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Note

Structural studies of *E. coli* K26 capsular polysaccharide, using g.l.c.–c.i.-m.s.*

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The successful analysis of oligosaccharides, produced by various chemical techniques from polysaccharides, is dependent to a large extent on the purity and homogeneity of the sample. Separation and purification of oligosaccharide mixtures by the usual methods, *e.g.*, preparative paper chromatography and gel-permeation chromatography, can be time consuming and, where complex mixtures are involved, may be only partially successful. In addition, where the oligosaccharides are available in only small amounts, or have similar R_F values or molecular weights, separation is not feasible. Hence, a method for simultaneously separating, purifying, and characterizing mixtures of oligosaccharides is desirable, but few such techniques are available. Fast-atom-bombardment mass spectrometry (f.a.b.-m.s.) has been used to analyse directly mixtures of oligosaccharides; however, the sample must be pure and contain oligosaccharides of different molecular weights for successful characterization. Furthermore, the relative abundance of ions will not necessarily reflect the relative amounts of the components[1]. A different approach is to couple permethylated oligosaccharides with a fluorophore at their reducing ends, separate by h.p.l.c., and then sequence by d.c.i.-mass spectrometry[2].

The most readily available and simplest method for simultaneously separating, purifying, and characterizing a mixture of oligosaccharides is g.l.c.–m.s. There are many examples of the analysis of permethylated oligosaccharides and oligosaccharide-alditols by g.l.c.–e.i.-m.s.[3–5]. The sequence of monosaccharide units can be deduced from the e.i.-mass spectrum, and in some cases the position of specific glycosidic linkages can be inferred[6]. The use of g.l.c.–c.i.-m.s. affords higher intensities of the molecular ion and larger fragment ions relative to g.l.c.–e.i.-m.s., and thus improves the ability to discriminate between different oligosaccharide derivatives[7]. A recent review by Sweeley and Nunez[6] gave no references for the application of this combination of techniques to the structural characterization of oligosaccharides, but since the work presented here was completed, a paper has

*Dedicated to Professor Bengt Lindberg.

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been published[8] in which g.l.c.–c.i.-m.s. was used to analyse acidic and neutral oligosaccharides obtained from an anti-complementary acidic heteroglycan. However, the authors, prior to analysis by g.l.c.–c.i.-m.s., separated the acidic and neutral fractions, and the acidic methylated oligosaccharide-alditols were carboxyl-reduced. In the work presented here, the prior separation of acidic and neutral fractions was found not to be essential in the characterization of oligosaccharide mixtures by g.l.c.–c.i.-m.s. Thus, the analysis of a relatively small amount of material, consisting of a mixture of three disaccharides, allowed the sequence of sugars in the polymeric backbone of *E. coli* K26 capsular polysaccharide to be determined.

