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STRUCTURE OF THE CAPSULAR POLYSACCHARIDE OF *Klebsiella* TYPE 73 (*Enterobacter aerogenes*)

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ABSTRACT

The capsular polysaccharide from *Klebsiella* Type 73 (*Enterobacter aerogenes*) was found to contain equimolar amounts of D-galactose, D-glucose, L-rhamnose, and D-glucuronic acid. Acid hydrolysis of the polysaccharide gave one aldobio- and one aldotrio-uronic acid, whose structures were established by acid hydrolysis and by methylation analysis. The anomeric configurations of the different sugar residues were determined from the specific rotations of the polysaccharide and the aldobio- and aldotrio-uronic acids, and also by oxidation of the native and the carboxyl-reduced polysaccharide with chromium trioxide. Methylation analysis of the polysaccharide provided information about the linkages of the different sugar residues. Based on all of these results, the structure assigned to the repeating unit of the polysaccharide is as follows.

$$\rightarrow 3)\text{-}\beta\text{-L-Rha}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Gal}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow$$
$$\begin{array}{c} 3 \\ \uparrow \\ 1 \end{array}$$
$$\beta\text{-D-Glc}p\text{A}$$

INTRODUCTION

A type-specific, capsular polysaccharide from the Gram-negative bacteria designated *Klebsiella* Type 73 was isolated by Nimmich[1]. This strain has recently been deleted[2] as being *Klebsiella*, because of its motility, and has been identified as *Enterobacter aerogenes*. It was reported[1] that, on acid hydrolysis, the polysaccharide gave glucose, galactose, rhamnose, and glucuronic acid. We now report structural studies of this polysaccharide.

RESULTS AND DISCUSSION

The polysaccharide (K-73) from *Klebsiella* Type 73 was purified by passing a

solution of it through a column of Sephadex G-100. The major portion of the material (80%) emerged from the column as a broad peak, and had $[\alpha]_{589.6}^{23}$ 0°. The homogeneity of the polysaccharide was revealed by electrophoresis in borate buffer and by ultracentrifugal analysis using Schlieren optics. Hydrolysis of the polysaccharide with 0.5M sulfuric acid for 20 h at 100°, followed by paper chromatography in solvent system *A*, gave spots corresponding to rhamnose, galactose, glucose, and glucuronic acid, and a trace of a slower-moving component (R_{Glc} 0.42) which appeared to be an aldobiouronic acid. G.l.c. analysis of the alditol acetates from the components of the hydrolyzate gave results corresponding to rhamnose, galactose, and glucose. The sugar components were isolated by preparative paper-chromatography, and their configurations, as determined by their specific rotations, were found to be D-galactose, D-glucose, L-rhamnose, and D-glucuronic acid.

Having identified the sugar components in the polysaccharide, it was necessary to estimate them. The uronic acid was determined spectrophotometrically by the carbazole method[3] and its proportion was found to be 22%. The polysaccharide was reduced with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate. The alditol acetate obtained from the acid hydrolyzate of the carboxyl-reduced K-73 showed the presence of galactose, glucose, and rhamnose in the ratios of 1:2:1. In another experiment, K-73 was acetylated, and the product was reduced with diborane[4]. Analysis, by g.l.c., of the acid hydrolyzate of this reduced K-73 showed the same ratio of the sugar components. The results are shown in Table I.

The K-73 polysaccharide was now hydrolyzed with 0.5M sulfuric acid for different times (up to 20 h) at 100°. Analysis of the various hydrolyzates by g.l.c. showed that the percentage of galactose had increased with time from 12.9% in 4 h to 26.3% in 20 h (see Table I). It is, therefore, probable that the galactose residue is linked to C-1 of glucuronic acid.

