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STRUCTURAL STUDIES OF AN EXTRACELLULAR POLYSACCHARIDE (S-130) ELABORATED BY *Alcaligenes* ATCC 31555

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ABSTRACT

The structure of an extracellular polysaccharide (S-130) elaborated by *Alcaligenes* ATCC 31555, has been investigated. It is concluded that the polysaccharide is composed of pentasaccharide units having the following structure:

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

Of these units, approximately two-thirds contain terminal α-L-rhamnopyranosyl groups; the remainder contain α-L-mannopyranosyl groups. Approximately 50% of the units contain an *O*-acetyl group.

INTRODUCTION

During the search for bacterial polysaccharides of potential industrial application, an extracellular polysaccharide from an *Alcaligenes* species, ATCC 31555, was prepared[1–4]. This polysaccharide, which was given the working name S-130, gave highly viscous aqueous solutions, the viscosity being maintained also at high temperature. We now report structural studies of S-130.

RESULTS AND DISCUSSION

An acid hydrolysate of S-130 contained glucose, rhamnose, and mannose in the relative proportions 43:46:11. It further contained glucuronic acid, identified by g.l.c. of a sample that had been methanolysed and trimethylsilylated[5].

It was first suspected that the mannose, because of its low percentage, was

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derived from a contaminating mannan. After purification of S-130 by precipitation with cetyltrimethylammonium bromide, however, the composition was unchanged. Other evidence, presented below, demonstrates that mannose is actually a component of S-130.

The absolute configurations of the component sugars were determined by g.l.c. of the glycosides obtained on solvolysis of S-130 with chiral 2-butanol followed by trimethylsilylation, as devised by Gerwig *et al.*[6]. As expected, the glucose and glucuronic acid have the D configuration and the rhamnose has the L configuration, whereas, unexpectedly, the mannose has the L configuration. This conclusion was confirmed by g.l.c. of the glycosides obtained on solvolysis of S-130 with chiral 2-octanol followed by acetylation, as devised by Leontein *et al.*[7].

The ^{1}H- and ^{13}C-n.m.r. spectra of S-130 were of low quality, due to the highly viscous solutions, but revealed the presence of *O*-acetyl groups (^{13}C, δ 22.0; ^{1}H, δ 2.16). The integral of the latter signal was approximately one third of that for the methyl group of L-rhamnose. The content of *O*-acetyl was not constant for different preparations. The presence of ^{13}C-n.m.r. signals at δ 62.0 and 62.5 (CH_2OH) and none at δ ~65 indicated that the primary hydroxyl groups were not acetylated.

Somewhat better spectra were obtained from a sample which had been partially degraded by treatment with a small amount of bromine at pH 7 and then with base. The ^{13}C-n.m.r. spectrum showed, *inter alia*, signals at δ 18.5 (CH_3), 62.0 and 62.5 (CH_2OH), 101.9, 102.2, 103.3, 103.8 and 104.6 (anomeric carbons), and ~176 (CO_2^-). The ^{1}H-n.m.r. spectrum contained signals for methyl protons at δ 1.23 ($J_{5,6}$ 6 Hz) and 1.29 ($J_{5,6}$ 6 Hz), and signals for anomeric protons at δ 4.49 ($J_{1,2}$ 7 Hz), 4.52 ($J_{1,2}$ 7 Hz), 4.68 ($J_{1,2}$ 7 Hz), 5.13 (not resolved), 5.24 (not resolved), and 5.36

TABLE I

METHYLATION ANALYSIS OF S-130 AND SOME DEGRADATION PRODUCTS[a]

Sugar[b]	T[c]	*Mole %*				
		A	*B*	*C*	*D*	*E*
1,2,3,5-Rhamnitol	0.38				13	22
2,3,4-Rha	0.59	12	7	16		
2,3-Rha	0 94	26	21	18		
2,3,4,6-Glc	1.00				54	36
2,3,4,6-Man	1.00	10	7	19		5
2,4,6-Glc	1.67	26	23	43[d]		
2,3,6-Glc	1.92				33	34
2,6-Glc	2.79	26	23	4		3
2,3-Glc	3.56		19			

[a]Key: A, methylated polysaccharide; B, methylated and carboxyl-reduced polysaccharide; C, uronic acid-degraded polysaccharide; D, acidic tetrasaccharide; E, acidic penta- and tetra-saccharide. [b]2,3,4-Rha = 2,3,4-tri-*O*-methyl-L-rhamnose, *etc.* [c]Retention time of the corresponding alditol acetate, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on an SP-1000 glass-capillary column at 200°. [d]>90% Trideuteriomethyl at O-4.

