5%), D-galactose (18.0%), and an uronic acid (21.5%). In the carboxyl-reduced OPS (OPS-I), the amount of galactose, determined with D-galactose oxidase, increased almost twice (to 31.0%), thus demonstrating the D-*galacto* configuration of the uronic acid.

Hydrolysis of the OPS and OPS-I with 10 M HCl (80 °C, 30 min) followed by GLC–MS analysis of the derived alditol acetates revealed glucose, galactose, and 2-amino-2-deoxyglucose in the molar ratios 1.0:0.8:2.2 and 1.0:1.4:2.5, respectively. Higher amount of galactose in the OPS-I compared to OPS confirmed the presence of galacturonic acid as a component of the O-specific polysaccharide. When hydrolysis of the OPS was performed with 2 M CF_3CO_2H (120 °C, 2 h), GlcN was not completely released, and the Glc:Gal:GlcN molar ratios were 1.0:1.1:1.0.

The D configuration of GlcN was determined in the OPS hydrolysate (4 M HCl, 100 °C, 18 h) with hexokinase, which completely phosphorylated this sugar in the presence of ATP, as shown by GLC–MS.

The ^{13}C NMR spectrum of the OPS (Fig. 1) showed signals for five anomeric carbons at δ 99.0–102.7, two nonsubstituted (δ 61.8, 2 C) and two substituted (δ 66.3 and 69.1) CH_2O groups (C-6, data of the DEPT spectrum), two carbons bearing nitrogen at δ 54.4 and 55.4 (C-2 of GlcNAc), 18 other sugar ring carbons linked to oxygen in the region δ 69.4–82.4, one carboxyl group at δ 174.0, and two *N*-acetyl groups (CH_3 at δ 23.0 and 23.1; CO at δ 175.6, 2 C). The ^1H NMR spectrum of the OPS (parts of it are shown in Fig. 2) contained signals for five anomeric protons at δ 4.46–5.37, other sugar protons at δ 3.4–4.3, and two *N*-acetyl groups at δ 1.99 and 2.09. Two from the anomeric proton signals, at δ 4.46 (d, $J_{1,2}$ 7 Hz) and 4.85 (d, $J_{1,2}$ 8 Hz), belonged to β-linked sugars and the remaining three, at 4.93, 4.95 and 5.37 (all d, $J_{1,2} \sim 3.5$ Hz), to α-linked sugars.

Therefore, the chemical and NMR spectroscopic data suggested that the O-specific polysaccharide of *H. alvei* 1196 has a pentasaccharide repeating unit containing two residues of D-GlcNAc and one residue each of D-Gal, D-Glc and D-GalA.

Methylation analysis of the OPS (Table 1) revealed derivatives from 2-substituted galactose, 6-substituted glucose, 3-substituted GlcNAc, and 6-substituted GlcNAc. When the OPS-I was methylated (Table 1), a derivative of 2,3,6-tri-*O*-methylgalactose was identified in addition to the above sugars, and, hence, GalA in the OPS is 4-substituted.

The ^1H NMR spectrum of the OPS was assigned using gsCOSY, TOCSY, and gsNOESY (Fig. 2(a)) experiments. Then, the ^{13}C NMR spectrum of the OPS could be unambiguously assigned using a ^1H,^{13}C gsHSQC experiment. Based on the ^1H and ^{13}C NMR chemical shift data (Table 2) and coupling constant values estimated from the 2D NMR

