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PURIFICATION OF THE LIPOPOLYSACCHARIDE FRACTION FROM *KLEBSIELLA PNEUMONIAE* O_1 K_2 BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An high-performance liquid chromatography technique was applied to purify the lipopolysaccharide fraction from a lysate of *Klebsiella pneumoniae* O_1 K_2 (NCTC 5055). The separation of the lipopolysaccharide fraction from the proteins was carried out with a reversed-phase column. By this method the lipopolysaccharide fraction was obtained in a pure state, devoid of proteins but possessing the same biological properties as the lipopolysaccharide fraction prepared by the classical phenol–water technique.

INTRODUCTION

Studies of the effects of endotoxins, have been greatly enhanced by the development of procedures which allow one to obtain a relatively pure endotoxin from whole bacteria. The first method used for obtaining endotoxins involved the extraction of bacteria with trichloroacetic acid.[1] Many other methods have been described, *e.g.*, the ethylene glycol technique[2], extraction with hot phenol–water[3,4], the dimethyl sulphoxide (DMSO)[5] and the phenol–chloroform–light petroleum[6] methods. With these techniques, the lipopolysaccharide (LPS) fraction was still contaminated with protein. In 1983, Darveau and Hancock[7] used digestion with DNase, RNase and pronase and ethanol precipitation to obtain LPS with an high degree of purity. However this method appears to be rather time-consuming.

In this paper we describe a method for the preparation of a pure lipopolysaccharide fraction by preparative high-performance liquid chromatography (HPLC) of a bacterial extract on a reversed-phase column.

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EXPERIMENTAL

Materials

Klebsiella pneumoniae O_1 K_2 (NCTC 5055) was cultured and a lysate extract (RU 41740) obtained as described[8]. The lysate extract containing acidic polysaccharide, proteins and lipopolysaccharides was treated with cethyltrimethylammonium bromide (cetavlon) according to the procedure of Scott[9]. The addition of six volumes of ethanol to the supernatant gave a precipitate denoted F_1.

HPLC of lipopolysaccharide

HPLC was performed on a semi-preparative 10-μm C_8 RP-300 aquapore column (25 cm × 0.7 cm I.D.; Brownlee Labs., Santa Clara, CA, U.S.A.) with a Spectra-Physics Model 8700 liquid chromatograph equipped with a Model 8400 variable-wavelength detector connected to a Model 4100 computing integrator.

An 120-mg amount of the material F_1 was dissolved in 4 ml of 1% sodium dodecyl sulphate (SDS) solution containing [^{14}C]SDS (1 μCi) and was heated at 100°C for 5 min. After cooling, the solution was centrifuged at 3000 *g* for 15 min and filtered on a 0.45-μm Millipore filter. Fractions of 0.5 ml were subjected to HPLC using a water–acetonitrile gradient as follows: elution with distilled water for 10 min; then a linear gradient to 20% acetonitrile in 10 min; isocratic elution for 15 min; linear gradient to 50% acetonitrile in 10 min; isocratic elution for 15 min; linear gradient to 100% acetonitrile in 20 min and isocratic elution for 5 min. The flow-rate was 2 ml/min and 1-ml fractions were collected. Protein- and sugar-containing fractions were detected at 200 nm. The sugar-containing fractions were detected with phenol–sulphuric acid reagent according to the procedure of Dubois *et al.*[10]. The SDS-containing fraction was detected by liquid scintillation.

Sugar and fatty acid analysis

The molar composition of monosaccharides was determined by gas–liquid chromatography (GLC) of trifluoroacetylated methyl glycosides according to Zanetta *et al.*[11].

The total lipids were estimated as fatty acid methyl esters by GLC after transesterification with methanol–0.5 *M* hydrochloric acid at 80°C for 18 h in the presence of heptadecanoic acid as an internal standard. GLC was performed with a temperature programme (100°C increasing to 240°C at 2°C/min) on a Girdel Serie 300 gas chromatograph equipped with a flame ionization detector connected to a Shimadzu integrator Model CR3A. A capillary column CP-Sil 5 CB (25 m × 0.32 mm I.D., Chrompack-France) was used with helium as carrier gas (0.5 bar). The fatty acids were identified by comparison of their relative retention times with authentic standards and by mass spectrometry.

Chemical methods

The 2-keto-3-deoxy-D-manno-octonic acid (KDO) content was estimated by the thiobarbituric acid assay method according to the procedure of Karkhanis *et al.*[12].

