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STRUCTURE OF THE EXTRACELLULAR GELLING POLYSACCHARIDE PRODUCED BY *Enterobacter* (NCIB 11870) SPECIES

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ABSTRACT

The gelling polysaccharide produced by a species of *Enterobacter* (NCIB 11870) contains L-fucose, D-glucose, and D-glucuronic acid in the ratios 1:2:1. Analysis of the methylated and methylated, carboxyl-reduced polysaccharide revealed terminal non-reducing glucose, (1→3)-linked fucose, (1→3,1→4)-linked glucose, and (1→4)-linked glucuronic acid in the ratios 1:1:1.2:0.8. From the results of Smith degradation of the polysaccharide and spectroscopic studies of the acidic tetra- and octa-saccharides produced by bacteriophage-induced enzymic depolymerisation of the polysaccharide, the following tetrasaccharide repeating-unit is proposed.

```
→4)-α-D-GlcpA-(1→3)-α-L-Fucp-(1→3)-β-D-Glcp-(1→
                                      4
                                      ↑
                                      1
                                   β-D-Glcp
```

This repeating-unit is identical to that of the capsular polysaccharide produced by *Klebsiella aerogenes* serotype K54 except for the absence of *O*-acetyl groups. The effects of the *O*-acetyl groups on the secondary structure and rheological properties of these polysaccharides are discussed.

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INTRODUCTION

The bacterium *Enterobacter* (NCIB 11870) produces an extracellular anionic polysaccharide that has unusual gelation properties of potential industrial value[1]. Limited chemical analysis showed[1] it to contain fucose, glucose, and glucuronic acid, but no *O*-acetyl groups or acetal-linked pyruvic acid[1].

In order to relate rheological properties[1] and preliminary X-ray fibre diffraction data[2,3] to structure, a detailed chemical analysis of the polysaccharide has been carried out.

RESULTS

Monosaccharide composition. — The polysaccharide contained fucose, glucose, and glucuronic acid in the ratios 1.0:4.5:2.0 and had no i.r. absorption at ~1730 cm^{-1} for *O*-acetyl groups. The fucose was underestimated because of the stability of the glucosyluronic acid→fucose linkage towards acid hydrolysis (see below).

Periodate oxidation of the polysaccharide followed by borohydride reduction and acid hydrolysis gave approximately equimolar proportions of fucose and glucose. Thus, the fucosyl and some of the glucosyl residues were resistant[1] to periodate oxidation.

Linkage analysis. — Analysis of the methylated and methylated, carboxyl-reduced polysaccharide (Table I, columns 1 and 2) revealed terminal non-reducing glucose, (1→3)-linked fucose, (1→3,1→4)-linked glucose, and (1→4)-linked glucuronic acid in the ratios 1.0:1.0:1.2:0.8. Methylation analysis of the poly-alcohol obtained during the Smith degradation revealed approximately equal proportions of (1→3)-linked fucose and (1→3,1→4)-linked glucose (Table I,

TABLE I

METHYLATION ANALYSIS OF THE *Enterobacter* POLYSACCHARIDE

Methylated alditol acetate[a]	*1*[e]	*2*	*3*	*4*	*Linkage*
2,3,4-Me_3-Fuc[b]	—	—	—	54	Fuc*p*
2,4-Me_2-Fuc	25[c]	25	47	—	(1→3)-Fuc*p*
1,2,4,5,6-Me_5-Glc	—	—	—	27	3-Glucitol
2,3,4,6-Me_4-Glc	35	25	—	—	Glc*p*
2,4,6-Me_3-Glc	—	—	—	19	(1→3)-Glc*p*
2,6-Me_2-Glc	40	29	53	—	(1→3,1→4)-Glc*p*
2,3-Me_2-Glc[d]	—	21	—	—	(1→4)-Glc*p*A

[a]Values expressed as relative peak-area %. [b]2,3,4-Me_3-Fuc = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylfucitol, *etc.* [c]Low proportions, due to incomplete hydrolysis of the aldobiouronic acid linkages. [d]Corresponds to 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl(6,6′-$^2H_2$)glucitol. [e]1, Methylated polysaccharide; 2, methylated and carboxyl-reduced polysaccharide; 3, periodate-degraded and methylated polysaccharide; 4, Smith-degraded and methylated polysaccharide.

column 3). Smith degradation[4] of the polysaccharide followed by reduction of the product and methylation analysis (Table I, column 4) revealed (1→3)-linked glucitol, (1→3)-linked glucose, and terminal non-reducing fucose in the ratios 1.0:0.7:2.0. The presence of (1→3)-linked glucose and glucitol was probably due to incomplete hydrolysis during the Smith degradation. These data are consistent with the repeating-unit **1**, which has also been found[4] for the capsular polysaccharide produced by *Klebsiella* serotype K54 but which is also *O*-acetylated at positions 2 or 4 of the fucosyl residue[4]. Further evidence that the polysaccharides had identical repeating-units was provided as follows.

```
→4)-α-D-GlcpA-(1→3)-α-L-Fucp-(1→3)-β-D-Glcp-(1→
                                        4
                                        ↑
                                        1
                                     β-D-Glcp
                 1
```

Phage-induced depolymerisation of the Enterobacter *(NCIB 11870) and* Klebsiella *K54 polysaccharides.* — Depolymerisation with an endo-glucanase from the host bacteriophage ϕ31, followed by preparative p.c.[5], yielded the octa- and tetra-saccharides in the ratios 1.0:2.6 *(Enterobacter)* and 1.0:4.1 (*Klebsiella* K54). Both the *O*-acetylated and non-acetylated tetrasaccharides were obtained from the *Klebsiella* K54 polysaccharide in the ratio 1:1.