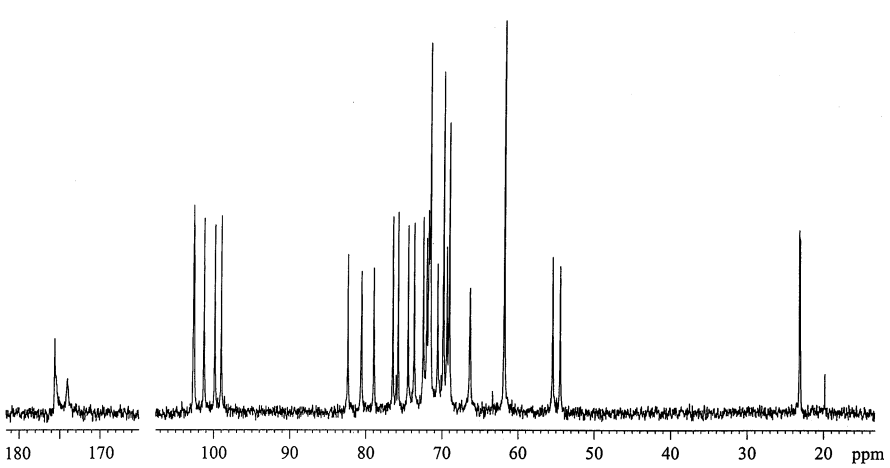

Fig. 1. 125-MHz ^{13}C NMR spectrum of the polysaccharide.

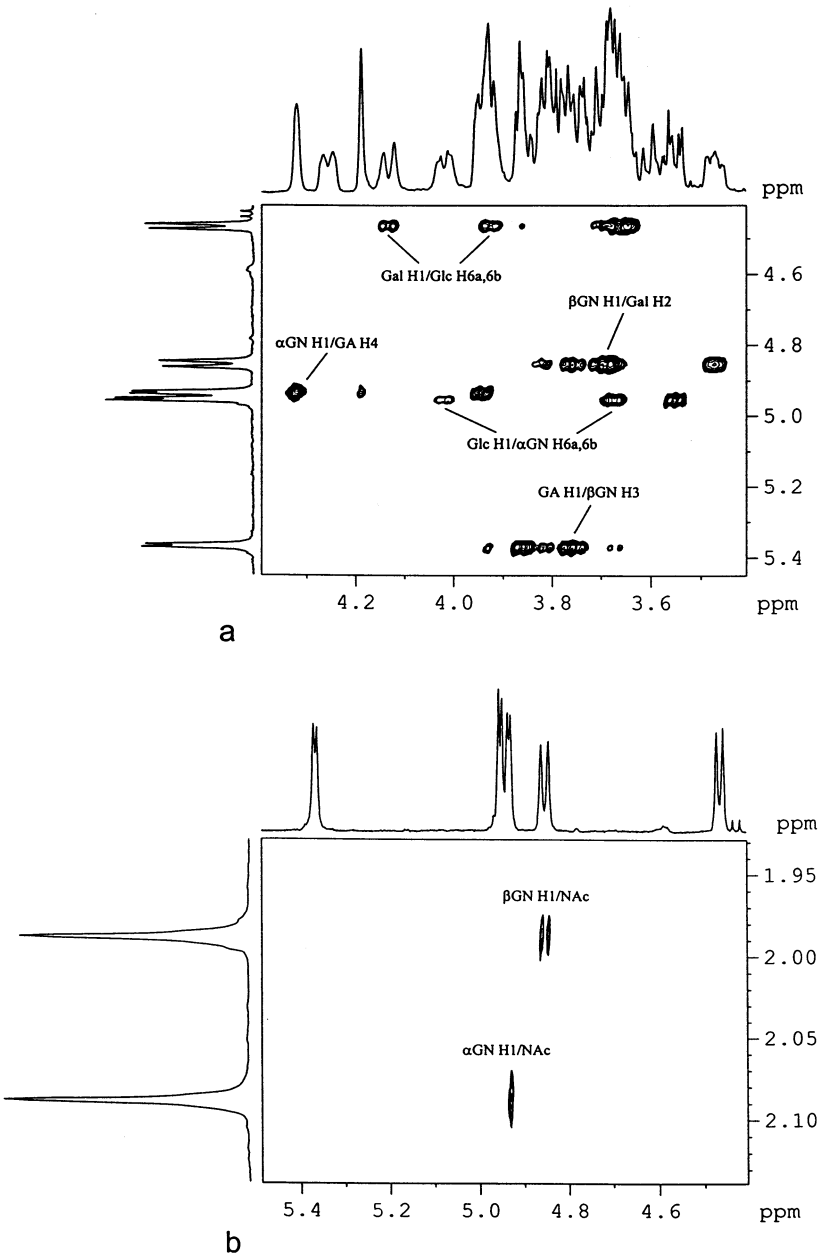

Fig. 2. Parts of a 500-MHz NOESY spectrum of the polysaccharide. The corresponding parts of the ^{1}H NMR spectrum are displayed along the axes. GN and GA stand for GlcN and GalA, respectively.