The structural complexity of *E. coli* K26 capsular polysaccharide arises from its high content of rhamnose and the nature of the linkages in its backbone, which are exclusively (1→3). It is known to contain four rhamnose residues as the only deoxy residues, all are α-linked and all except a terminal residue are 3-substituted. The capsular polysaccharide also contains a 3,4-linked glucuronic acid residue as the branch point, and a 3-linked galactose residue, both β-linked. Selective hydrolysis of the polysaccharide had indicated that the side chain consists of a single rhamnosyl group linked to position 4 of the glucuronic acid[9]. Thus, the remaining three rhamnose residues, one glucose residue, and one galactose residue were assigned to the main chain. A mild partial hydrolysis of the capsular polysaccharide with acid, followed by separation of the products by gel-permeation chromatography, gave, among others, fraction I, which was then either methylated or reduced with sodium borohydride prior to methylation. G.l.c.–c.i.-m.s. revealed that the methylated fraction I consisted of three disaccharides I_a, I_b, and I_c with relative retention times of 0.70, 1.06, and 1.21, respectively (Table I). Previous work undertaken on the characterization by g.l.c.–c.i.-m.s. of a series of methylated standards (di-, tri-, and tetra-saccharides), using DB 5, DB 17, and DB 225 columns, had made available relative retention times which could be used to give an indication of the monosaccharide composition of disaccharides[9]. The mass spectrum of I_a had a pseudomolecular ion $(M + NH_4)^+$ at *m/z* 412 and fragments ions at *m/z* 380, 363, and 189 (Table I). Both the relative retention time and mass spectrum were consistent with a deoxyhexose–deoxyhexose disaccharide. The mass spectum of I_b exhibited a pseudomolecular ion $(M + NH_4)^+$ at *m/z* 442 and fragmentations at *m/z* 410, 393, 219, and 189 (Table I), indicating that I_b was composed of a hexose and a deoxyhexose residue. The reducing sugar was identified when, on conversion of I_b into its alditol ($I_{b'}$), no fragment ion at *m/z* 219 was observed and a new fragment ion appeared at *m/z* 235. Therefore, I_b was identified as a deoxyhexose–hexose disaccharide. The third component I_c gave a mass spectrum with a pseudomolecular ion $(M + NH_4)^+$ at *m/z* 456 and two prominent fragment ions at *m/z* 201 and 233. Thus, I_c was identified as the aldobiouronic acid which had been previously shown to be present in *E. coli* K26 capsular polysaccharide[10] (Table I). The results of this g.l.c.–c.i.-m.s. analysis together with methylation data from the capsular polysaccharide allowed the following structures to be assigned to the components of

TABLE I

RELATIVE RETENTION TIMES AND IONS OBTAINED IN G.L.C.–C.I.-M.S. OF METHYLATED DISACCHARIDES OBTAINED FROM *E. coli* K26 CAPSULAR POLYSACCHARIDE

Oligosaccharide fraction	*Relative retention time*[a] *(min)*	*Chemical-ionization mass-spectral ions* [m/z] *(Relative abundance)*			
		$[M+NH_4]^+$	$[M+H]^+$	$[M+NH_4-MeOH]^+$	$[M+H-MeOH]^+$
I_a α-Rha-(1→3)-Rha	0.70	412 (17)		380 (18)	363 (35)
$I_{a'}$ α-Rha-(1→3)-Rha-ol	0.58		411 (8)		379 (51)
I_b α-Rha-(1→3)-Gal	1.06	442 (12)		410 (10)	393 (38)
$I_{b'}$ α-Rha-(1→3)-Gal-ol	0.84		441 (19)		409 (10)
I_c β-GlcA-(1→3)-Rha	1.21	456 (68)		424 (12)	407 (8)
$I_{c'}$ β-GlcA-(1→3)-Rha-ol	0.94	472 (26)	455 (10)		423 (20)

Oligosaccharide fraction	*Relative retention time*[a] *(min)*	*Chemical-ionization mass-spectral ions* [m/z] *(Relative abundance)*						
		AOH_2^+	A^+	H'^{+b}	H^{+b}	$[A-MeOH]^+$	$[H'-MeOH]^+$	$[H-MeOH]^+$
I_a α-Rha-(1→3)-Rha	0.70				189 (100)			157 (95)
$I_{a'}$ α-Rha-(1→3)-Rha-ol	0.58	223 (78)	205 (16)		189 (100)	173 (9)		157 (45)
I_b α-Rha-(1→3)-Gal	1.06			219 (93)	189 (94)		187 (68)	157 (86)
$I_{b'}$ α-Rha-(1→3)-Gal-ol	0.84	253 (93)	235 (72)		189 (100)	203 (2)		157 (60)
I_c β-GlcA-(1→3)-Rha	1.21			189 (9)	233 (30)		157 (40)	201 (100)
$I_{c'}$ β-GlcA-(1→3)-Rha-ol	0.94	223 (69)	205 (13)		233 (30)	173 (28)		201 (100)

[a]DB 17, 210° for 1 min, 4°/min → 240°; retention times relative to that of sucrose (5.98 min). [b]H and H' represent the non-reducing and reducing sugars, respectively, in samples.

fraction I: I_a, 3-*O*-α-L-rhamnosyl-L-rhamnose; I_b, 3-*O*-α-L-rhamnosyl-D-galactose; I_c, 3-*O*-(β-D-glucopyranosyluronic acid)-L-rhamnose. Making the assumption that the mild hydrolytic conditions did not cleave the aldobiouronic acid unit, the following structure was assigned to the backbone of the biopolymer:

→3)-β-GlcA-(1→3)-α-Rha-(1→3)-α-Rha-(1→3)-α-Rha-(1→3)-β-Gal-(1→

This assignment was confirmed by subsequent analyses of other oligosaccharides produced during the partial acid hydrolysis[9].

