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STRUCTURAL STUDIES OF AN EXTRACELLULAR POLYSACCHARIDE, S-657, ELABORATED BY *Xanthomonas* ATCC 53159*

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ABSTRACT

The structure of an extracellular polysaccharide, S-657, elaborated by *Xanthomonas* ATCC 53159 was investigated by methylation analysis, specific degradations, and ^{1}H-n.m.r. spectroscopy. It is concluded that the polysaccharide is composed of hexasaccharide repeating-units having the structure

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

INTRODUCTION

Several extracellular polysaccharides elaborated by bacteria have potential industrial application as gelling or thickening agents[1]. Five such polysaccharides, elaborated by *Pseudomonas* and *Alcaligenes* species, proved to have closely related structures[2]. They are composed of oligosaccharide repeating-units containing the same linear component (**1**), either without side chain or with a mono- or disaccharide side chain

$$\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{A-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow$$
$$\mathbf{1}$$

*Dedicated to the memory of Karl Freudenberg on the centenary of his birth.
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linked to one of the two β-D-glucopyranosyl residues. In some of these polysaccharides, L-rhamnose is partially replaced by L-mannose.

We now report structural studies of a polysaccharide, S-567, elaborated by *Xanthomonas* ATCC 53159, which was shown to be a sixth member of this group.

RESULTS AND DISCUSSION

The crude polysaccharide (S-657), heavily contaminated by protein, was purified by partition between water and phenol. In this procedure, widely used for the purification of bacterial lipopolysaccharides[3], the polysaccharide accumulates in the aqueous phase and the protein in the phenolic phase. A hydrolysate of S-657 contained D-glucose and L-rhamnose in the ratio 36:64. Additional components were D-glucuronic acid (~20%) and *O*-acetyl groups[4]. The absolute configurations of the sugars were determined as devised by Gerwig *et al.*[5].

Both S-657 and deacetylated S-657 formed highly viscous solutions and therefore gave poor n.m.r. spectra. Better spectra were obtained with a deacetylated sample which had been somewhat degraded by brief heating with dilute acid, a treatment which caused the loss of some L-rhamnosyl residues. The ^{1}H-n.m.r. spectrum of this material showed, *inter alia*, signals for H-1 of α-L-rhamnosyl residues at δ 5.30 and 5.15, not resolved (2.25 H together), and for H-1 of pyranosides having the *β-gluco* configuration at δ 4.71 ($J_{1,2}$ 7.3 Hz), 4.68 ($J_{1,2}$ 7.5 Hz) and 4.66 ($J_{1,2}$ 7.6 Hz) (2 H together), and δ 4.57 (1 H, $J_{1,2}$ 7.9 Hz). It further contained signals for H-6 of L-rhamnosyl residues at δ 1.33 ($J_{5,6}$ 6.3 Hz) and 1.29 ($J_{5,6}$ 5.9 Hz) (7 H together).

Methylation analysis of S-657, without and with carboxyl reduction of the methylated product, gave the sugars listed in Table I, columns A and B. These analyses, together with the ^{1}H-n.m.r. spectrum, indicate that the polysaccharide is composed of hexasaccharide repeating-units containing one terminal α-L-rhamnopyranosyl group, two α-L-rhamnopyranosyl residues linked through O-4, one β-D-glucopyranosyl residue linked through O-3, one β-D-glucopyranosyl residue linked through O-3 and O-4, and one β-D-glucopyranosyluronic acid residue linked through O-4.

The deacetylated S-657 was hydrolysed with acid to a mixture of mono- and oligosaccharides which were fractionated by ion-exchange chromatography on a DEAE-Trisacryl M column. The mixture of acidic oligosaccharides obtained was desalted and fractionated into two components by chromatography on a Bio-Gel P-2 column.

One component, eluted in the trisaccharide region on gel-permeation chromatography, on hydrolysis with acid yielded equimolecular amounts of D-glucose and L-rhamnose. Methylation analysis of its alditol, with carboxyl reduction, yielded the sugars listed in Table I, column C. The ^{1}H-n.m.r. spectrum showed, *inter alia*, signals for H-1 of a reducing L-rhamnose residue at δ 5.09 ($J_{1,2}$ 1.5 Hz) and 4.83 (not resolved) (1 H together), and for H-1 of two β-pyranosides