(not resolved). The integrals of the signals below δ 5 and that at δ 5.13 were similar, whereas those of the signals at δ 5.24 and 5.36 were smaller and much smaller, respectively. A sample of S-130 was solvolysed with anhydrous hydrogen fluoride at −70° for 15 min and the product was isolated by precipitation with ether. The polymeric material obtained by chromatography on Sephadex G-25 had $[\alpha]_{578}$ −40°. This value, together with the n.m.r. evidence, indicated that the D-sugar residues in S-130 have the β configuration and that the L-sugar residues have the α configuration.

Methylation analysis of S-130, without and with carboxyl-reduction of the methylated polysaccharide, gave the products listed in Table I, columns A and B, respectively. These results, in conjunction with the n.m.r. evidence, indicate that all sugar residues in S-130 are pyranosidic and that it contains the structural elements **1–6**.

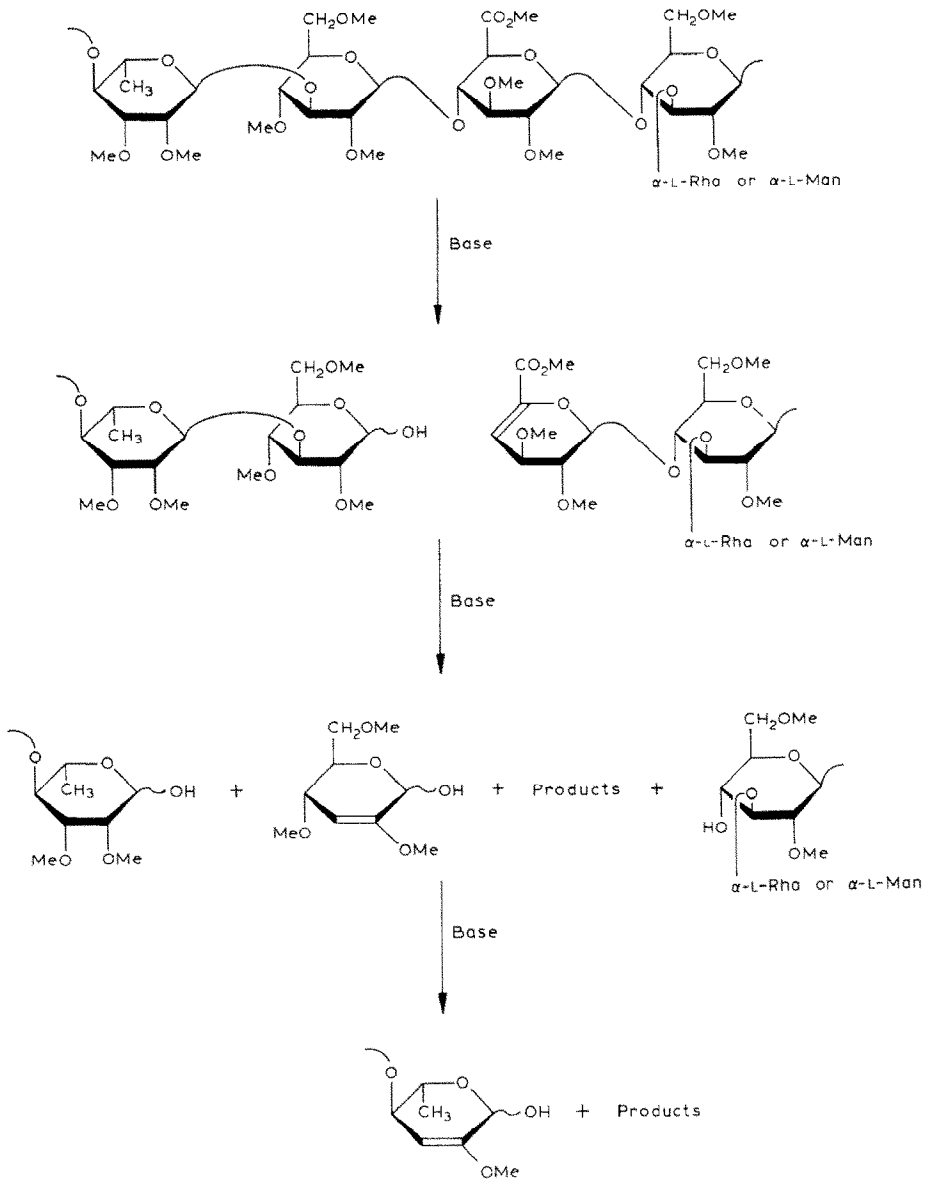
L-Rha*p*-(1→	L-Man*p*-(1→	→4)-L-Rha*p*-(1→
1	**2**	**3**
→3)-D-Glc*p*-(1→	→4)-D-Glc*p*-(1→	→4)-D-Glc*p*A-(1→
	3 ↑	
4	**5**	**6**

The mixture of methylated sugars was analysed by g.l.c.–m.s. of their alditol acetates. As the 2,3,4,6-tetra-*O*-methyl-D-glucose and 2,3,4,6-tetra-*O*-methyl-L-mannose derivatives were not separated on the column used, they were identified and quantified by g.l.c.–m.s. of the acetylated sugars[8]. The presence of only one L-mannose derivative, derived from terminal groups, lends further support to the assumption that L-mannose is actually a component of S-130.

