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Structure of the neutral O-polysaccharide and biological activities of the lipopolysaccharide of *Proteus mirabilis* O20

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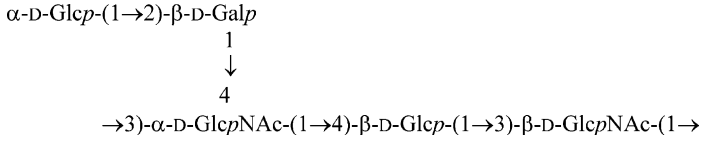
Abstract—Mild acid degradation of the lipopolysaccharide (LPS) of *Proteus mirabilis* O20 resulted in depolymerisation of the O-polysaccharide to give a repeating-unit pentasaccharide. A polysaccharide was obtained by O-deacylation of the LPS followed by nitrous acid deamination. The derived pentasaccharide and polysaccharide were studied by NMR spectroscopy, including 2D $^{1}H,^{1}H$ COSY, TOCSY, ROESY, $^{1}H,^{13}C$ HMQC and HMQC–TOSCY experiments, along with chemical methods, and the following structure of the repeating unit of the O-polysaccharide was established:



As opposite to most other *P. mirabilis* O-polysaccharides studied, that of *P. mirabilis* O20 is neutral. A week serological cross-reactivity was observed between anti-*P. mirabilis* O20 serum and LPS of a number of *Proteus* serogroups with known O-polysaccharide structure. The ability of LPS of *P. mirabilis* O20 to activate the serine protease cascade was tested in *Limulus* amoebocyte lysate and in human blood plasma and compared with that of *P. mirabilis* O14a,14c having an acidic O-polysaccharide. The LPS of *P. mirabilis* O20 was found to be less active in both assays than the LPS of *P. mirabilis* O14a,14c and, therefore, the structurally variable O-polysaccharide may influenced the biological activity of the conserved lipid A moiety of the LPS.

Keywords: *Proteus mirabilis*; O-Polysaccharide structure; Lipopolysaccharide; Serological cross-reactivity; Blood serum

1. Introduction

Bacteria of the genus *Proteus* are facultative human pathogens and cause septicaemia and urinary tract infections, which often result in acute or chronic pyelonephritis, bacteriemia and formation of kidney and bladder stones.[1–4] Outer-membrane lipopolysaccharide (LPS) is considered as a one of important virulent factors of *Proteus*. The polysaccharide chain (O-polysaccharide or O-antigen) and sometimes the core region of the LPS define the serological specificity of *Proteus*. Currently, *Proteus* strains are classified into more than 80 O-serogroups.[5–7] In most O-serogroups, the O-polysaccharide is acidic due to the presence of uronic and aldulosonic acids, amino acids, phosphate and other acidic non-sugar components.[8]

Aiming at establishing a correlation between the serological specificity and the LPS structure as the

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molecular basis for classification of *Proteus* strains, we studied the LPS of *Proteus mirabilis* O20, demonstrated that it has a neutral acid-labile O-polysaccharide and determined the structure of the pentasaccharide repeating unit of the O-polysaccharide. In addition, we studied serological cross-reactivity of anti-*P. mirabilis* O20 serum with other *Proteus* strains and tested the role of LPS with neutral O-polysaccharide of *P. mirabilis* O20 in activation of the proteolytic cascade in *Limulus* amoebocyte lysate[9,10] and in human blood plasma in comparison with the effect of the acidic O-polysaccharide of *P. mirabilis* O14a,14c.[11]

