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STRUCTURAL INVESTIGATION OF THE CAPSULAR POLYSACCHARIDE OF *Klebsiella* SEROTYPE K23*

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ABSTRACT

Klebsiella K23 capsular polysaccharide has been investigated by the techniques of hydrolysis, methylation, Smith degradation–periodate oxidation, and base-catalysed degradation, either on the original or the carboxyl-reduced polysaccharide. The structure was found to consist of a tetrasaccharide repeating-unit, as shown below. The anomeric configurations of the sugar residues were determined by ^{1}H- and ^{13}C-n.m.r. spectroscopy on the original and degraded polysaccharides.

$$\begin{array}{l} \rightarrow 3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow \\ \qquad\qquad\quad 2 \\ \qquad\qquad\quad | \\ \qquad\qquad\quad 1 \\ \beta\text{-D-GlcA}p\text{-}(1\rightarrow 6)\text{-}\alpha\text{-D-Glc}p \end{array}$$

INTRODUCTION

The genus *Klebsiella*, belonging to the family Enterobacteraceae, has been classified by Ørskov[1] into approximately 80 different serotypes that are distinguished by their capsular polysaccharides. Nimmich[2,3] has analysed qualitatively the polysaccharide from each strain, and Heidelberger and Nimmich[4] have summarised the structures of the capsular polysaccharides that have been determined to date.

Many of the strains thus far examined possess capsules having different qualitative compositions. There are, however, several groups of *Klebsiella* bacteria that have capsules that give the same qualitative analysis but display different serological reactions. One such group includes the strains of K-type K17, K23, and K44, whose capsules are composed of residues of D-glucuronic acid, D-glucose, and L-rhamnose.

We now report on the structure of the capsular polysaccharide of type K23, as part of our program to correlate chemical structure with immunological response.

*Dedicated to Dr. Elizabeth Percival.

RESULTS AND DISCUSSION

N.m.r. spectra and composition. Klebsiella K23 bacteria were grown on an agar medium, and the capsular polysaccharide isolated was purified by one precipitation with Cetavlon.

The p.m.r. spectrum of the polysaccharide in D_2O at 90°, after mild hydrolysis to reduce the viscosity, showed a doublet at τ 8.71 consistent with the signal for the methyl group of an ω-deoxy sugar. Four discernible signals were observed in the anomeric region (see Table I), and their chemical shifts indicate[5,6] that the repeating

TABLE I

P.M.R. DATA FOR *Klebsiella* K23 CAPSULAR POLYSACCHARIDES

Compound	*Repeating unit*	τ^a	$J_{1,2}$ (*Hz*)	*Proton assignment*
Original polysaccharide	→3)-α-L-Rha*p*-(1→3)-β-D-Glc*p*-(1→ 2 | 1 α-D-Glc*p* 6 | 1 β-D-GlcA*p*	8.71 5.38 5.24 4.92 4.69	6 ($J_{5,6}$) 7 7 2 1	CH_3 of Rha β-GlcA β-Glc α-Glc α-Rha
Smith-degraded polysaccharide	→3)-α-L-Rha*p*-(1→3)-β-D-Glc*p*-(1→	8.71 5.27 4.80	6 ($J_{5,6}$) 7 1	CH_3 of Rha β-Glc α-Rha

[a]Shifts are quoted relative to acetone as the internal standard; τ 7.77 downfield from sodium 4,4-dimethyl-4-silapentane-1-sulphonate.

unit consists of four monosaccharide residues, two of which are α-linked and two are β-linked. More precise assignment of these signals was achieved after studying the p.m.r. spectra of the residual polysaccharide obtained by the Smith–periodate treatment and by base-catalysed degradation; see later, and Tables I and III. The ^{13}C-n.m.r. spectra were consistent with these assignments.

Carboxyl-reduced[7] K23 polysaccharide was hydrolysed, and the presence of glucose and rhamnose, in the ratio of 3:1, was demonstrated by gas–liquid chromatography (g.l.c.) of their alditol acetates. The configurations of the glucitol hexa-acetate and the rhamnitol penta-acetate, as determined by circular dichroism (c.d.)[8], were shown to be D and L, respectively.