spectra, the spin systems were identified for α-Glc, β-Gal, α-GalA, α-GlcNAc, and β-GlcNAc, all occurring as pyranosides. These findings were confirmed by the gsNOESY experiment, which showed intra-residue correlations between signals for H-1 and H-2 of the α-linked monosaccharides and for H-1 and H-3, 5 of the β-linked monosaccharides. The gsNOESY spectrum also revealed weak connectivities between signals for the NAc groups and anomeric protons of both amino sugars (Fig. 2(b)) and thus allowed assignment of the former (Table 2).

Downfield displacements of the signals for Gal C-2, β-GlcNAc C-3, GalA C-4, Glc C-6, and α-GlcNAc C-6 to δ 79.0, 82.4, 80.6, 69.1, and 66.3, respectively, compared to their positions in the spectra of the corresponding non-substituted monosaccharides,[7] revealed the modes of substitution of the sugar residues in the OPS.

The glycosylation pattern was confirmed and the sugar sequence in the repeating unit was determined using gsNOESY and gsHMBC experiments. The gsNOESY spec-

trum of the OPS (Fig. 2(a)) showed the following inter-residue cross-peaks between the transglycosidic protons: GalA H-1/β-GlcNAc H-3, β-GlcNAc H-1/Gal H-2, Gal H-1/Glc H-6a,6b, α-Glc H-1/α-GlcNAc H-6a,6b, α-GlcNAc H-1/GalA H-4 at δ 5.37/3.76, 4.85/3.69, 4.46/4.13, 3.92, at 4.95/4.02, 3.68, and 4.93/4.32, respectively. The corresponding connectivities between the anomeric protons and the linkage carbons were also observed in the gsHMBC spectrum (data not shown).

On the basis of the data obtained, it was concluded that the pentasaccharide repeating unit of the polysaccharide has the following structure:

→4)-α-D-Gal*p*A-(1→3)-β-D-Glc*p*NAc-(1→2)-β-D-Gal*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Glc*p*NAc-(1→

(A, B, C, D, E)

1. Experimental

Preparation of lipopolysaccharide, O-specific polysaccharide, and carboxyl-reduced polysaccharide.—*H. alvei* PCM 1196 derived from the collection of the Institute of Immunology and Experimental Therapy (Wrocław, Poland). Bacteria were cultivated in the liquid medium for 24 h as described previously.[8] The

Table 1
Methylation analysis data [a]

Partially methylated sugar	Relative retention time	GLC detector response related to 3,4,6-Me_3Gal		
		OPS		OPS-I
		CF_3CO_2H	HCl	HCl
2,3,6-Me_3Gal	1.23			1.0
3,4,6-Me_3Gal	1.26	1.0	1.0	1.0
2,3,4-Me_3Glc	1.3	0.9	1.0	1.1
4,6-Me_2GlcN	1.95	0.3	1.1	1.6
3,4-Me_2GlcN	1.98	0.3	0.8	1.5

[a] Retention time of the alditol acetates is related to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol (2,3,4,6-Me_4Glc). Hydrolysis of the methylated polysaccharides was performed with 10 M HCl at 80 °C for 30 min (for OPS and OPS-I) or with 2 M CF_3CO_2H at 120 °C for 2 h (for OPS).

Table 2
500-MHz ^{1}H and 125-MHz ^{13}C NMR data of the O-specific polysaccharide (δ in ppm)