Hydrolysis of K-73 with 0.5M sulfuric acid for 4 h gave a mixture, paper chromatography of which, in solvent system *A*, revealed the presence of glucose, galactose, rhamnose, and two slower-moving spots. One of the slower-moving spots (Component *1*) was the same as that already observed having R_{Glc} 0.42, and it was

TABLE I

RESULTS OF ACID HYDROLYSIS OF K-73 AND CARBOXYL-REDUCED K-73

Sugars as alditol acetates	*Mole percent of sugars from K-73*			*Mole percent of sugars in carboxyl-reduced K-73*	
	Time of hydrolysis (h)				
	4	*8*	*20*	*Diborane-reduced*	*Carbodiimide-reduced*
Rhamnose	47.5	37.1	33.1	26.4	27.9
Galactose	12.9	17.1	26.3	24.7	24.2
Glucose	39.5	45.7	39.8	48.9	47.9
myo-Inositol				38	22

TABLE II

METHYLATION ANALYSIS OF K-73 AND DERIVED POLY- AND OLIGO-SACCHARIDES

Methylated[a] sugars (as alditol acetates)	*Retention time (minutes)*		*Mole %*				m/z							
	Column 1	*Column 2*	*I*	*II*	*III*	*IV*	*45* %	*87*	*101*	*113*	*117*	*129*	*131*	*233*
2,4-Rha	0.99	0.94	38.7	28.0				17	60		100		22	4
2,4,6-Gal	2.28	2.03			52.6	36.2								
2,3,4-Glc	2.49	2.22		20.5	47.4	28.6								
2,3,6-Glc	2.50	2.32	32.6	26.3		35.2	60	35	43	52	100	18		27
2,6-Gal	3.70	3.11	29.1	25.2			26	18			100	26		

[a]2,4 Rha = 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-L-rhamnitol, etc. I, methylated, original K-73 polysaccharide; II, methylated and reduced K-73 polysaccharide; III, methylated and reduced component *1*; IV, methylated and reduced component *2*.

probably an aldobiouronic acid. The other slower-moving spot (Component *2*) had R_{Glc} 0.22, and was probably an aldotriouronic acid. (The mobility of these spots in solvent system *B* was almost zero.) The acidic and the neutral components were separated from the hydrolysis mixture by means of a column of Dowex-1 X4 (OAc^-) ion-exchange resin. From the acidic part, Components *1* and *2* were isolated by preparative paper-chromatography in solvent *A*.

Component *1*, $[\alpha]_{589.6}^{23}$ +11°, had an equivalent weight of 352, and was, therefore, an aldobiouronic acid. Acid hydrolysis of the material with 0.5M sulfuric acid for 20 h, followed by paper chromatography in solvent system *A*, showed the presence of glucuronic acid, galactose, and some unreacted starting-material. Analysis of the hydrolyzate by g.l.c. (column *1*) showed galactose as the only sugar present. Component *1* was reduced with diborane, and acid hydrolysis of the resulting, neutral disaccharide showed the presence of glucose and galactose in the ratio of 1:1; this indicated that the aldobiouronic acid was GlcA→Gal. Methylation[5] of the aldobiouronic acid gave a fully methylated product that, on hydrolysis, gave a single peak of 2,4,6-tri-*O*-methylgalactose as its alditol acetate. In another experiment, the methylated aldobiouronic acid was reduced with lithium aluminum hydride[6], the product was hydrolyzed, and the hydrolyzate analyzed by g.l.c. (as the alditol acetates). 2,4,6-Tri-*O*-methylgalactose and 2,3,4-tri-*O*-methylglucose were obtained in the ratio of 1:1 (see Table II). All these findings confirmed that component *1* is an aldobiouronic acid having the structure 3-*O*-(β-D-glucopyranosyluronic acid)-D-galactose.

