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Note

Separation by high-performance liquid chromatography of oligosaccharides obtained after mild acid hydrolysis of *Klebsiella pneumoniae* O_1K_2 (NCTC 5055) lipopolysaccharides

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Cell wall lipopolysaccharides (LPS) are characteristic components of all Gramnegative bacteria[1–4] and confer antigenic properties to the cell. The complete LPS are constituted of three distinct structural parts: the O-specific side-chain, the core oligosaccharide and the lipid A.

The lipopolysaccharides can easily be cleaved by mild acid hydrolysis with 1% acetic acid[5]. The free water-insoluble lipid A precipitates and can be separated by centrifugation. The acetic acid supernatant contains the water-soluble degraded polysaccharides. Gel permeation chromatography is the most commonly used method to fractionate these components into polysaccharides (O-specific side-chains) and oligosaccharides (core oligosaccharides)[6,7]. In this paper we describe a high-performance liquid chromatographic (HPLC) method for the separation of the water-soluble fraction components obtained by the mild acetic acid hydrolysis of *Klebsiella pneumoniae* O_1K_2 lipopolysaccharides.

EXPERIMENTAL

Materials

Bacterial culture of *Klebsiella pneumoniae* O_1K_2 (NCTC 5055) was carried out as described previously[8]. The lipopolysaccharides (LPS) were extracted by the hot phenol–water procedure[9] and purified by ultracentrifugation.

Mild acid degradation of lipopolysaccharides and fractionation of oligosaccharides

The lipopolysaccharides (334 mg) were dissolved in 1% acetic acid at a concentration of 10 mg/ml and heated at 100°C for 90 min. The precipitated lipid A was separated by centrifugation. The supernatant was freeze-dried and fractionated by HPLC on Magnum 9 SAX (10 μm) anion-exchange column (50 cm × 9.4 mm I.D.)

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(Whatman, Clifton, NJ, U.S.A.) with a Spectra-Physics Model 8700 liquid chromatograph equipped with a Model 8400 variable-wavelength detector and connected to a Model 4100 computing integrator.

The lyophilizate of the supernatant (198 mg) obtained by the 1% acetic acid hydrolysis of LPS was dissolved in 4 ml of water. The solution was filtered on a 0.45-μm Millipore filter and 1-ml fractions were injected on to the HPLC column using a water–0.5 *M* KH_2PO_4 gradient as follows: elution with distilled water for 30 min, then a linear gradient to 2.5% of 0.5 *M* KH_2PO_4 for 10 min; isocratic gradient with the latter buffer for 30 min; linear gradient to 5% of 0.5 *M* KH_2PO_4 for 10 min; isocratic gradient with the same buffer for 30 min and then a linear gradient to 100% 0.5 *M* KH_2PO_4 was applied for 50 min. The flow-rate was 2 ml/min and 2-ml fractions were collected. Each fraction was assayed for neutral sugar by the phenol–sulphuric acid method[10]. The sugar-containing fractions were pooled, desalted on a Sephadex G-10 column (100 cm × 1.5 cm I.D.) and freeze-dried.

Determination of monosaccharide composition

The molar composition of monosaccharides was determined by gas chromatography of trifluoroacetylated methyl glycosides according to Zanetta *et al.*[11] or of trimethylsilylated methyl glycosides according to Kamerling *et al.*[12] as modified by Montreuil *et al.*[13].

Chemical methods

The 2-keto-3-deoxy-D-mannooctonic acid (KDO) content was determined by the thiobarbituric acid method according to Karkhanis *et al.*[14]. The total phosphorus content was determined by the method described by Lowry *et al.*[15] with Na_2HPO_4 as a standard. The total protein content was determined by the Lowry method[16]. The total neutral carbohydrate content was determined by the orcinol–sulphuric acid method as described by Rimington[17].

RESULTS

Isolation of lipopolysaccharides

The lipopolysaccharides were extracted from *Klebsiella pneumoniae* O_1K_2 by the hot phenol–water procedure[9] and purified by repeated ultracentrifugation. Nucleic acids were removed by precipitation with cetyltrimethylammonium bromide (Cetavlon)[18]. After lyophilization, the lipopolysaccharides were obtained with a yield of 2% based on the dry bacterial weight.

As shown in Table I, the lipopolysaccharide fraction contained neutral sugars (62.6%), glucosamine (1.9%), KDO (5.6%), phosphate (3.6%) and fatty acids (7.7%). The LPS preparation was found to be free from proteins ($<0.5\%$) as estimated by the method of Lowry *et al.*[16].

Mild acid hydrolysis of lipopolysaccharides

The lipopolysaccharides were subjected to weak acid hydrolysis (1% acetic acid, 100°C, 1.5 h). The insoluble lipid A was separated from the water-soluble fraction by centrifugation. Starting from 334 mg of lipopolysaccharides, 85.7 mg (25.4%) of lipid A and 199 mg (59.5%) of water soluble materials were obtained. As shown in Table I,

TABLE I

COMPOSITION OF NATIVE LIPOPOLYSACCHARIDE, THE WATER-SOLUBLE FRACTION AND THE LIPID A FRACTION

Gal = galactose; Glc = glucose; Man = mannose; Hep = heptose; KDO = 2-keto-3-deoxy-D-manno-octonic acid; GlcN = glucosamine. –, Not detected.