	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	CH_3CON
→4)-α-D-Gal*p*A-(1→ (**A**)	5.37	3.86	3.93	4.32	4.19			
→3)-β-D-Glc*p*NAc-(1→ (**B**)	4.85	3.82	3.76	3.66	3.47	3.93	3.77	1.99
→2)-β-D-Gal*p*-(1→ (**C**)	4.46	3.69	3.68	3.86	3.64	3.80	3.73	
→6)-α-D-Glc*p*-(1→ (**D**)	4.95	3.55	3.81	3.67	3.81	4.13	3.92	
→6)-α-D-Glc*p*NAc-(1→ (**E**)	4.93	3.94	3.71	4.26	3.59	4.02	3.68	2.09
	C-1	C-2	C-3	C-4	C-5	C-6	CH_3CON	CH_3CON
→4)-α-D-Gal*p*A-(1→ (**A**)	101.3	69.4	69.8	80.6	72.0	174.0		
→3)-β-D-Glc*p*NAc-(1→ (**B**)	102.6	55.4	82.4	71.6	76.5	61.8	23.0	175.6
→2)-β-D-Gal*p*-(1→ (**C**)	102.7	79.0	74.5	69.8	75.8	61.8		
→6)-α-D-Glc*p*-(1→ (**D**)	99.0	72.5	73.7	69.8	71.6	69.1		
→6)-α-D-Glc*p*NAc-(1→ (**E**)	99.8	54.4	71.8	71.8	70.6	66.3	23.1	175.6

lipopolysaccharide was extracted from dry bacterial mass by the phenol–water procedure[9] and purified from nucleic acids by GPC on Sepharose 2B.[10]

The lipopolysaccharide was hydrolyzed with 1% HOAc (100 °C, 60 min), a lipid precipitate was removed by centrifugation, and a water-soluble material was fractionated by GPC on Sephadex G-50 to give the O-specific polysaccharide (OPS).

Carboxyl reduction of the OPS (12 mg) was carried out according to Taylor et al.,[11] and the reduced polysaccharide (OPS-I, 8.0 mg) was isolated by dialysis against distilled water.

Compositional and methylation analyses.—For sugar analysis, the OPS and OPS-I were hydrolyzed with 2 M CF_3CO_2H at 120 °C for 2 h or 10 M HCl at 80 °C for 30 min. Monosaccharides were converted into the alditol acetates by reduction with $NaBH_4$ followed by acetylation with Ac_2O in pyridine and analyzed by GLC and GLC–MS.

The absolute configuration of glucose and galactose was established with D-glucose oxidase[12] and D-galactose oxidase,[13] respectively. Uronic acid was determined directly in the OPS by published colorimetric method,[14] and its absolute configuration was established by identification of D-galactose after carboxyl reduction of the OPS to the OPS-I (the latter contained twice as much galactose as the former). The D configuration of GlcN was demonstrated in the OPS hydrolysate (4 M HCl, 100 °C, 18 h) using hexokinase test.[15]

Methylation of the OPS and OPS-I was performed with methyl iodide in dimethyl sulphoxide in the presence of solid base according to Gunnarsson.[16] Methylated products were hydrolyzed as in sugar analysis, conventionally reduced with $NaBH_4$, acetylated, and analyzed by GLC and GLC–MS.

Chromatography and mass spectrometry.—GPC was performed on a column (2×100 cm) of Sephadex G-50 equilibrated with 0.05 M pyridinium acetate pH 5.6. Elution was monitored by the phenol–H_2SO_4 reaction.

GLC and GLC–MS were carried out with a Hewlett–Packard 5971A chromatograph equipped with a HP-1 glass capillary column ($12\ m \times 0.2$ mm) using a temperature program of 150–270 °C at 8 °C/min.

NMR spectroscopy.—The ^1H and ^{13}C NMR spectra were recorded with a Bruker DRX-500 spectrometer for solutions in D_2O at 40 °C. Chemical shifts are reported with internal acetone (δ_H 2.225, δ_C 31.45) as reference. Bruker software XWINNMR 2.6 was used to acquire and process the NMR data.

Standard pulse sequences were used in gradient-selected COSY (gsCOSY), TOCSY (MLEV-17), gsNOESY, and gsHMBC. A pulse sequence with multiplicity editing was applied in H-detected ^1H,^{13}C HSQC.[17] The following parameters were used: sweep width 2500 Hz (^1H) and 13,500 Hz (^{13}C), hard pulses 11.5 and 16 μs for ^1H and ^{13}C, respectively, and spin-lock 8 kHz. In gradient experiments, 1 ms sine-shaped gradients of maximum amplitude 10 G/cm followed by 60 μs ring-down delay were used. A mixing time of 200 ms was used in TOCSY and gsNOESY. Long-range correlation in gsHMBC was optimized for coupling constant of 8 Hz.

Acknowledgements

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