2. Results and discussion

2.1. Chemical and NMR spectroscopic studies

The LPS was isolated from dried bacterial cells of *P. mirabilis* O20 by the phenol–water procedure.[12] Mild acid degradation of the LPS with dilute acetic acid, followed by GPC on Sephadex G-50, resulted in a mixture of oligosaccharides and no high-molecular-mass polysaccharide. Further fractionation of the oligosaccharides by GPC on TSK HW-40 gave oligosaccharide **1**, which was suggested to be the repeating unit of the O-polysaccharide. To obtain a high-molecular-mass polysaccharide, the LPS was O-deacylated by aq ammonia and cleaved by deamination with nitrous acid. This approach is based on the presence in the outer core region of *Proteus* LPS of an amino sugar with a free amino group (GlcN or GalN), whose linkage is split upon deamination to release the polysaccharide.[13] The resultant polysaccharide was isolated by GPC on Sephadex G-50. Comparison of the NMR spectra of oligosaccharide **1** and the polysaccharide showed that no substituent was lost in the course of degradation and isolation by either procedure.

The ^{13}C NMR spectrum of the polysaccharide (Fig. 1) contained signals for five anomeric carbons at δ 98.0 (2C), 100.1, 103.4 and 103.9, two nitrogen-bearing carbons (C-2 of amino sugars) at δ 54.1 and 55.8, five non-substituted HO*C*H$_2$–C groups (C-6 of hexoses and hexosamines) at δ 61.1–62.1 and other oxygen-bearing carbons in region of δ 68.6–82.7. The ^{1}H NMR spectrum of the polysaccharide contained, among other signals, five anomeric protons in the region of δ 4.45–5.33. These data suggest that the polysaccharide has a pentasaccharide repeating unit containing three residues of hexoses and two residues of *N*-acetylhexosamines.

Sugar analysis by GLC of the alditol acetates showed that the polysaccharide contains glucose, galactose and GlcN. GLC of the acetylated (*S*)-2-octyl glycosides demonstrated the D configuration of all monosaccharides.

The ^{13}C and ^{1}H NMR spectra of the polysaccharide were assigned using 2D ^{1}H,^{1}H COSY, TOCSY and H-detected ^{1}H,^{13}C HMQC experiments (Tables 1 and 2). The spin systems of two residues each of Glc*p* and Glc*p*NAc were distinguished by relatively large $J_{3,4}$ and $J_{4,5}$ coupling constant values of 9–10 Hz, whereas those of Gal*p* were <3 Hz. Two Glc*p*NAc residues were demonstrated by correlation of the protons at the nitrogen-bearing carbons at δ 3.84 and 4.12 to the corresponding carbons at δ 55.8 and 54.1, respectively. $J_{1,2}$ values of 7–8 Hz showed that one residue each of Glc*p*, Glc*p*NAc and Gal*p* is β-linked, whereas the values of 3–4 Hz indicated that the second residues of Glc*p* and Glc*p*NAc are α-linked.

The following signals were shifted downfield by 4.0–8.3 ppm as compared with their positions in the spectra of the corresponding non-substituted monosaccharides:[14] C-2 of Gal*p* to δ 76.5, C-3 of β-Glc*p*NAc to δ 82.7, C-4 of α-Glc*p*NAc to δ 75.3 and C-4 of β-Glc*p* to δ 77.5. The ^{13}C NMR chemical shifts for Glc*p* were close to those of the unsubstituted α-D-glucopyranose,[14] thus