Methylation of the original polysaccharide. Methylation[9] of K23 polysaccharide, followed by reduction of the uronic ester, hydrolysis, conversion of the products into alditol acetates, and g.l.c.–m.s. analysis[10,11], indicated that K23 is composed of a

tetrasaccharide repeating-unit (see Table II), in which one rhamnosyl residue constitutes a branch-point and glucuronic acid occupies the terminal position in a side chain.

TABLE II

METHYLATION ANALYSIS OF THE ORIGINAL AND THE SMITH-DEGRADED *Klebsiella* K23 CAPSULAR POLYSACCHARIDE

Methylated sugars[a] (*as alditol acetates*)	T[b]	T[c]	*I*[d] (*mol %*[e])	*II*[d]	*III*[d]
4-Rha	0.91	0.92	26.0[f]		34.1[g]
2,4-Rha	0.66	0.67		49.9[g]	
2,3,4-Glc	1.13	1.10	48.1		
2,4,6-Glc	1.00	1.00	25.9	50.1	33.5
2,3,4,6-Glc[h]	0.72				32.4

[a]4-Rha = 1,2,3,5-tetra-*O*-acetyl-4-*O*-methylrhamnitol, *etc.* [b]Retention time relative to alditol acetate of 2,4,6-tri-*O*-methyl-D-glucose on OV-225, and [c]on HIEFF-1B. [d]I, original polysaccharide, methylated and uronic ester-reduced; II, degraded polymer obtained by Smith degradation; III, degraded polymer obtained after β-elimination (see text for details). [e]Values are corrected by use of the effective carbon-response factors given by Albersheim *et al.*[12]. [f]Column *A*. [g]Column *B*. [h]1,5-Di-*O*-acetyl-6-*O*-ethyl-2,3,4-tri-*O*-methylglucitol.

Periodate oxidation of the carboxyl-reduced polysaccharide. The carboxyl-reduced polysaccharide[7] was subjected to a Smith-degradation[13], and a polymeric product was obtained following dialysis and lyophilisation. Methylation of this material showed it to consist of a disaccharide repeating-unit composed of one glucosyl and one rhamnosyl residue (see Table II). It thus follows that the capsular polysaccharide of *Klebsiella* K23 has a two-unit side-chain which is attached to O-2 of the rhamnosyl residue.

The p.m.r. spectrum of the unmethylated, degraded polymer showed two signals due to anomeric protons; one of these is attributable to a β-linked D-glucosyl residue and the other to an α-linked L-rhamnosyl residue (see Table I). The structure of the degraded polymer is therefore established as 3)-α-L-Rha*p*-(1→3)-β-D-Glc*p*-(1→. The only remaining point to be established is the anomeric configuration of the two units in the side chain; this was achieved by base-catalysed degradation.

Base-catalysed degradation of methylated K23 polysaccharide[14,15]. The methylated polysaccharide was subjected to a β-elimination reaction, and the product was directly alkylated with ethyl iodide. Comparison of the p.m.r. spectra of the methylated starting-material and the ethylated product demonstrated the absence of a signal in the anomeric region at τ 5.66, corresponding to the loss of a β-linked glucuronic acid residue (see Table III). Hydrolysis and analysis by g.l.c.–m.s. of the ethylated product showed that only the glucuronic acid residue had been removed and that it was attached to O-6 of the side-chain glucosyl residue (see Table II).

TABLE III

P.M.R. DATA FOR *Klebsiella* K23 METHYLATED POLYSACCHARIDES

Compound	*Repeating unit*	τ^a	*Proton assignment*
Methylated polysaccharide	→3)-α-L-Rha*p*-(1→3)-β-D-Glc*p*-(1→	8.69	CH_3 of Rha
	2	5.66	β-GlcA
	|	5.48	β-Glc
	1	4.92	α-Glc
	α-D-Glc*p*	4.79	α-Rha
	6		
	|		
	1		
	β-D-GlcA*p*		
Methylated/ethylated, degraded polysaccharide	→3)-α-L-Rha*p*-(1→3)-β-D-Glc*p*-(1→	8.79	CH_3 of ethyl
	2	8.69	CH_3 of Rha
	|	5.45	β-Glc
	1	4.90	α-Glc
	α-D-Glc*p*	4.76	α-Rha

[a]Shifts are quoted relative to Me_4Si as internal standard ($\tau = 10.0$). Spectra were run in $CDCl_3$, at 100 and 270 MHz, at room temperature.