Component *2*, $[\alpha]_{589.6}^{23}$ 0°, had an equivalent weight of 512, and was probably an aldotriouronic acid. Hydrolysis of component *2* with 0.5M sulfuric acid for 20 h at 100° gave galactose and glucose, the proportion of glucose being greater than that of galactose; consequently, component *2* was composed of glucuronic acid, galactose, and glucose. The aldotriouronic acid was methylated by the Kuhn method[5]. The

hydrolyzate of the permethylated product gave, in g.l.c., peaks corresponding to 2,4,6-tri-*O*-methylgalactose and 2,3,6-tri-*O*-methylglucose. In another experiment, the permethylated aldotriouronic acid was reduced with lithium aluminum hydride[6]. G.l.c. of the alditol acetates obtained on hydrolysis of the reduced product gave peaks for 2,4,6-tri-*O*-methylgalactose, 2,3,6-tri-*O*-methylglucose, and 2,3,4-tri-*O*-methylglucose in the ratios of 1 : 1 : 0.9 (see Table II); therefore, the aldotriouronic acid must be 4-*O*-[3-*O*-(*β*-D-glucopyranosyluronic acid)-*β*-D-galactopyranosyl]-D-glucose.

The K-73 polysaccharide was methylated first by the Hakomori procedure[7,8] and then by the Kuhn procedure[5]. The i.r. spectrum of the permethylated product showed no hydroxyl band, indicating complete methylation. A portion of the permethylated polysaccharide was hydrolyzed, first with formic acid for 2 h and then with 0.5M sulfuric acid for 20 h. Alditol acetates were prepared from the hydrolyzate. Analysis by g.l.c. (columns *1* and *2*) and g.l.c.–m.s. (column *2*) showed 2,4-di-*O*-methylrhamnose, 2,3,6-tri-*O*-methylglucose, and 2,6-di-*O*-methylgalactose (see Table II). The other part of the permethylated K-73 was reduced with lithium aluminum hydride[6]. Alditol acetates were prepared from the reduced material, and these were analyzed by g.l.c. The analysis showed the presence of 2,4-di-*O*-methylrhamnose, 2,3,4-tri-*O*-methylglucose, 2,3,6-tri-*O*-methylglucose, and 2,6-di-*O*-methylgalactose in almost equimolar proportions. The results are listed in Table II. The 2,3,4-tri-*O*-methylglucose must have derived from D-glucuronic acid, because this peak was absent before reduction with lithium aluminum hydride. Moreover, it was also evident that the D-glucosyluronic acid groups are attached to O-3 of galactose as nonreducing end-groups. The galactose unit must, in turn, be attached to O-4 of the glucose residue through its reducing end, in order to fit the aldotriouronic acid into the repeating unit. The only sugar residue left to be incorporated into the repeating unit, namely, rhamnose, must be attached to O-4 of galactose and must also be linked to the glucose unit through its O-3 atom.

It was now desirable to determine the anomeric configurations of the different sugar residues of K-73. The polysaccharide has $[\alpha]_{589.6}^{23}$ 0°. The low specific rotation of the aldobiouronic acid strongly indicated that the uronic acid is *β*-linked. The aldotriouronic acid has $[\alpha]_{589.6}^{23}$ 0°, indicating that the galactose residue also has the *β* configuration. In order to obtain further information about the configuration of the sugar residues, K-73 and the carboxyl-reduced K-73 were acetylated, and the peracetates were subjected to oxidation with chromium trioxide[7]. During such an oxidation, only an axially oriented 1-proton is abstracted, to yield a 5-hexulosonic acid, thus leading to the disappearance of the sugar residues having *β*-glycosidic linkages. In both of the foregoing experiments (see Table III), the amounts of all of the sugar components decreased very sharply with time, indicating that all of the sugar residues have *β*-glycosidic linkages. The *β*-rhamnosyl residue would contribute to the specific rotation in the positive direction; and the other sugars, namely, galactose, glucose, and glucuronic acid, all having the *β* configuration, would contribute to the specific rotation in the negative direction. It was, therefore, to be expected that the specific rotation of the polysaccharide would be close to zero.