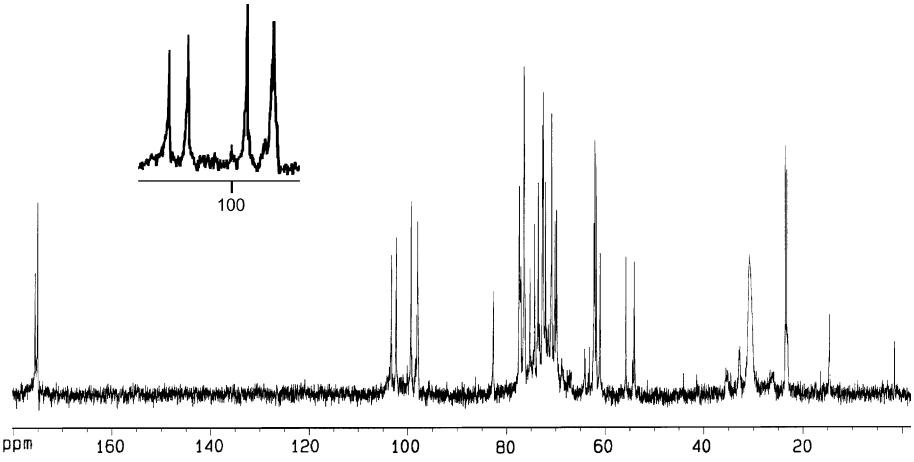

Figure 1. ^{13}C NMR spectrum of the polysaccharide obtained by deamination of the LPS of *P. mirabilis* O20. Inset shows the extension of the region for anomeric carbons.

Table 1. ^{13}C NMR chemical shifts (δ) of the O-polysaccharide of *P. mirabilis* O20[a]

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
α-Glc*p*-(1→	100.1	72.1	73.6	70.2	72.6	61.1
→2)-β-Gal*p*-(1→	103.4	76.5	72.8	69.9	76.5	61.8
→3,4)-α-Glc*p*NAc-(1→	98.0	54.1	70.2	75.3	72.6	61.1
→4)-β-Glc*p*-(1→	103.9	74.4	77.5	77.5	77.4	62.1
→3)-β-Glc*p*NAc-(1→	98.0	55.8	82.7	68.6	76.5	61.8

[a]Signals for NAc are at δ 23.2, 23.5 (both Me), 175.1 and 175.6 (both CO).

Table 2. ^{1}H NMR chemical shifts (δ) of the O-polysaccharide of *P. mirabilis* O20[a]

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6a,6b
α-Glc*p*-(1→	5.33	3.58	3.12	3.43	4.02	3.82, 3.89
→2)-β-Gal*p*-(1→	4.70	3.70	3.72	3.94	3.68	3.79, 3.81
→3,4)-α-Glc*p*NAc-(1→	5.27	4.12	4.19	4.12	3.94	3.80, 3.94
→4)-β-Glc*p*-(1→	4.45	3.31	3.62	3.73	3.79	3.75, 3.82
→3)-β-Glc*p*NAc-(1→	4.94	3.84	3.76	3.44	3.39	3.67, 3.85

[a]Signals for NAc are δ 2.03 and 2.05.

indicating the terminal position of this sugar. Further studies showed that α-GlcNAc is at the branching point and is substituted at positions 3 and 4. These data revealed the glycosylation pattern in the repeating unit.

The 2D ROESY spectrum showed the following correlations between the anomeric protons and the protons at the linkage carbons: α-Glc H-1,β-Gal H-2 at δ 5.33/3.70; α-GlcNAc H-1,β-Glc H-4 at δ 5.27/3.73 and β-Glc H-1,β-GlcNAc H-3 at δ 4.45/3.76. Each of β-Gal H-1 and β-GlcNAc H-1 showed a correlation with α-GlcNAc H-4 at δ 4.70/4.12 and 4.94/4.12, respectively. In addition, the former gave a correlation with α-GlcNAc H-6a,6b at δ 4.70/3.80 and 4.70/3.94, which is typical of a 1→4-linkage, and the latter with α-GlcNAc H-3 at δ 4.94/4.19, indicating a 1→3-linkage between the monosaccharides. Based on these data, it was concluded that the O-polysaccharide of *P. mirabilis* O20 has the structure shown in Figure 2. This structure was

Proteus mirabilis O20 (this work)

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

Proteus mirabilis O7[15]

β-D-Qui*p*4NMal
1
↓
6
→2)-β-D-Gal*p*-(1→4)-**β-D-Glc*p*-(1→3)-β-D-Glc*p*NAc**-(1→

Proteus mirabilis O18[16]

α-D-Glc*p*
Cho*P* 1
| ↓
4 4
→3)-α-D-Glc*p*NAc-1-*P*-(O→6)-β-D-Glc*p*-(1→3)-**β-D-Gal*p*-(1→3)-β-D-GlcpNAc**-(1→

Figure 2. Structures of the O-polysaccharides of *P. mirabilis* O20 and two cross-reactive *Proteus* O-serogroups. Similar disaccharide fragments are shown in bold type. Qui4NMal stands for 4,6-dideoxy-4-malonylaminoglucose, and Cho*P* stands for phosphocholine.

confirmed by similar NMR spectroscopic studies of the pentasaccharide **1**, which established the structure shown below. Oligosaccharide **1** was found to be linear, and, hence, depolymerisation in the course of mild acid degradation of the LPS occurred by cleavage of the linkage at the branching point between two GlcNAc residues.

