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Structural investigation of *Klebsiella* serotype K10 capsular polysaccharide

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The primary structure of *Klebsiella* serotype K10 capsular polysaccharide has been investigated using mainly the techniques of methylation, partial hydrolysis, and ^{1}H and ^{13}C NMR spectroscopy. The polysaccharide was found to consist of hexasaccharide repeating units having the following structure:

α-D-Gal*p*
$^{1}\downarrow_{4}$
β-D-Glc*p*
$^{1}\downarrow_{4}$
β-D-Glc*p*A
$^{1}\downarrow_{2}$
→4)-β-D-Gal*p*-(1→3)-α-D-Man*p*-(1→4)-α-D-Gal*p*-(1→

Of the 81 serologically different strains of *Klebsiella* bacteria [1–3] the capsular polysaccharides isolated from almost all the strains have now been reported for their structural studies. The serotype K10 glycan is one of the few remaining ones, and the structure of this polysaccharide is now reported.

Klebsiella K10 belongs to the group containing 20 chemotypes, all of which contain mannose, galactose, glucose and glucuronic acid; 10 of these 20 polysaccharides also contain pyruvic acid. The K10 capsular polysaccharide does not contain any pyruvate or *O*-acetyl substituent.

EXPERIMENTAL PROCEDURE

The serological test strain [4] for *Klebsiella* K10 capsular antigen was kindly supplied by Prof. S. Stirm of the University of Giessen (FRG). The strain was grown on nutrient agar medium [5], harvested, dried, and the capsular polysaccharide was isolated by the phenol/water/Cetavlon method [6]. A portion of the native polysaccharide was mildly alkali-treated [7].

All the methods used during the present investigation have been reported or cited previously [7, 8].

Descending paper chromatography was carried out using Whatman nos 1 and 3 paper, and the following solvent systems (by vol.): (a) ethyl acetate/acetic acid/formic acid/water (18:3:1:4) and (b) ethyl acetate/pyridine/water (4:1:1).

The equivalent weight of the polysaccharide was determined by a conductometric titration method [9].

Sedimentation analysis was carried out in an analytical ultracentrifuge using a 0.4% solution in phosphate-buffered physiological saline at pH 7.

Quantitative constituent analyses of sugars as their alditol acetates were performed with a Chemito-3800 gas chromatograph instrument using ECNSS-M and OV-17 columns [10]. Hexuronic acid was estimated by the carbazole/sulphuric acid method [11].

Reduction of the carboxyl groups in the polysaccharide and the oligosaccharides was carried out using sodium borohydride and carbodiimide (CMC) according to the method of Taylor and Conrad [12].

The reducing end group of the oligosaccharide was detected [13] by GLC of the acetylated alditol and the derived aldononitriles.

Methylation was carried out by the method of Hakomori [14] following the experimental details of Hellerqvist et al. [15]. Mass spectrometry [16] of the fragments of the permethylated products was performed with the Finningan combined GLC-MS instrument.

Uronic acid degradation [17] was performed using the methylsulfinyl carbanion.

Oligosaccharides were isolated by partial acid hydrolysis of the polysaccharide with 0.5 M H_2SO_4. Acidic oligosaccharides were separated by high-voltage paper electrophoresis [18] and neutral oligosaccharides were separated by paper chromatography (solvent a) of the neutral fraction of the hydrolysate obtained after passing through Amberlite AG-1-X_2 (formate) resin.

NMR spectroscopic analysis was performed with the Bruker WM-300 spectrometer with D_2O as the solvent at room temperature using sodium 4,4-dimethyl-4-sila(2,2,3,3,-$^{2}H_4$)pentanoate as the external standard. ^{1}H NMR experiments were carried out at 300.13 MHz and ^{13}C NMR at 75.47 MHz.

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Abbreviation. CMC, 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate.