EXPERIMENTAL

General methods. — Deionizations were performed on a column (20 × 1.5 cm) of Amberlite IR-120 (H^+) resin. Solutions were concentrated under diminished pressure at 37°.

Analytical paper chromatography was performed by the descending method, using Whatman No. 1 paper with the following solvent systems: *A*, 18:3:1:4 ethyl acetate–acetic acid–formic acid–water; *B*, 8:2:1 ethyl acetate–pyridine–water; *C*, 4:1:5 1-butanol–acetic acid–water. Chromatograms were developed using alkaline silver nitrate. Separation of oligosaccharides was achieved by gel-permeation chromatography performed on a column (95 × 3 cm) of Bio-gel P2 with acidified distilled water (1 drop of HCOOH per litre of H_2O) as the eluant. Oligosaccharide fractions were located by the phenol–sulphuric acid method[11].

Methylation. — The methylation of samples (5 mg) was performed by the procedure of Hakomori[12], as modified by Sandford and Conrad[13].

Reduction. — To an aqueous solution of the oligosaccharide was added excess of sodium borohydride, and the mixture was stirred for 3 h at room temperature. The excess of $NaBH_4$ was removed by IR-120 (H^+) resin, and the solution was filtered and co-evaporated with methanol (3×).

Partial acid hydrolysis. — A solution of *E. coli* K26 capsular polysaccharide (1.6 g) in H_2SO_4 (200 mL, 0.025M) was heated on a steam bath for 1.5 h. The acid was then neutralized with lead carbonate and the precipitate was removed by centrifugation. The supernatant solution was dialysed against distilled water, and the dialysate was concentrated, deionized, and analysed by paper chromatography (solvents *A* and *B*). Rhamnose appeared to be the only component in the dialysate. The retentate was lyophilized and subjected to a second mild hydrolysis with acid (0.5M CF_3CO_2H, 1.5 h, 95°). The excess of acid was removed under diminished pressure and the final traces were neutralized with sodium hydrogencarbonate. The hydrolysate was de-salted on a column (86 × 3 cm) of Sephadex G10 and subjected to gel-permeation chromatography. Analysis by paper chromatography (solvent *C*) revealed a relatively pure fraction I (R_{Gal} 0.49). G.l.c.–c.i.-m.s. data for the methylated fraction I and for reduced and methylated fraction I are shown in Table I.

Capillary g.l.c. — Analytical g.l.c. was performed using a Hewlett–Packard 5890A gas chromatograph fitted with a dual flame-ionization detector and a 3392A recording integrator. Methylated oligosaccharides were separated on a capillary column (15 m × 0.25 μm i.d.) (fused silica; DB 17; J & W Scientific, Rancho Cardova, CA 95670, U.S.A.). The programme used was as follows: initial temperature 210° for 1 min, then a rate of 4°/min to bring the temperature to 240°. The retention times were determined relative to methylated sucrose and are listed in Table I.

G.l.c.–c.i.-m.s. — Analysis of disaccharides was performed on a Varian Vista 6000 gas chromatograph coupled directly to a Delsi Nermag R10-10C quadrupole mass spectrometer, or a Carlo Erba 4160 gas chromatograph coupled directly to a

Kratos MS 80RFA double-focusing mass spectrometer. Each mass spectrometer was fitted with a chemical-ionization source, with ammonia as the reagent gas, a source pressure of 0.1 Torr, an ion-source temperature of 175°, and an electron voltage of 72 eV.

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