TABLE I

METHYLATION ANALYSES OF S-657 AND ITS DEGRADATION PRODUCTS

Methylated sugar[a]	T[b]	*Proportion (mol %) in product*[c]				
		A	*B*	*C*	*D*	*E*
1,2,3,5-Rhamnitol	0.31	—	—	14.5	5.2	—
2,3,4-Rha	0.48	30.7	18.4	—	—	40.1
2,3-Rha	0.94	52.0	37.8	—	—	39.0
2,3,4,6-Glc	1.00	—	—	—	31.9	—
2,4,6-Glc	1.76	9.0	13.4	—	—	15.7[d]
2,3,4-Glc	2.04	—	—	44.8	—	—
2,3,6-Glc	2.16	—	—	40.8	31.8	—
2,6-Glc	3.10	8.3	15.5	—	—	5.2
2,3-Glc	4.00	—	14.9	—	31.1	—

[a]2,3,4-Rha = 2,3,4-tri-*O*-methyl-L-rhamnose, *etc.* [b]Retention time of the derived alditol acetate, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, on a DB-225 column at 190°. [c]*A*, Methylated polysaccharide; *B*, methylated and carboxyl-reduced polysaccharide; *C*, acidic trisaccharide; *D*, acidic tetrasaccharide; *E*, product from uronic acid degradation. [d]Trideuteriomethyl group at O-4.

having the *β-gluco* configuration at δ 4.71 (1 H, $J_{1,2}$ 7.8 Hz) and 4.53 (1 H, $J_{1,2}$ 7.8 Hz), and for H-6 of the rhamnose residue at δ 1.37 (3 H, $J_{5,6}$ 5.7 Hz). These results demonstrate that the trisaccharide has structure **2**.

β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4)-L-Rha

2

The other acidic oligosaccharide was eluted in the tetrasaccharide region on gel-permeation chromatography. Methylation analysis of its alditol, with carboxyl reduction, gave the sugars listed in Table I, column D. This analysis demonstrates that the tetrasaccharide contains the same sugar residues as the trisaccharide plus a terminal glucopyranosyl group linked to O-4 of the glucuronic acid residue. The ^{1}H-n.m.r. spectrum of the tetrasaccharide showed, in the region for anomeric protons, signals which almost coincided with those given by the trisaccharide and further a signal for H-1 of the terminal β-D-glucopyranosyl group at δ 4.63 (1 H, $J_{1,2}$ 7.3 Hz). The tetrasaccharide thus has the structure **3**.

β-D-Glc*p*-(1→4)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4)-L-Rha

3

In order to determine which of the two β-D-glucopyranosyl residues carries the side chain, S-657 was subjected to a uronic-acid degradation. The fully methylated polysaccharide was treated with sodium methylsulfinylmethylide in dimethyl sulfoxide, then trideuteriomethylated. A hydrolysate of the product contained the

sugars listed in Table I, column E. The course of the degradation is analogous to that described for another polysaccharide, S-88, having a related structure[6]. The 2,6-di-*O*-methyl-D-glucose found in the original methylation analysis of S-657 has been replaced by 2,6-di-*O*-methyl-4-*O*-(trideuterio)methyl-D-glucose, demonstrating that the uronic acid is linked to O-4 of the branching β-D-glucopyranosyl residue. The disappearance of 2,4,6-tri-*O*-methyl-D-glucose and part of the 2,3-di-*O*-methyl-L-rhamnose is in agreement with the partial structure **4**.

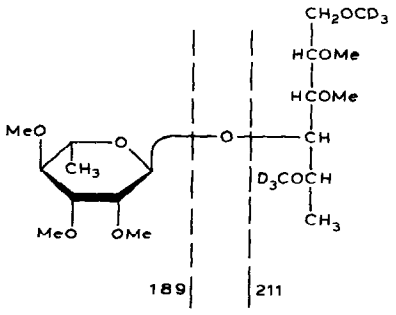
→4)-α-L-Rha*p*-(1→3)-β-D-Glc*p*-(1→4)-β-D-Glc*p*A-(1→
4

as the corresponding sugar residues should be unmasked consecutively as reducing sugar residues, and degraded during the treatment with base.

From the results discussed above, it seemed most likely that S-657 is composed of hexasaccharide repeating-units having the structure **5**, and is thus a sixth member of the group of polysaccharides discussed in the Introduction.

→3)-β-D-Glc*p*-(1→4)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4)-α-L-Rha*p*-(1→
3
↑
1
α-L-Rha*p*-(1→4)-α-L-Rha*p*
5

It could not, however, be excluded that one of the α-L-rhamnopyranosyl residues placed in the side chain was instead part of the main chain. In order to decide between these alternatives, methylated S-657 was treated with acid under mild conditions, which should mainly cause the cleavage of α-L-rhamnopyranosyl linkages. The product was then reduced with sodium borohydride, trideuteriomethylated and investigated by g.l.c.–m.s. A component with the retention time of a disaccharide derivative gave the mass spectrum expected for a rhamnitol



6

rhamnoside (**6**). The cleavages leading to the aA_1 (*m/z* 189) and bA_1 (*m/z* 211) fragments are indicated in the formula. As this disaccharide derivative should be formed from **5** but not from an alternative structure with two α-L-rhamnopyranosyl residues in the main chain, the latter possibility is eliminated.