Table 1. *Sugar analysis of* Klebsiella *K10 capsular polysaccharide*
Hexoses were determined by GLC of the alditol acetates [10], hexuronic acid by the carbazole/sulphuric acid method [11]; native polysaccharide was treated [7] for 4 h at 56°C with 0.25 M NaOH; polysaccharide was carboxyl-reduced using carbodiimide (CMC) and sodium borohydride [12]; the M_r of the repeating unit was determined by conductometric titration of the acidic polysaccharide with 0.1 M NaOH. n.d. = not determined

Polysaccharide	Molar ratios of constituent sugars				M_r
	D-Glc	D-Gal	D-Man	D-GlcA	
Native	1.00	2.80	0.90	1.05	1008 ± 5
Alkali-treated	1.00	2.70	0.90	1.00	1015 ± 5
Carboxyl-reduced	1.80	3.00	1.00	0.25	n.d.

RESULTS

Isolation, homogeneity and composition of the capsular polysaccharide

About 4.6% (w/w) of the *Klebsiella* K10 capsular polysaccharide was isolated from the dry bacterial mass by the phenol/water/Cetavlon (cetyltrimethylammonium bromide) procedure [6]. The polysaccharide sedimented uniformly in the analytical ultracentrifuge showing a single peak (s°_{20} = 2.80 S). The native polysaccharide was used for the spectral analysis and the mildly alkali-treated [7] material was used for methylation and other experiments. Optical rotations of the sugars, isolated from the hydrolysate, indicated that all of them had the D configuration. The M_r of the repeating unit of the native and the alkali-treated products, determined by conductometric titration, were found to be 1008 ± 5 and 1015 ± 5 respectively.

The analysis of the type-10 polysaccharide showed the presence of D-glucose, D-galactose, D-mannose and D-glucuronic acid in the molar ratios 1.00:2.80:0.90:1.05 (Table 1). The neutral sugars were analysed by GLC as alditol acetates and glucuronic acid by the modified carbazole method. A portion of the polysaccharide was carboxyl-reduced with 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate (CMC) and sodium borohydride according to the method of Taylor et al. [12]. The carboxyl-reduced product contained D-glucose, D-galactose, D-mannose and D-glucuronic acid in the molar ratios 1.80:3.00:1.00:0.25.

Isolation of the oligosaccharides

Partial acid hydrolysis of the polysaccharide yielded a disaccharide (H2), trisaccharide (H3) and tetrasaccharide (H4), containing glucuronic acid and a neutral disaccharide (N2). Mobilities for the H2, H3 and H4 relative to D-glucuronic acid (R_{GlcA}) in high-voltage paper electrophoresis (45 V/cm, 2 h) in the solvent pyridine/glacial acetic acid/water (10:4:86) at pH 5.3 were found to be 0.66, 0.48 and 0.37 respectively. The neutral disaccharide (N2) was separated by paper chromatography (R_{Glc} = 0.32, solvent b). The sugar composition and some other properties of the oligosaccharides are shown in Table 2. The disaccharide (N2) contained D-galactose and D-glucose (1.00:0.90), and glucose was the reducing-end sugar. The disaccharide (H2) was composed of D-glucuronic acid and D-mannose (1.00:0.80) with mannose as the reducing end. The trisaccharide contained D-glucuronic acid, D-mannose and D-galactose in the molar ratios 1.00:0.90:1.00, and galactose occupied the reducing end. The tetrasaccharide (H4) was composed of D-glucuronic acid, D-mannose and D-galactose in the molar ratios 1.00:0.90:1.70, and galactose was the reducing-end sugar.

Methylation analysis

The type-10 polysaccharide and the derived oligosaccharides were methylated according to Hakomori [14], and the permethylated products were analysed by GLC and GLC-MS. The results are shown in Table 3. The methylated polysaccharide contained 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol and 1,2,3,5-tetra-*O*-acetyl-4,6-di-*O*-methylmannitol in the molar ratios 1.00:2.20:0.90:0.85 (Table 3, column I). When the methylated product was reduced/dideuterated [19] before hydrolysis, one molar proportion of additional 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methylglucitol was obtained (column II). The dideuterated fragment, obtained from the permethylated glycan, was identified by the appearance of the mass spectral peak at m/z = 263 instead of the normal peak at m/z = 261.