TABLE III

OXIDATION OF PERACETYLATED K-73 AND CARBOXYL-REDUCED K-73 WITH CHROMIUM TRIOXIDE

Material	*Time of oxidation (h)*	*Rhamnose*	*Galactose*	*Glucose*	myo-*Inositol*
K-73	0	79.0	60.0	90.0	100
	1	8.5	5.4	8.3	100
	2	1.3	1.3	5.1	100
Carboxyl-reduced K-73	0	20.1	21.1	37.0	20
	1	6.7	5.8	11.8	20
	2	1.2	1.5	4.1	20

We conclude that the polysaccharide from *Klebsiella* Type 73 (*Enterobacter aerogenes*) has the tetrasaccharide **1** as its repeating unit.

$$\rightarrow 3)\text{-}\beta\text{-L-Rha}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Gal}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow$$
$$\begin{array}{c} 3 \\ \uparrow \\ 1 \end{array}$$
$$\beta\text{-D-Glc}p\text{A}$$

1

EXPERIMENTAL

Materials and methods. — Paper partition-chromatography was performed on Whatman Nos. 1 and 3 MM papers. Solvent systems (v/v) used were (*A*) 4:1:5 1-butanol–acetic acid–water (upper layer) and (*B*) 8:2:1 ethyl acetate–pyridine–water; the spray reagent used was alkaline silver nitrate[9]. All solvents were distilled before use, and all evaporations were conducted at 50°, unless stated otherwise. Optical rotations were measured with a Perkin–Elmer Model 241MC spectropolarimeter. Colorimetric estimates were conducted with a Yanaco Model SP-1 spectrophotometer.

Gas–liquid chromatography (g.l.c.) was performed with a Hewlett–Packard Model 5730A gas chromatograph having a flame-ionization detector, and glass columns (1.83 m × 6 mm) with (*1*) 3% of ECNSS-M on Gas Chrom Q (100–120 mesh) and (*2*) 3% of OV-225 on Gas Chrom Q (100–120 mesh). All g.l.c. analyses were conducted (at 185° for unmethylated sugars, and at 170° for methylated sugars) by converting the sugars into their alditol acetates[10]. Retention times of partially methylated alditol acetates were measured with respect to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol as unity. G.l.c.–m.s. analyses were performed with a VG-Micromass spectrometer at an ionization potential of 70 eV.

Purification of the polysaccharide. — The polysaccharide (K-73) was isolated by Nimmich[1]. The material was purified in 50-mg batches by passage through a column

of Sephadex G-100. The column was eluted with 0.5M ammonium hydrogencarbonate solution (pH 8.0), and 70 fractions (5 mL each) were collected. The fractions were automatically monitored with a Waters Associates' Differential Refractometer Model 403 fitted with a recorder. Fractions 24–48 contained the polysaccharide, which emerged as a broad peak. The fractions containing the polysaccharide were combined, and lyophilized; yield 41 mg, $[\alpha]_{589.6}^{23}$ 0° (*c* 0.5, water). The homogeneity of the polysaccharide was established by means of (*1*) a Beckman L5-65 Ultracentrifuge having Schlieren optics, utilizing a 1% solution of the polysaccharide in 0.1M phosphate buffer for 45 min, and (*2*) high-voltage electrophoresis in borate buffer in a shandon Model L-24 apparatus.

Acid hydrolysis of the polysaccharide. — The polysaccharide (2 mg) was hydrolyzed with 0.5M sulfuric acid for 20 h at 100°. The acid was neutralized with barium carbonate, the slurry filtered through a Celite bed, and the filtrate was divided into two parts. One part was treated with Amberlite IR-120 ion-exchange resin, the suspension filtered, and the filtrate concentrated to a small volume and examined by paper chromatography in solvent *A*; spots for rhamnose, glucose, galactose, glucuronic acid, and a trace of a slower-moving component having R_{Glc} 0.42 were found. To the other part of the filtrate was added an equal volume of 40% sodium borohydride solution, and the alditol acetates were prepared in the usual way[8] and analyzed by g.l.c. (column *1*).