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

The structure of the O-polysaccharide of *P. mirabilis* O20 is unique among the known structures of *Proteus* O-antigens, which is in accordance with classification of the strain studied into a separate O-serogroup. It is also worth mentioning that, as opposite to the majority of the *Proteus* O-antigens studied so far,[8] the O-polysaccharide of *P. mirabilis* O20 is neutral.

2.2. Biological studies

A number of *Proteus* lipopolysaccharides with known O-polysaccharide structure were tested with rabbit polyclonal anti-*P. mirabilis* O20 serum in an enzyme immunosorbent assay (EIA). As expected, a strong reaction was observed with the homologous LPS, whereas, when it occurred, the cross-reaction was only week (Table 3). In a Western blot (Fig. 3), anti-*P. mirabilis* O20 serum reacted strongly with both slow- and fast-migrating bands of the homologous LPS, which correspond to high- and low-molecular-mass LPS species (Fig. 3, lane 5). The banding pattern was characteristic for smooth-type LPS with a variable number of repeating units in the O-chain. A faint staining was observed with high- and low-molecular-mass LPS species of *P. mirabilis* O34 (lane 3) and with only high-molecular-mass LPS species of *P. mirabilis* O7, *P. mirabilis* O18, *Proteus vulgaris* O19 and *P. vulgaris* O21 (Fig. 3, lanes 1, 2, 6 and 7, respectively). A common epitope in the LPS core region may be responsible for the cross-reaction of low-molecular-mass LPS species of *P. mirabilis* O34. The O-polysaccharides of *P. mirabilis* O20 and O7 share a β-D-Glc*p*-(1 → 3)-β-D-Glc*p*NAc disaccharide fragment,[15] and that of *P. mirabilis* O18 possesses a similar β-D-Gal*p*-(1 → 3)-β-D-Glc*p*NAc fragment[16] (Fig. 2), which could be responsible for the cross-reactivity of the LPSs with anti-*P. mirabilis* O20 serum. No common fragments are present in the O-polysaccharides of the other cross-reactive strains, and the molecular basis for their serological relationship with *P. mirabilis* O20 remains unknown. However, as mentioned above, the cross-reactivity observed was only weak and does not prejudice the classification of *P. mirabilis* O20 in a separate O-serogroup.

Table 3. Reactivity in EIA of *Proteus* LPSs with rabbit polyclonal anti-*P. mirabilis* O20 serum

Antigen (LPS)	Reciprocal titer
P. mirabilis O20[a]	**1,024,000**[a]
P. mirabilis O7	8000
P. mirabilis O18	4000
P. mirabilis O34	16,000
P. mirabilis O38	1000
P. vulgaris O19	4000
P. vulgaris O21	8000
P. penneri 75	2000

[a]Data of the homologous LPS are shown in bold type.