The total phosphorus content was determined by the method described by Lowry *et al.*[13].

The total protein content was estimated by the Lowry method[14] with bovine serum albumin as the standard.

The total neutral carbohydrate content was determined by the orcinol–sulphuric acid method reported by Rimington[15].

RESULTS AND DISCUSSION

Determination of the chemical composition of F_1

The fermentation of 1 l of *Klebsiella pneumoniae* O_1 K_2 culture leads to the isolation of 1.5 g of bacterial lysate. Cetavlon precipitation of 100 mg of bacterial extract (Fig. 1) yields 32.5 mg of acidic precipitate. The ethanol precipitation of the supernatant gives 26.6 mg of a neutral fraction called F_1.

The percentage composition of the fraction F_1 is given in Table I. The carbohydrate composition of the F_1 fraction (Table II) shows the presence of three neutral monosaccharides (galactose, glucose and glycero-D-mannoheptose), an acidic monosaccharide (2-keto-3-deoxy-D-manno-octonic acid) and glucosamine. As shown

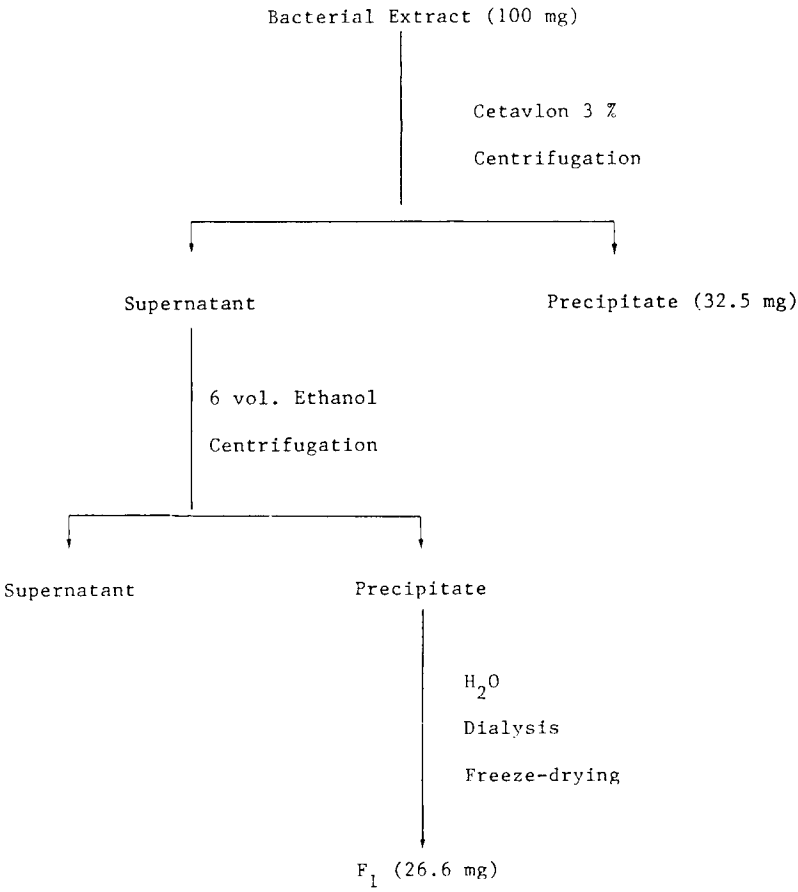

Fig. 1. The extraction of fraction F_1 from the bacterial extract.

TABLE I

PERCENTAGE COMPOSITION AND WEIGHT OF THE FRACTION AG-1 OBTAINED BY SEMI-PREPARATIVE HPLC OF FRACTION F_1

Fraction	*Composition (%)*				*Weight (mg)*
	Protein	*Sugar*	*Lipid*	*Phosphate*	
F_1	33	42.5	5.5	1.55	120
AG-1 (LPS)	0.8	80	8.5	1.97	34.6

in Table III, fraction F_1 also contains fatty acids: *n*-tetradecanoic, 2-hydroxytetradecanoic, 3-hydroxytetradecanoic and *n*-hexadecanoic.

These results indicate that the fraction F_1 was made up of lipopolysaccharide contaminated with proteins.