Isolation of aldobio- and aldotrio-uronic acids. — In a pilot experiment, one-third of a solution of the polysaccharide (6 mg) in 0.5M sulfuric acid (1.5 mL) was poured into each of three ampules, and the ampules were respectively heated for 4, 8, and 20 h at 100°. The alditol acetates were prepared from the three hydrolyzates, and these were analyzed by g.l.c. The results are shown in Table I. The hydrolyzates from the aforementioned fractions were examined by paper chromatography (solvent *A*); the first fraction (hydrolysis time, 4 h) gave good spots of two slower–moving components having R_{Glc} 0.42 and 0.22, respectively. The mobility of these components in solvent *B* was almost zero.

In a separate experiment, the polysaccharide (90 mg) was hydrolyzed with 0.5M sulfuric acid for 4 h at 100°. The acid was neutralized with barium carbonate, and the solution was treated with Amberlite IR-120 resin, and concentrated to a small volume. The mixture was then passed through a column (20 × 1 cm) of Dowex-1 X4 (OAc^-) anion-exchange resin. The neutral sugars were obtained by eluting the column with water, and the eluate was evaporated to dryness. The acidic sugars were then isolated by eluting the column with 30% acetic acid, and the eluate was evaporated to dryness (31 mg). By preparative paper-chromatography of the neutral and acid mixtures, the following sugars were isolated and their specific rotations determined: rhamnose (12 mg), galactose (4 mg), glucose (8 mg), glucuronic acid (3 mg), aldobiouronic acid (component *1*; 11.8 mg) and aldotriouronic acid (component *2*, 7 mg).

Preparation of carboxyl-reduced K-73 polysaccharide. — To a solution of K-73 polysaccharide (15 mg) in water (12 mL) was added 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (450 mg), with stirring. Cyclohexanol

(3 drops) was added as an antifoaming agent, and the pH was kept at 4.75 by addition of 0.01M hydrochloric acid. After 2 h, aqueous 2M sodium borohydride (8 mL) was added during 1 h, and the pH was kept at 7 by simultaneous addition of 4M hydrochloric acid. The solution was dialyzed for 24 h against distilled water, and freeze-dried. The procedure was repeated twice on the same material, to ensure complete reduction of the carboxyl groups, giving 9.5 mg of product having $[\alpha]_{589.6}^{23}$ 0° (*c* 0.2, water).

In a separate experiment[9], K-73 (10 mg) was dissolved in formamide (1 mL), and pyridine (4 mL) and acetic anhydride (2.5 mL) were added. The mixture was stirred for 60 h at room temperature and evaporated to dryness *in vacuo* at 40°. The product was reacetylated with pyridine and acetic anhydride. The material was dissolved in oxolane, an excess of diborane in the same solvent was added, and the mixture was stirred for 20 h at room temperature. The excess of diborane was decomposed by adding methanol dropwise, and methyl borate was removed by repeated addition and evaporation of methanol. The product was deacetylated with 0.1M sodium methoxide, sodium ions were removed with Dowex 50-W X8 cation-exchange resin, the suspension was filtered, and the filtrate was lyophilized, to give carboxyl-reduced K-73 (7 mg). The i.r. spectrum of the compound (KBr pellet) showed a very small peak for carbonyl stretching-vibration.

Determination of sugar components in the K-73 polysaccharide. — Carboxyl-reduced polysaccharide (1.5 mg) from the carbodiimide reduction was mixed with *myo*-inositol (0.3 mg), the mixture was heated with 0.5M sulfuric acid (1 mL) for 20 h at 100°, and the alditol acetates were prepared. Examination by g.l.c. in column *1* showed the ratios of galactose:glucose:rhamnose to be 1:2:1. The results are given in Table I. In a separate experiment, the carboxyl-reduced polysaccharide (1.2 mg) obtained by diborane reduction was mixed with *myo*-inositol (0.42 mg), the mixture was heated with 0.5M sulfuric acid (1 mL) for 20 h at 100°, and the alditol acetates were prepared. Examination by g.l.c. (column *1*) gave the same result as before. The results are given in Table I. The uronic acid content in the native, K-73 polysaccharide, estimated by the carbazole method[3], was 20%.