The structure of S-657 is most closely related to those of welan gum[7] (S-130) and S-88 (ref. 8), in both of which an α-L-rhamnopyranosyl side chain is linked to the same position as the disaccharide side chain on S-657. In welan gum, this terminal α-L-rhamnopyranosyl group is partially replaced by an α-L-mannopyranosyl group. In S-88, the α-L-rhamnopyranosyl residue in the main chain is partially replaced by an α-L-mannopyranosyl residue.

EXPERIMENTAL

General methods. — These were the same as used in the investigation of S-88 (ref. 6). Methylation analyses were performed essentially as previously described[8]. Methylated products were purified on Sep-Pac C_{18} cartridges, using the procedure described by Waeghe *et al.*[9]. Hydrolysis in connection with sugar and methylation analyses was performed with 2M trifluoroacetic acid for 2 h at 120° or with 0.5M trifluoroacetic acid for 16 h at 100°.

Purification of S-657. — The crude polysaccharide (300 mg) was suspended in water (300 mL) at 66–68°. Aqueous phenol (90%, 300 mL), also at 66–68°, was added and the solution was kept at this temperature and vigorously stirred for 30 min. Phase separation was achieved by centrifugation and the phenolic phase was extracted with water (200 mL) as described above. The combined aqueous phases were dialysed against deionised water and concentrated, and the polysaccharide (155 mg) was recovered by freeze drying.

Deacetylation of this material was performed by treatment with 0.1M sodium hydroxide for 16 h at 50°. The polysaccharide was recovered by dialysis, concentration, and freeze drying.

Partial hydrolysis of S-657. — The deacetylated polysaccharide was dissolved in water at 100° and 2M trifluoroacetic acid was added to a concentration of 0.1M. The solution was kept for 15 min at 100° and the polysaccharide recovered by dialysis, concentration, and freeze drying. This material was used for ^{1}H-n.m.r. spectroscopy.

For the isolation of oligosaccharides, deacetylated S-657 (60 mg) was treated as above, but hydrolysed for 3 h. The product, in 0.01M sodium dihydrogenphosphate, was added to a column of DEAE-Trisacryl M (350 × 25 mm) and was eluted with a gradient of sodium chloride (0–0.5M) in the same phosphate solution (1 L). A mixture of acidic tri- and tetra-saccharides was eluted at ~0.21M sodium chloride. This material was desalted and fractionated into pure components by chromatography on a Bio-Gel P-2 column.

Partial hydrolysis of methylated S-657. — Methylated S-657 (5 mg) was dissolved in water (25 mL) at 100°, trifluoroacetic acid was added to a concentration

of 2M, and the solution was kept for 30 min at 100° with stirring. The solution was then cooled and concentrated, and the product dried by distillation with methanol. It was then reduced with sodium borohydride and trideuteriomethylated, using standard procedures, and investigated by g.l.c.–m.s.

A main component had a retention time on the SE-54 column, operated at 190°, of 0.65, relative to fully methylated maltitol. Its mass spectrum showed, *inter alia*, the following fragments: *m/z* (relative intensities) 88 (100), 99 (12), 101 (24), 104 (12), 105 (5), 125 (4), 129 (5), 144 (4), 157 (5), 179 (3), 189 (18), 211 (18), 222 (3), 244 (2), 226 (6).

Uronic degradation of S-657. — To a solution of the methylated polysaccharide (2 mg) in dimethyl sulfoxide (1.5 mL) was added *p*-toluenesulfonic acid (a trace) and 2,2-dimethoxypropane (0.1 mL) in order to eliminate any water present. Sodium methylsulfinylmethylide in dimethyl sulfoxide (2M, 1 mL) was added, and the mixture was agitated in an ultrasonic bath at room temperature for 30 min, then kept standing for 15 h. Trideuteriomethyl iodide (0.5 mL) was added with external cooling, and the mixture was agitated in the ultrasonic bath for 30 min. The excess of methyl iodide was removed by flushing with nitrogen, and the solution was diluted with water and transferred to a Sep-Pak C_{18} cartridge. This was washed with water and the product eluted with acetonitrile.

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