The methylation results obtained from the neutral disaccharide N2, and the uronic acids H2, H3 and H4 are also shown in Table 3 (columns III – VII). Methylated H4 yielded 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol and 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylmannitol in the molar ratios 1.70:1.00 (column VI). But when the methylated product was carboxyl-reduced/dideuterated before hydrolysis, one molar proportion of additional 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol was obtained (column VII). The dideuterated fragment was identified by the appearance of the peaks of m/z = 191 and 235 in the mass spectra instead of the normal peaks at m/z = 189 and 233.

Uronic acid degradation

The methylated polysaccharide was subjected to base degradation [17]. The resulting products contained 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol, 1,2,3,5-tetra-*O*-acetyl-4,6-di-*O*-methylmannitol in the molar ratios 0.20:2.00:0.25:1.00 (Table 3, column VIII).

NMR spectral analysis

The results of ^{1}H NMR and ^{13}C NMR spectra were summarised in Table 4. The ^{1}H NMR spectroscopy of the polysaccharide showed the presence of six anomeric protons, of which three were α linkages and three β linkages. From the signal at δ = 5.42 ppm (J, 3.5 Hz) in the spectrum of N2, α linkage was assigned to galactose. In the spectrum of H2 the anomeric signals at δ = 4.92 ppm and 4.83 ppm were integrated together for 1.5 integrals of protons, hence indicating that glucuronic acid was β-linked. The signal at δ = 5.21 ppm in the spectrum of H3 was accounted for by the α linkage of the mannose. In the spectrum of H4, the signal at δ = 5.35 ppm (J, 3.5 Hz) was assigned to the α-galactose attached to the reducing-end galactose. From the signals at δ = 4.82 ppm (J, 7.0 Hz) and 4.55 (J, 7.0 Hz) in the spectrum of the polymer, the glucose and the second galactose of the repeating unit were deduced to be β-linked.

Table 2. *Analysis of oligosaccharides obtained by partial acid hydrolysis of* Klebsiella *K10 capsular polysaccharide*
N2, neutral disaccharide, isolated by paper chromatography (solvent a; $R_{Glc} = 0.32$); H2, hydrolysis of the polysaccharide with 0.5 M H_2SO_4, 100 °C, 2.5 h; H3, 1 h hydrolysis; H4, 1 h hydrolysis (acidic oligosaccharides were separated by high-voltage electrophoresis [18]); ratio of reducing/non-reducing hexoses was determined from the ratio of acetylated alditol/aldononitriles [13], the GlcA derivative was not recorded in GLC analysis. $R_{D\text{-}GlcA}$, mobility in paper electrophoresis relative to D-GlcA

Oligosaccharide	Yield	Approx. molar ratio of sugars				Reducing-end sugar	Ratio of reducing/non-reducing hexoses	$R_{D\text{-}GlcA}$
		D-GlcA	D-Man	D-Gal	D-Glc			
	%	mol/mol						
N2	2.5	–	–	1.00	0.90	D-Glc	1.0:1.0	–
H2	5.4	1.00	0.80	–	–	D-Man	–	0.66
H3	4.2	1.00	0.90	1.00	–	D-Gal	1.0:1.0	0.48
H4	12.5	1.00	0.90	1.70	–	D-Gal	1.0:1.8	0.37