Constituent[a]	*Concentration (%)*		
	Native LPS	*Water-soluble fraction*	*Lipid A fraction*
Gal	34.5	51.4	–
Glc	6.1	7.9	–
Man	Traces	Traces	–
Hep	7.2	8.9	–
KDO	5.6	1.1	–
GlcN	1.9	0.1	16.1
Phosphate	3.6	3.7	5.4
Fatty acids	7.7	–	52
Proteins	<0.5	–	–

glucosamine and fatty acids were absent from the water-soluble fraction but were present in the lipid A fraction. The KDO content decreased after acid hydrolysis. Phosphate was detected in both water-soluble and lipid A fractions. The presence of this compound with neutral sugars in the water-soluble fraction led us to fractionate negatively charged compounds by HPLC with an anion-exchange column.

Fractionation of oligosaccharides by HPLC

As shown in Fig. 1, six fractions (S_1–S_6) were obtained. The fractionation of 198 mg of the water-soluble materials obtained from the mild acid hydrolysis of LPS gave 55.2 mg of fraction S_1, which was not retained by the column and was eluted at the void volume with a retention time of 9 min; 98.9 mg of retained fractions (S_2–S_6) were eluted by the water–0.5 *M* KH_2PO_4 gradient. The overall chromatographic yield was 73.6%.

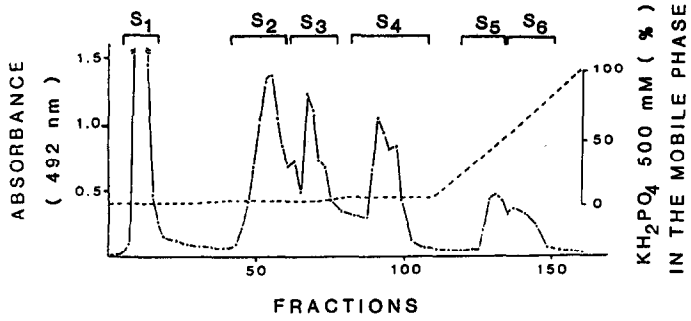

Fig. 1. HPLC on a Magnum 9 SAX anion-exchange column of the acetic acid-soluble fraction obtained by mild acid hydrolysis of *Klebsiella pneumoniae* O_1K_2 lipopolysaccharides.

TABLE II

COMPOSITION AND WEIGHTS OF FRACTIONS OBTAINED BY PREPARATIVE HPLC OF THE WATER-SOLUBLE FRACTION OBTAINED BY MILD ACID HYDROLYSIS OF *KLEBSIELLA PNEUMONIAE* O_1K_2 LIPOPOLYSACCHARIDES

–, Not detected.

Fraction	*Weight (mg)*	*Yield (%)*	*Retention time (min)*	*Phosphate (%)*	*Molar ratios*[a]			
					Gal	*Man*	*Glc*	*Hep*[b]
Water-soluble fraction	198	73.6	–	3.7	6.52	Trace	1	0.96
S_1	55.2	27.8	9	–	M[c]	–	–	–
S_2	26.8	13.4	55	1.7	14.10	–	1	0.67
S_3	19.3	9.6	68	2.2	5.28	–	1	1.00
S_4	28.0	14.1	89	2.9	0.34	–	1	1.09
S_5	7.4	3.6	130	4.3	2.98	–	1	0.80
S_6	9.2	4.6	140	4.5	0.78	0.16	1	0.69

[a] On the basis of one glucose residue.
[b] Hep = heptose.
[c] M = Main component.

Monosaccharide compositions

The monosaccharide compositions of the fractions obtained by HPLC are given in Table II. Fraction S_1 contained only galactose residues whereas all the other fractions contained variable amounts of galactose and approximately the same heptose:glucose ratios (0.7:1 to 1:1). Phosphate was absent from S_1 but was present in all of the other fractions. Fraction S_6 also contained mannose, which was detected in a very small amount in native LPS.

DISCUSSION

The oligosaccharides released from LPS by mild acid hydrolysis have usually been fractionated by gel permeation chromatography. However, with this method we did not obtain a good separation of oligosaccharides released from the *Klebsiella pneumoniae* O_1K_2 LPS. By using HPLC with an anion-exchange column we obtained six distinct fractions, S_1–S_6.

Fraction S_1 contained only neutral sugar (galactose) and was not retained by the anion-exchange column. In comparison with the structure described by Kenne and Lindberg[19], this fraction corresponds to the O-antigen polysaccharide of *Klebsiella* serogroup O_1 lipopolysaccharide.

The other fractions (S_2–S_6) were retained on the column by their negative charges. Their fixed glucose and heptose composition for variable amounts of galactose can be explained by the length heterogeneity of the O-antigen[20–22] and by the incomplete cleavage of this O-antigen from the core oligosaccharide. Structural studies are currently being undertaken that should shed light on the nature of the LPS architecture.

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