It thus follows that the *Klebsiella* K23 polysaccharide has the following structure.

→3)-α-L-Rha*p*-(1→3)-β-D-Glc*p*-(1→
2
|
1
β-D-GlcA*p*-(1→6)-α-D-Glc*p*

Of the *Klebsiella* polysaccharide structures known to date, those from K20[16], K55[17], and K83[18] are the closest analogues of K23.

EXPERIMENTAL

General methods. — Concentrations were carried out under diminished pressure at bath temperatures not exceeding 40°. Analytical g.l.c. separations were performed with a Hewlett Packard 5700 instrument fitted with dual flame-ionisation detectors. An Infotronics CRS-100 electronic integrator was used to measure peak areas. Stainless-steel columns (1.8 m × 3 mm) were used, with a carrier-gas flow-rate of 20 ml/min. Columns used were (*A*) 3% of HIEFF-1B on Gas Chrom Q (100–120 mesh); (*B*) 3% of OV-225 on the same support; and (*C*) 3% of SP2340 on Supelcoport (100–120 mesh). Preparative g.l.c. was performed on an F & M Model 720 instrument equipped with dual thermal-conductivity detectors, using a column (1.8 m × 6.5 mm) analogous to column *C*. G.l.c.–m.s. was performed with a Micromass 12 instrument fitted with a Watson–Biemann separator. Spectra were recorded at 70 eV with an ionisation current of 100 μA and an ion-source temperature of 200°

P.m.r. spectra were recorded on a Varian XL-100 instrument. Samples dissolved in D_2O were exchanged and freeze-dried three or four times in 99.7% D_2O, and then finally dissolved in 99.9% D_2O. Acetone was used as the internal standard. Spectra were recorded at ~90°. Spectra of the methylated polysaccharides ($CDCl_3$, internal Me_4Si) were recorded at 100 and 270 MHz at 24°. ^{13}C-N.m.r. spectra were recorded with a Varian CFT-20 instrument and were obtained at ambient temperature in 50% D_2O, with acetone (31.07 p.p.m. from aqueous sodium 4,4-dimethyl-4-silapentane-1-sulphonate) as the internal standard. Circular dichroism (c.d.) spectra were recorded on a Jasco J20 automatic recording spectropolarimeter. Optical rotations were measured at 23 ±2° on a Perkin–Elmer model 141 polarimeter, with a 1-cm cell. I.r. spectra were recorded with a Perkin–Elmer 457 spectrophotometer.

Preparation and properties of K23 capsular polysaccharide. — A culture of *Klebsiella* K23 (2813/50) was obtained from Dr. I. Ørskov (Copenhagen) and was grown on a medium of sodium chloride (8 g), dipotassium hydrogenphosphate (4 g), magnesium sulphate heptahydrate (1 g), calcium carbonate (2 g), sucrose (120 g), Bacto yeast extract (8 g), and agar (60 g) in 4 litres of water for 3 days. The cells were harvested, and diluted to 1000 ml with water containing 1% of phenol. This suspension was then centrifuged for 3 h at 34,000 r.p.m. in a Beckman T4 zonal rotor. Crude polysaccharide, obtained by precipitation into ethanol (4 litres), was redissolved in 250 ml of water, precipitated with 10% of Cetavlon, redissolved in 4M sodium chloride (250 ml), and then dialysed against tap water for 2 days. Lyophilisation of this solution yielded 5.5 g of the polysaccharide, $[\alpha]_D$ +28° (*c* 1.0, water).

Analysis of constituent sugars. — Methanolysis of a sample (23 mg) of K23 polysaccharide with 3% methanolic hydrogen chloride and subsequent treatment with sodium borohydride in methanol reduced the uronic acid residues in the native polysaccharide. Hydrolysis with 2M trifluoroacetic acid overnight at 95°, followed by conversion of the liberated monosaccharides into alditol acetates, gave rhamnitol penta-acetate and glucitol hexa-acetate (m.p. 94–96°) in the ratio of 1.00:2.90 (column *C*; programmed at 195° for 4 min and then at 2°/min to 240°). The c.d. spectrum of the rhamnitol penta-acetate showed ε_{212}^{MeCN} −1.85, and that of the glucose hexa-acetate ε_{213}^{MeCN} +0.19.