Table 3. *Analysis of* O-*acetyl*-O-*methyl alditols obtained from* Klebsiella *serotype K10 capsular polysaccharide and derived products*
2,3,4,6-GalOH indicates 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, etc. I, type-10 polysaccharide, permethylated; II, type-10 polysaccharide, permethylated and reduced with $Ca(BD_4)_2$ before hydrolysis; III, neutral disaccharide (N2), permethylated; IV, H2, permethylated; V, H3, permethylated; VI, H4, permethylated; VII, H4, permethylated carboxyl-reduced/dideuterated; VIII, permethylated type-10 polysaccharide, base-degraded [17]

Methylated sugars (as alditol acetates)	Molar ratios of sugars (ratio of peak integrals)							
	I	II	III	IV	V	VI	VII	VIII
	mol/mol							
2,3,4,6-GalOH	1.00	1.00	1.00	–	–	–	–	0.20
2,3,6-GalOH	2.20	2.00	–	–	0.80	1.70	1.80	2.00
2,3,6-GlcOH	0.90	1.05	0.90	–	–	–	–	0.25
3,4,6-ManOH	–	–	–	1.00	1.00	1.00	1.00	–
2,3,4-GlcOH	–	–	–	–	–	–	0.75	–
4,6-ManOH	0.85	0.90	–	–	–	–	–	1.00
2,3-GlcOH	–	0.80	–	–	–	–	–	–

The ^{13}C NMR spectrum of the polymer also clearly indicated six signals in the anomeric region, of which three (95.31, 96.76, and 97.03 ppm) were assigned for α linkages and three (101.90, 103.48, and 104.08 ppm) for β linkages. The signal at 175.31 ppm was attributed to C-6 of the glucuronic acid and those at 61.13 and 61.81 ppm were assigned for C-6 of the hexose residuces. The signals of the spectrum of the polymer in the anomeric region of the ^{13}C NMR were assigned in accordance with the assignment of the anomeric configuration by the ^1H NMR spectral data.

Enzymic hydrolysis

Results of the exoglycosidase treatment showed that on treatment of the α-D-galactosidase (Sigma) with the neutral disaccharide (N2), galactose and glucose were separated in paper chromatography, but β-D-galactosidase (Sigma) could not split the disaccharide. On incubation of H2 with β-D-glucuronidase from *Helix pomatia,* glucuronic acid was liberated.

DISCUSSION

The native glycan contained D-glucose, D-galactose, D-mannose and D-glucuronic acid in approximate molar ratios 1:3:1:1 (Table 1). Alkali treatment did not change the composition. The carboxyl-reduced product yielded D-glucose, D-galactose and D-mannose (2:3:1), which indicated that the additional proportion of D-glucose was derived from the D-glucuronic acid of the original polymer. The amount of D-glucuronic acid was proportionately reduced in the carboxyl-reduced product. The M_r (1008 ± 5) of the polymer repeating unit was found to be in good agreement with the results of sugar composition.

The composition (Table 1), the methylation results (Table 3) and the NMR spectral data (Table 4) of the polymer suggested that the type-10 glycan consisted of hexasaccharide repeating units containing one residues each of 2,3-disubstituted D-mannose, 4-substituted D-glucose, 4-substituted D-glucuronic acid and unsubstituted D-galactose, and two residues of 4-substituted D-galactose. From methylation data it also followed that the repeating unit was branched with a chain mannosyl residue carrying the branch that terminated in a galactosyl unit.

Six distinct signals in the ^1H NMR spectrum of the polymer (Table 4) indicated the presence of six anomeric protons. The spectrum did not show any signal at $\delta = 1.5$ ppm or 2.2 ppm thereby indicating the absence of pyruvic acetal [20] and *O*-acetyl groups in the polymer. The ^{13}C NMR spectrum of the polymer also showed the presence of six anomeric carbon atoms in the polymer.

The sequence of the constituents in the isolated oligosaccharides was determined from the methylation data (Table 4, columns III–VII). From the results of the sugar composition and other properties (Table 2), and methylation analysis, the following structures of the oligosaccharides were determined:

N2: D-Gal-(1→4)-D-Glc
H2: D-GlcA-(1→2)-D-Man
H3: D-GlcA-(1→2)-D-Man-(1→4)-D-Gal
H4: D-GlcA-(1→2)-D-Man-(1→4)-D-Gal-(1→4)-D-Gal.