Separation of LPS from proteins

Separation of the LPS from the contaminating proteins was achieved by HPLC using a reversed-phase RP-300 aquapore column after dissociation by SDS. Elution with water yields a single sugar-containing fraction (AG-1) which was slightly retained by the column with a retention time of 4 min (Fig. 2A and B). As shown in Fig. 2C this fraction is not contaminated by SDS which was eluted by the water–acetonitrile gradient with a retention time of 45 min. Other peaks eluted by this

TABLE II

CARBOHYDRATE COMPOSITION OF THE FRACTION AG-1 OBTAINED BY SEMI-PREPARATIVE HPLC OF FRACTION F_1

Fraction	*Molar ratio**				
	Gal	*Glc*	*Heptose*	*$GlcNH_2$*	*KDO*
F_1	4.6	1	1.4	0.10	0.9
AG-1 (LPS)	4.2	1	1.2	0.18	0.8

* The molar ratio of glucose (Glc) was taken as 1.

TABLE III

FATTY ACID COMPOSITION OF THE FRACTION AG-1 OBTAINED BY SEMI-PREPARATIVE HPLC OF FRACTION F_1

Fraction	*Molar ratio**			
	C_{14}	*α-OH-C_{14}*	*β-OH-C_{14}*	C_{16}
F_1	0.34	0.24	1	0.1
AG-1 (LPS)	0.30	0.20	1	0.09

* The molar ratio of β-OH-C_{14} was taken as 1.

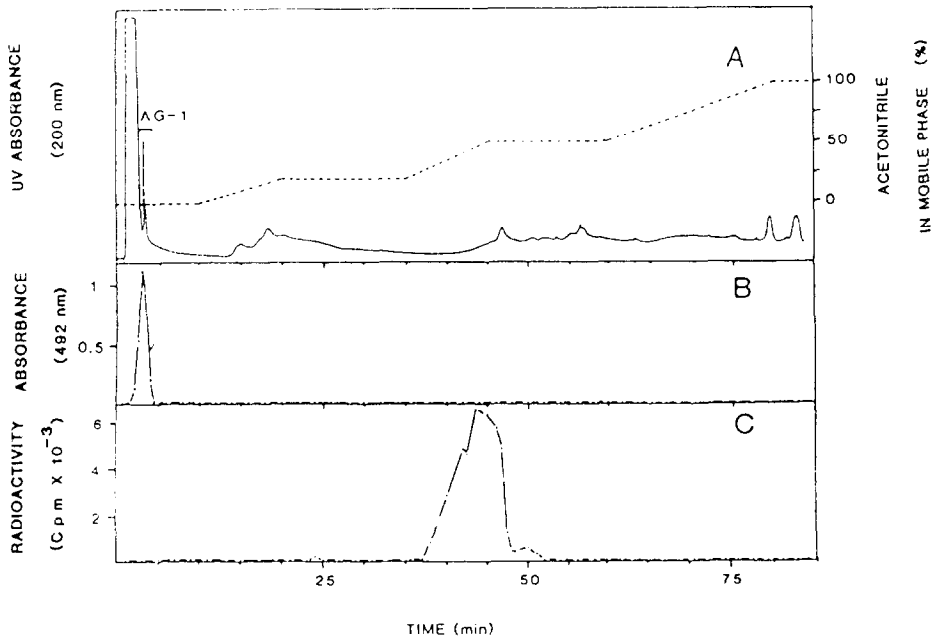

Fig. 2. HPLC of F_1 on a semi-preparative RP-300 aquapore column. For details see Experimental. (A) Absorbance at 200 nm; (B) phenol–sulphuric acid test, absorbance at 492 nm; (C) radioactivity.

gradient (Fig. 2A) absorb at 200 nm but do not contain any sugar. The chromatography of 120 mg of F_1 gives 34.6 mg of lipopolysaccharide with a yield of 29%. The carbohydrate composition of the fraction AG-1 (LPS) was determined by GLC (Table II). No difference was observed in the molar ratio of the total carbohydrate fraction from fraction F_1 and that of the lipopolysaccharide (AG-1) obtained by HPLC. However, as shown in Table I, the lipopolysaccharide fraction (AG-1) obtained by HPLC is practically free from proteins (0.8%), while sugar and lipids increased respectively from 42.5 to 80% and from 5.5 to 8.5%.

These results indicate that it is possible to purify LPS from proteins by HPLC within a short time. These separations are due to the difference in the interactions of LPS and proteins with the reversed phase, proteins being more hydrophobic than LPS. The lipopolysaccharide fraction obtained by this technique possesses a similar composition and the same biological properties as the LPS extracted from *Klebsiella pneumoniae* O_1 K_2 by the phenol–water method.

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