Methylation of the capsular polysaccharide. — Methylation of K23 under the Hakomori conditions, followed by subsequent Purdie[19] treatment, yielded a product that showed no hydroxyl absorption in the i.r. spectrum. This material was reduced overnight with sodium borohydride in tetrahydrofuran–ethanol (8:3) and, following hydrolysis with 2M trifluoroacetic acid for 16 h at 95°, the mixture was reduced with sodium borohydride and then acetylated. G.l.c. (column *A*, programmed at 165° for 8 min and then at 2°/min to 200°; column *B*, programmed at 180° for 4 min and then at 2°/min to 230°) and m.s. of the collected components gave the results shown in Table II.

Periodate oxidation of carboxyl-reduced polysaccharide. — A sample of the polysaccharide was reduced by the procedure of Taylor and Conrad[7]. Two treatments were required to achieve complete reduction, as monitored by i.r. spectroscopy.

Reduced, capsular polysaccharide (400 mg) was dissolved in 150 ml of water, to which a solution (150 ml) of 0.1M sodium periodate was then added[20]. The solution was kept in the dark at 3°. After 3 days, ethylene glycol (10 ml) was added, the mixture was dialysed, and the product was reduced with sodium borohydride. The polyol was isolated by dialysis and lyophilisation.

A portion of the polymeric product (10 mg) was hydrolysed (2M trifluoroacetic acid) at 95° overnight. Conversion of the hydrolysis products into the corresponding alditol acetates gave (g.l.c.) glycerol triacetate, rhamnitol penta-acetate, and glucitol hexa-acetate in the ratios of 2:1:1.

Smith hydrolysis (0.5M trifluoroacetic acid overnight at room temperature) of the remaining periodate-treated material, followed by dialysis and lyophilisation, yielded 160 mg of a polymeric product. P.m.r. spectroscopy (D_2O, 90°) of this degraded polysaccharide showed anomeric signals at τ 5.36 (1 H, $J_{1,2}$ 7 Hz) and 4.90 (1 H). The ^{13}C-n.m.r. spectrum displayed signals in the anomeric region at 104.4 and 101.4 p.p.m.

The fully methylated, degraded polysaccharide had $[\alpha]_D$ −94.0° (*c* 1.0, chloroform), and hydrolysis (2M trifluoroacetic acid, 18 h, 95°), followed by derivatisation of the products and analysis by g.l.c., gave the results shown in Table II. The substitution pattern of each component was confirmed by m.s.

Base-catalysed degradation of methylated polysaccharide. — A solution of carefully dried, methylated polysaccharide (100 mg) and toluene-*p*-sulphonic acid (trace) in a mixture (20 ml) of methyl sulphoxide and 2,2-dimethoxypropane (19:1) was prepared in a serum vial that was sealed with a rubber cap. The vial was flushed with nitrogen, and the solution was stirred for 3 h. Sodium methylsulphinylmethanide (2M) in methyl sulphoxide (10 ml) was then added with the aid of a syringe, and the solution was stirred at room temperature overnight. After cooling to 10°, ethyl iodide (3 ml) was added dropwise, using a syringe. The solution was stirred for a further 30 min, excess of ethyl iodide was removed by using a rotary evaporator, and the solution was dialysed overnight against tap water. After concentration to dryness, the product (80 mg) was purified by decoloration with carbon, centrifugation, and precipitation into petroleum ether (30–60°), yielding 65 mg of polymeric material.

A sample (10 mg) of this material was hydrolysed with 90% (v/v) aqueous formic acid (2 ml) at 95° for 2 h, followed by hydrolysis overnight with trifluoroacetic acid (2M, 2 ml) at 95°. The product was reduced with sodium borohydride and acetylated. G.l.c. analysis (column *B*) gave the results shown in Table II. The substitution pattern of each component was confirmed by using m.s. P.m.r. data are given in Table III.

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