When the methylated polysaccharide is subjected to base degradation [17], the 4-substituent on the glycosyluronic acid residue is eliminated along with the uronic acid. The results of the base degradation reaction (Table 3, column VIII) indicated that β-elimination caused the loss of both 1,5-di-*O*-

Table 4. *NMR data for* Klebsiella *K10 capsular polysaccharide and its oligosaccharides*
^{1}H NMR spectra were run at 300.13MHz at room temperature, and ^{13}C NMR at 75.47 MHz, room temperature; sodium 2,2,3,3-tetradeutero-4,4-dimethylsilapentanoate was used as the external standard. In ^{1}H NMR chemical shifts were measured relative to acetone (δ 2.22 ppm), and coupling constants, when available, in Hz; in ^{13}C NMR, chemical shifts were measured relative to acetone (31.07 ppm)

Oligosaccharide or polysaccharide	^{1}H				^{13}C	
	δ	(J)	Ratio of integrals	Proton assignment	δ	Anomeric carbon assignment
	ppm	(Hz)			ppm	
$Gal_{\alpha}^{1,4}$→Glc-OH	5.42	(3.5)	1.0	α-Gal		
Neutral oligosaccharide (N2)	5.32		0.6	α-Glc-OH		
	4.68		0.4	β-Glc-OH		
$GlcA_{\beta}^{1,2}$→Man-OH (H2)	5.33		0.5	α-Man-OH		
	4.92	(7.0)	1.6	β-Man-OH		
	4.83	(7.0)		β-GlcA		
$GlcA_{\beta}^{1,2}$→$Man_{\alpha}^{1,4}$→Gal-OH (H3)	5.41	(3.0)	0.6	α-Gal-OH		
	5.21		1.0	α-Man		
	4.88		1.5	β-Gal-OH		
	4.78			β-GlcA		
$GlcA_{\beta}^{1,2}$→$Man_{\alpha}^{1,4}$→$Gal_{\alpha}^{1,4}$→Gal-OH (H4)	5.35	(3.5)	1.0	α-Gal		
	5.27		1.5	α-Man		
	5.22	(3.5)		α-Gal-OH		
	4.65	(7.0)	1.6	β-GlcA		
	4.58			β-Gal-OH		
α-Gal	5.48	(3.5)	1.0	α-Gal	175.31	C-6 of GlcA
$^1\downarrow_4$	5.32	(3.0)	1.0	α-Gal	104.08	β-GlcA
β-Glc	5.14		1.0	α-Man	103.48	β-Gal
$^1\downarrow_4$	4.88		2.0	β-GlcA	101.90	β-Glc
β-GlcA	4.82	(7.0)		β-Gal	97.03	α-Gal
$^1\downarrow_2$	4.55	(7.0)		β-Glc	96.76	α-Gal
→$^3Man_{\alpha}^{1,4}$→$Gal_{\alpha}^{1,4}$→Gal_{β}^{1}→			1.0		95.31	α-Man
Type-10 polysaccharide					61.81	C-6 of hexose
					61.13	

acetyl-2,3,4,6-tetra-*O*-methylgalactitol and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol. This indicated that the 4-substituted glucose was linked to the C-4 of the glucuronic acid, and the terminal galactose residue was linked to C-4 position of the glucose. The β-elimination reaction further indicated that the glucuronic acid formed a part of the branch, and it was, therefore, evident that the branch in the repeating unit consisted of D-Gal-(1→4)-D-Glc-(1→4)-D-GlcA-(1→. From the structures of N2, H2, H3 and H4, and from the information that the chain mannosyl residue carried the branch, it could be deduced that the mannose was substituted by the branch at C-2, and the chain of the repeating unit comprised →3)-D-Man-(1→4)-D-Gal-(1→4)-D-Gal-(1→. The sequence of all the constituents in the hexasaccharide repeating unit was thus established.