The LPSs of *P. mirabilis* O20 with a neutral O-polysaccharide and of *P. mirabilis* O14a,14c with an acidic O-polysaccharide[11] were compared for their ability to activate serine protease cascade in *Limulus* amoebocyte lysate (LAL) and in human blood. For this reaction, which is often used in studies of biological activities of

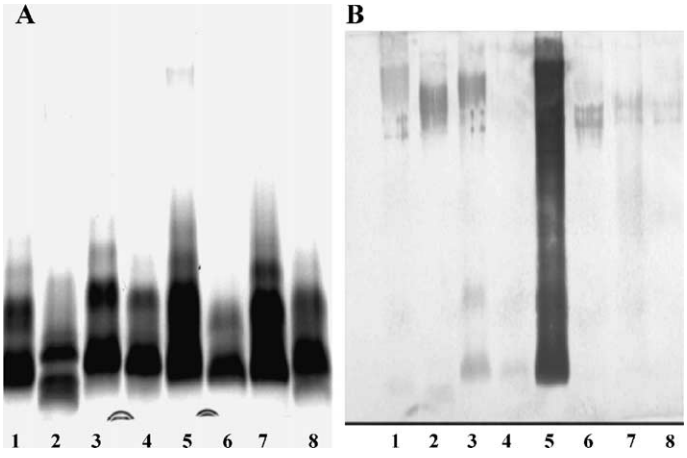

Figure 3. Silver-stained SDS-PAGE (A) and anti-parallel Western blot with anti-*P. mirabilis* O20 serum (B) of the LPSs of *P. mirabilis* O7 (lane 1), *P. mirabilis* O18 (lane 2), *P. mirabilis* O34 (lane 3), *P. mirabilis* O38 (lane 4), *P. mirabilis* O20 (lane 5), *P. vulgaris* O19 (lane 6) and *P. vulgaris* O21 (lane 7) and *P. penneri* 75 (lane 8).

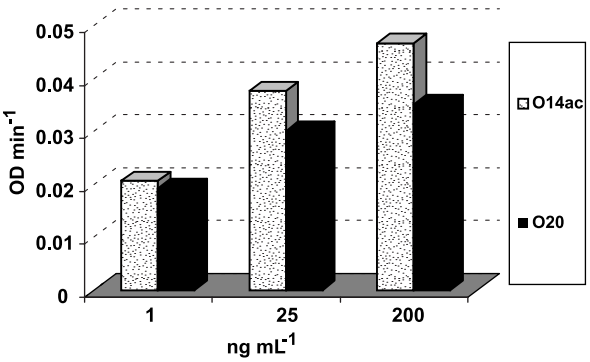

Figure 4. Activation of the proteolytic cascade in LAL by LPSs of *P. mirabilis* O20 and O14a,14c measured in chromogenic test (*$p < 0.05$, Mann–Whitney *U* test).

LPS, the lipid A portion is crucial.[9] The LPS of *P. mirabilis* O14a,14c does not cross-react with anti-*P. mirabilis* O20 serum. That is important to avoid the interference of naturally occurring human antibodies against the LPSs of the two strains. No significant difference in the activity was observed in LAL assay at low concentration of the LPS (1 ng mL^{-1}), whereas at higher concentrations (25 and 200 ng mL^{-1}) the LPS of *P. mirabilis* O14a,14c was considerably more active than the LPS of *P. mirabilis* O20 (Fig. 4). Similarly, in human blood plasma the former LPS activated the proteolytical cascade stronger than the latter LPS (118 ± 8.9 vs 61.2 ± 2.4 U L^{-1}, respectively). A possible explanation of the observed differences is the distinction in the O-polysaccharide structures, which may influence the biological activities of the conserved lipid A moiety of the LPS.

3. Experimental

3.1. Bacteria, growth and isolation of the lipopolysaccharide

P. mirabilis O7 (PrK 15/57), O18 (PrK 34/57), O14a,14c (EU313), O20 (PrK 38/57), O38 (PrK 64/57), *P. vulgaris* O19, O21 and O34 were from the Czech National Collection of Type Cultures (Institute of Epidemiology and Microbiology, Prague). *P. mirabilis* O20 was cultivated under aerobic conditions in a fermenter (Chemap AG, Switzerland) in nutrient broth (BTL, Poland) under the controlled conditions (37 °C, pH 7.4–7.6, pO_2 75–85%). Cells were harvested at the end of the logarithmic growth phase, centrifuged (5000g, 30 min), washed with distilled water and lyophilised. The LPS was isolated by the phenol–water procedure[12] and purified by treatment with DNase and RNase (Boehringer Mannheim, Germany) as described.[17] The LPS preparations thus obtained were practically free of nucleic acid and contained <2.5% proteins. LPS stock solution (1 mg mL^{-1}) in phosphate-buffered saline was shaken thoroughly and sonicated prior to use.