Hydrolysis of the aldobiouronic acid and its carboxyl-reduced product. — The aldobiouronic acid (0.5 mg) was hydrolyzed with 0.5M sulfuric acid for 20 h, and the products were analyzed, as the alditol acetates, by g.l.c. (column *1*). The neutral sugar observed was galactose. In another experiment, the aldobiouronic acid (1 mg) was acetylated with pyridene and acetic anhydride, and the acetate was reduced with an excess of diborane[4]. The neutral disaccharide was hydrolyzed, and the alditol acetates were prepared; analysis by g.l.c. (column *1*) showed glucose and galactose in the ratio of 0.9:1.

Methylation analysis of the aldobiouronic acid. — The aldobiouronic acid (2 mg) was methylated by the method already described[10]. Examination by g.l.c. (columns *1* and *2* at 170°) of the alditol acetates prepared from a portion of the methylated product showed 2,4,6-tri-*O*-methylgalactose. Another portion of the methylated aldobiouronic acid was reduced[10] with lithium aluminum hydride, and

the product hydrolyzed; the alditol acetates were prepared, and analyzed by g.l.c. as before. The analysis showed 2,4,6-tri-*O*-methylgalactose and 2,3,4-tri-*O*-methyl glucose in the ratio of 1 : 1. The results are given in Table II.

Hydrolysis of aldotriouronic acid. — The aldotriouronic acid (0.5 mg) was hydrolyzed, and the alditol acetates were prepared in the usual way and analyzed by g.l.c.

Methylation analysis of aldotriouronic acid. — The aldotriouronic acid was methylated[10]. The alditol acetates obtained from a portion of the methylated product were analyzed by g.l.c. as before. Another portion of the permethylated aldotriouronic acid was reduced with lithium aluminum hydride as described[10], and the alditol acetates prepared from this material were analyzed by g.l.c. (see Table II).

Methylation analysis of K-73 polysaccharide. — The K-73 polysaccharide (23 mg) was dissolved in dry dimethyl sulfoxide (15 mL) by stirring overnight in a serum vial. To the solution, under a nitrogen atmosphere, was added methylsulfinyl carbanion[7,8] (15 mL) and the mixture was stirred for 1 h, and kept overnight. The vial was then cooled in an ice bath, methyl iodide (15 mL) was added, and the mixture was stirred for 2 h. The vial was opened, the excess of methyl iodide was removed by passing nitrogen through the solution, and the mixture was dialyzed against distilled water for 2 days. The solution from the dialysis bag was lyophilized, to afford methylated K-73, the i.r. spectrum of which showed a small band for hydroxyl stretching-vibration. The material was therefore remethylated by the Kuhn method[5], and the product (16 mg) then showed no hydroxyl band in the i.r. spectrum. A portion (4 mg) of the methylated K-73 was hydrolyzed, first with 90% formic acid for 2 h on a boiling-water bath, and then with 0.5M sulfuric acid for 20 h at 100°. The alditol acetates were prepared from the hydrolyzate, and examined by g.l.c. (columns *1* and *2*) and g.l.c.–m.s. (column *2*). Another portion (5 mg) of the methylated K-73 was dissolved in a mixture of dichloromethane (9 mL) and ethyl ether (6 mL). To this solution was added an excess of lithium aluminum hydride (45 mg); the mixture was boiled under reflux for 5 h in a hot-water bath. The excess of the hydride was decomposed by adding ethyl acetate and, finally, a few drops of water. The mixture was then filtered under suction, and the filtrate was evaporated to dryness. The product was hydrolyzed as usual, and the alditol acetates were prepared, and analyzed by g.l.c. (columns *1* and *2*). The results are summarized in Table II.

Oxidation of K-73 and carboxyl-reduced K-73 with chromium trioxide[9]. — These two reaction sequences were performed exactly as described earlier[10]. The results are summarized in Table III.

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