On negative ion f.a.b.-m.s., each of the non-acetylated tetrasaccharides gave an ion at *m/z* 663, corresponding to $[M - H]^-$ from an oligosaccharide containing hexuronosyl, 6-deoxyhexosyl, and two hexosyl residues, and ions at *m/z* 501 $[(M - Glc) - H]^-$ and 339 $[(M - 2Glc) - H]^-$, corresponding to the consecutive loss of hexosyl residues. No signals were observed at the expected masses[6] ($[M - H]^-$, *m/z* 705) for the *O*-acetylated compounds. Likewise, the octasaccharide from the

233 407 628 614 440 219

a b ald a′

2

TABLE II

^{1}H-N.M.R. DATA FOR THE ANOMERIC PROTONS OF THE TETRASACCHARIDE DERIVED FROM THE *Enterobacter* POLYSACCHARIDE

H-1 (δ)	$J_{1,2}$ *(Hz)*	*Integral*	*Assignment*[a]
5.44	3.7	0.53	α-Fuc*p*
5.38	3.8	0.47	
5.23	3.4	1.00	α-Glc*p*A
5.18	3.9	0.54	α-Glc*p*-OH
4.65	8.0	0.46	β-Glc*p*-OH
4.49	7.0	1.00	β-Glc*p*
4.17	10.1	1.00	H-5 Glc*p*A

[a]Based, in part, on the assignments of Dutton and Merrifield[4].

Enterobacter polysaccharide gave an ion at *m/z* 1309, corresponding to $[M - H]^-$ from an oligosaccharide containing two hexuronosyl, two 6-deoxyhexosyl, and four hexosyl residues.

The methylated tetrasaccharide-alditol derivatives had identical retention times (*T* 16.3 min) on reverse-phase h.p.l.c. and, on positive ion f.a.b-m.s., each gave an ion at *m/z* 864 for $[M + H]^+$ from a methylated oligosaccharide-alditol methyl ester containing hexuronosyl, 6-deoxyhexosyl, hexosyl, and hexitol residues. Direct-insertion e.i.-m.s. gave the fragment ions at *m/z* 219 ($a'A_1$), 233 (aA_1), 407 (baA_1), 440 (*alda*'J_2), 486 (*alda*'J_0), 614 (b*alda*'J_2), and 628 (*ald*baJ_2), which define the sequence **2**. The nomenclature used is that of Kochetkov and Chizhov[7] except that the alditol moiety is designated[8] *ald*. The J_0 fragment ions are formed[9] from 3-substituted glycosyl residues.

The non-acetylated tetrasaccharides gave essentially identical ^{1}H-n.m.r. spectra. Six well-resolved signals were observed in the region for anomeric protons (Table II). The two doublets for H-1 of the α-Fuc*p* residue were present in the same ratio as that for the anomeric protons of the reducing terminus and probably reflected the mutarotational equilibrium[10,11] since there was only one doublet for H-1 of the α-Fuc*p* residue of the tetrasaccharide-alditol, at δ 5.27 ($J_{1,2}$ 3.7 Hz).

Positive and negative ion f.a.b.-m.s. of the *O*-acetylated tetrasaccharide obtained from the *Klebsiella* K54 polysaccharide showed that the *O*-acetyl group was not attached to the glucuronosyl or glucosyl residues, but was linked through positions 2 or 4 of the fucosyl residue.

DISCUSSION

The above data establish that the polysaccharides produced by *Enterobacter* (NCIB 11870) and *Klebsiella* K54 have the same repeating unit with the exception of *O*-acetyl groups in the latter.

Aqueous solutions[1] of the *Enterobacter* polysaccharide form cation-depen-

dent, thermally reversible gels. Although the partially *O*-acetylated polysaccharide produced by *Klebsiella* K54 gives viscous aqueous solutions[4], the ability to form cation-dependent gels has not been reported. This difference is also reflected in the X-ray fibre diffraction patterns[2,3,12]. Preliminary results indicated[2] that the *O*-deacetylated polysaccharide from *Klebsiella* K54 forms cation-dependent, thermally reversible gels, and suggested that the *O*-acetyl groups restricted the development of a secondary structure compatible with the formation of crystalline junction-zones. The *O*-deacetylated polysaccharide gave X-ray fibre diffraction patterns that are essentially the same as those given by the *Enterobacter* polysaccharide[2,3].