3.2. Mild acid degradation of the lipopolysaccharide

The LPS (103 mg) was hydrolysed with aq 2% HOAc at 100 °C for 2 h, and a lipid precipitate was removed by centrifugation at 13,000g for 20 min. The carbohydrate portion was fractionated by GPC on a column (56 × 2.6 cm) of Sephadex G-50 in 0.05 M pyridinium acetate buffer (pH 4.5) with monitoring by a Knauer differential refractometer to give an oligosaccharide fraction that was further separated on a TSK-40 column to give two oligosaccharides (9.7 and 4.9 mg, in order of elution).

3.3. Deacylation and deamination of the lipopolysaccharide

The LPS (100 mg) was treated with aq ammonia at 37 °C overnight and fractionated by GPC on a G-50 column as described above to give O-deacylated LPS (20 mg).

The O-deacylated LPS (20 mg) was treated with aq 1% $NaNO_2$ (5 mL) and aq 10% HOAc (5 mL) at 25 °C for 3 h. A lipid precipitate was removed by centrifugation, and the supernatant was fractionated by GPC on Sephadex G-50 as described above to give a high-molecular-mass polysaccharide (14 mg).

3.4. Sugar analysis

The polysaccharide was hydrolysed with 2 M CF_3COOH (120 °C, 2 h). Sugars were identified as the alditol acetates by GLC using a Hewlett–Packard 5989A instrument equipped with an HP-5 column and a temperature gradient 150 °C (3 min) → 320 °C at 5 °C min^{-1}. The absolute configurations of the monosaccharides were determined by GLC of the acetylated (*S*)-2-octyl glycosides prepared as described[18,19] using a Hewlett–Packard 5880 instrument with a DB-5 column and a temperature gradient of 160 °C (1 min) to 250 °C at 3 °C min^{-1}.

3.5. NMR spectroscopy

Samples were deuterium exchanged by freeze-drying three times from D_2O and then examined as solutions in 99.96% D_2O at 60 °C. Spectra were recorded on a Bruker DRX-500 MHz spectrometer equipped with an SGI INDY computer workstation. Two-dimensional spectra were obtained using standard Bruker software, and XWINNMR 2.1 program (Bruker) was used to acquire and process the NMR data. The parameters used for 2D experiments were essentially the same as described previously.[20] A mixing time of 200 ms was used in the TOCSY and ROESY experiments.

3.6. Serological techniques

Rabbit polyclonal anti-*P. mirabilis* O20 serum was obtained, and EIA was performed as described previously.[21] LPSs were separated by SDS-PAGE according to Laemmli.[22] The gels were silver-stained according to Tsai and Frasch[23] or electroblotted onto nitrocellulose plates (Schleicher & Schüll, Germany), which were incubated with diluted (1:250) immune rabbit sera as primary antibodies and then with horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma) as the secondary antibodies as described.[21]

3.7. Activation of serine proteolytical cascade

The ability of LPSs to activate the proteolytic cascade was tested in LAL and human blood plasma using chromogenic substrates S-2423 and S-2288 (Chromogenic), respectively. The reaction rate of substrate hydrolysis was followed spectrophotometrically by measuring changes in absorbance at 405 nm over time (OD min^{-1}) and expressed in enzyme activity units (U L^{-1}). Normal pooled citrated plasma was collected by centrifugation (3000 rpm, 4 °C) of blood samples obtained from healthy volunteers of the National Blood Service. Limuli were from the Department of Marine Resources, Marine Biological Laboratory, Woods Hole, MA, USA. Amoebocyte lysate was obtained from *Limulus polyphemus* as described.[9,10]

Acknowledgements

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