The anomeric configurations of all the glycosidic linkages were assigned from the results of ^{1}H NMR spectral data of the oligosaccharides and the native polysaccharide (Table 4). Of the six anomeric signals in the ^{1}H-NMR spectrum of the native polymer, the results indicated that three were derived from α-linked and three from β-linked sugars. The ^{13}C NMR spectrum of the polysaccharide also showed six signals in the anomeric region with an approximately equal ratio of integrals. The assignment of β linkage to the glucuronic acid and α linkage to the terminal galactose was also supported by the results of enzymic reactions.

From the results discussed above, it has been concluded that the *Klebsiella* K10 capsular polysaccharide consists of hexasaccharide repeating units having the following structure:

α-D-Gal*p*
$^1\downarrow_4$
β-D-Glc*p*
$^1\downarrow_4$
β-D-Glc*p*A
$^1\downarrow_2$
→4)-β-D-Gal*p*-(1→3)-α-D-Man*p*-(1→4)-α-D-Gal*p*-(1→

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REFERENCES

1. Nimmich, W. (1968) *Z. Med. Mikrobiol. Immunol. 154,* 117–131.
2. Nimmich, W. (1979) *Z. Allg. Mikrobiol. 19,* 343–347.
3. Nimmich, W. (1971) *Acta Biol. Med. Ger. 26,* 397–403.
4. Kauffmann, F. (1966) *The bacteriology of Enterobacteriaceae,* Munkasgaard, Copenhagen.

5. Thurow, H., Choy, Y. M., Frank, N., Niemann, H. & Stirm, S. (1975) *Carbohydr. Res. 41,* 241–255.
6. Westphal, O. & Jann, K. (1965) *Methods Carbohydr. Chem. 5,* 83–91.
7. Niemann, H., Frank, N. & Stirm, S. (1977) *Carbohydr. Res. 59,* 165–177.
8. Okutani, K. & Dutton, G. G. S. (1980) *Carbohydr. Res. 86,* 259–271.
9. Chakraborty, A. K. (1982) *Makromol. Chem. 183,* 2881–2887.
10. Sawardeker, J. S., Sloneker, J. H. & Jeanes, A. (1965) *Anal. Chem. 12,* 1602–1604.
11. Bitter, T. & Muir, M. H. (1962) *Anal. Biochem. 4,* 330–334.
12. Taylor, R. L. & Conrad, H. E. (1972) *Biochemistry 11,* 1383–1388.
13. Morrison, I. M. (1977) *J.Chromatogr. 108,* 165–177.
14. Hakomori, S. (1964) *J. Biochem. (Tokyo) 55,* 205–207.
15. Hellerqvist, C. G., Lindberg, B., Svensson, S., Holme, T. & Lindberg, A. A. (1968) *Carbohydr. Res. 8,* 43–55.
16. Jansson, P. E., Kenne, L., Liedgren, H., Lindberg, B. & Lönngren, J. (1976) *Chem. Commun. Univ. Stockholm 8,* 1–75.
17. Lindberg, B. & Lönngren (1976) *Methods Carbohydr. Chem. 7,* 142–148.
18. Kickhofen, B. & Westphal, O. (1952) *Z. Naturforsch. B 7,* 655–662.
19. Chakraborty, A. K., Friebolin, H., Niemann, H. & Stirm, S. (1977) *Carbohydr. Res. 59,* 525–530.
20. Choy, Y. M., Dutton, G. G. S., Stephen, A. M. & Yang, M. T. (1972) *Anal. Lett. 5,* 675–681.
21. Bebault, G. M., Choy, Y. M., Dutton, G. G. S., Funnel, N., Stephen, A. M. & Yang, M. T. (1973) *J. Bacteriol. 113,* 1345–1347.