Thus, the addition of *O*-acetyl groups to a polysaccharide can produce significant changes in its secondary and tertiary structures. Similar effects on tertiary structure have been reported[13,14] for the extracellular anionic polysaccharides produced by *Arthrobacter viscosus* and *Pseudomonas elodea*.

The range of data now available makes computer-aided model building possible. With a knowledge of the three-dimensional structures of the *Enterobacter* and *Klebsiella* K54 polysaccharides, the effect of *O*-acetyl groups on their rheological properties may be determined. This study may also stimulate an examination of polysaccharides that contain non-carbohydrate substituents, in order to evaluate the role of these groups in controlling functional properties.

EXPERIMENTAL

Bacterial cultures. — Cultures of the *Enterobacter* (NCIB 11870) species[1] and *Klebsiella aerogenes* serotype K54[5] were maintained on nutrient agar in screw-cap vials[5].

For large-scale production of the *Enterobacter* polysaccharide, seed cultures were grown for 16 h at 30° with shaking in a minimal salts medium supplemented with yeast extract (0.5%) and D-glucose (1%). The inoculum (200 mL) was transferred to a 500-L fermenter containing a minimal salts medium supplemented with yeast extract (0.1%), casamino acids (0.1%), D-glucose (1.5%), and M phosphoric acid (2.1% v/v), and buffered to pH 7.0 with aqueous 50% NH_4OH which also served as the nitrogen source. The culture was grown for 32 h at 30° with an air flow of 0.5 L/min. The crude polysaccharide (1.2 g/g of bacterial cells) was precipitated with methanol, and a 0.5% solution in water was centrifuged (50,000*g*, 2 h), dialysed against distilled water, and freeze-dried.

Cultures of *Klebsiella* K54 were grown on trays of solid medium[15], and the polysaccharide was isolated and purified as described by Dudman and Wilkinson[16].

Monosaccharide analysis. — Neutral sugars, released by hydrolysis with M H_2SO_4 (100°, 2.5 h), were determined[17] by g.l.c. of their alditol acetates. Uronic acid was determined colorimetrically[18], using D-glucuronic acid as the standard.

Periodate oxidation. — A viscous solution of the *Enterobacter* polysaccharide (100 mg) in 0.4M $NaClO_2$ (50 mL) was treated with 0.1M $NaIO_4$ for 72 h at 4° in the dark[4]. The product was treated with ethylene glycol, dialysed, and reduced with $NaBH_4$ to yield the polyalcohol (85 mg).

Smith degradation. — The polyalcohol (10 mg) was treated as described by Dutton and Merrifield[4] except that, after acid hydrolysis, the products were reduced with $NaBH_4$.

Methylation analysis. — A solution of the polysaccharide (15 mg) in methyl sulphoxide was methylated[19] and a portion (5 mg) was analysed[20] as the alditol acetates by g.l.c.–m.s. on a column (3 m × 2.2 mm) containing 3% of ECNSS-M[4]. The remaining portion (~10 mg) was carboxyl-reduced[21] with $LiAl^2H_4$ in refluxing dichloromethane–ether and the product was analysed[20] as the alditol acetates by g.l.c.–m.s. The products of Smith degradation (5 mg) in methyl sulphoxide were methylated[19], extracted into chloroform, and analysed[20] as the alditol acetates by g.l.c.–m.s.

Bacteriophage-induced enzymic depolymerisation. — Separate solutions of the *Enterobacter* and *Klebsiella* K54 polysaccharides (10 mg) in water (50 mL) were treated[5] for 24 h at 37° in the presence of toluene with 100 units of the endo-glucanase from the bacteriophage ϕ31. The enzymic digests were dialysed against distilled water (2 × 500 mL), and the diffusates were concentrated to ~5 mL and desalted on a column (55 × 1 cm) of Bio-Gel P-2. Carbohydrate was detected with phenol–sulphuric acid[22], and appropriate fractions were combined and freeze-dried. The partially purified oligosaccharides were isolated[5] by preparative p.c. and characterised by positive and negative ion f.a.b.-m.s. using a Kratos MS9 mass spectrometer. Xenon was used as the primary bombarding gas and the atom gun was operated at 8 kV[23].

The tetrasaccharides were reduced with NaB^2H_4, desalted, and methylated[24]. The methylated derivatives were isolated from Sep-Pak C_{18} cartridges (Waters Assoc.) by elution[25] with aqueous 60% acetonitrile and purified by reverse-phase h.p.l.c. on a Zorbax ODS column (25 cm × 4.6 mm) by isocratic elution[25] with aqueous 60% acetonitrile. The effluent was monitored by using a Waters 401 differential refractometer, the fractions were collected manually and concentrated to dryness under diminished pressure at 25°, and a solution of each residue in acetone (100 μL) was used for f.a.b.-m.s.[23] and direct-insertion e.i.-m.s.[26].

^{1}H-N.m.r.-spectra (300 MHz) were obtained under non-saturating conditions with a Bruker CXP-300 spectrometer. Chemical shifts (δ) are reported in p.p.m. from internal acetonitrile (δ 2.09 downfield from the signal for Me_4Si).

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