The proportions of the methylated sugars indicate that S-130 is composed of pentasaccharide units, in which the terminal sugar is either L-rhamnopyranose or L-mannopyranose.

In order to determine the sequence of the sugar residues, S-130 was subjected to a uronic acid-degradation[9,10]. The fully methylated polysaccharide was treated with sodium methylsulfinylmethanide in dimethyl sulfoxide, methylated (using trideuteriomethyl iodide), and hydrolysed, and the mixture of methylated sugars was analysed (Table I, column C). The result is in agreement with the sequence and the reactions indicated in Scheme 1. Thus, 2,6-di-*O*-methyl-4-*O*-trideuteriomethyl-D-glucose was derived from the branching D-glucopyranosyl residue, the 4-position of which was liberated on degradation of the uronic acid. The 3-substituted D-glucopyranosyl residue linked to O-4 of the uronic acid was released by β-elimination and further degraded by β-elimination, with release of the 4-substituted L-rhamnopyranosyl residue. A considerable part of this residue was also degraded.

The structure of S-130 is defined by the experiments discussed above. It seemed desirable, however, to have further evidence for the presence of two diffe-



Scheme 1. Uronic acid-degradation of S-130.

rent terminals, α-L-rhamnopyranosyl and α-L-mannopyranosyl groups linked to O-3 of the branching β-D-glucopyranosyl residue. The polysaccharide was therefore treated with acid under mild conditions, during which mainly α-L-rhamnosidic linkages should be cleaved, and the product was fractionated by gel-permeation chromatography. From the material eluted in the tetrasaccharide region, an early and a late fraction were investigated separately.

The latter fraction, on acid hydrolysis, yielded only D-glucose and L-rhamnose as the neutral sugars. Methylation analysis of the derived alditol yielded the sugars listed in Table I, column D. These data, in conjunction with the results of the uronic acid-degradation, demonstrate that the tetrasaccharide has structure **7**. In agreement with this conclusion, the ^{1}H-n.m.r. spectra of the alditol showed, *inter alia*, a signal for deoxy protons at δ 1.28 (3 H, $J_{5,6}$ 6.4 Hz) and three signals in the region for anomeric protons at δ 4.54 (1 H, $J_{1,2}$ 7.8 Hz), 4.61 (1 H, $J_{1,2}$ 7.9 Hz), and 4.62 (1 H, $J_{1,2}$ 7.8 Hz).

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

7

The early fraction eluted in the tetrasaccharide region yielded D-glucose, L-mannose, and L-rhamnose in the proportions 56:4:40 on acid hydrolysis. Methylation analysis of the derived alditol (Table I, column E) gave comparable amounts of 2,3,4,6-tetra-*O*-methyl-L-mannose and 2,6-di-*O*-methyl-D-glucose, but no 2,3,4-tri-*O*-methyl-L-rhamnose, demonstrating that L-mannose is linked to the branching D-glucopyranosyl residue. The ^{1}H-n.m.r. spectrum of the derived alditol was similar to that of the tetrasaccharide-alditol, but showed, in addition, a signal at δ 5.33, assigned to the anomeric proton of the L-mannopyranosyl group, which should be α-linked.

From the combined evidence, it is proposed that S-130 is composed of pentasaccharide "repeating-units" with the structure **8**. It has not been determined whether the distribution of the terminal α-L-rhamnopyranosyl and α-L-mannopyranosyl groups is random or more regular.

→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* or α-L-Man*p*

8

Of the great number of microbial polysaccharides that have been investigated, only a few have had such physical properties that an industrial application has been considered. One of the most promising candidates, gellan gum, is composed[11] of tetrasaccharide repeating-units with the structure **9**. This structure differs from that of S-130 only in the absence of the terminal glycosyl group.

→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→

9

L-Mannose is an unusual natural sugar. To the best of our knowledge, it has

only been observed once before, as a component of the polysaccharide associated with coccoliths of the alga *Emiliania huxleyi* (Lohmann) Kamptner[12].

EXPERIMENTAL

General methods. — Concentrations were performed under diminished pressure at ~40° (bath) or at room temperature by flushing with air. For g.l.c., a Hewlett–Packard 5830A instrument fitted with a flame-ionisation detector was used. Separation of alditol acetates was performed on an SE-54 fused-silica capillary column using a temperature programme 150→220° at 2°/min. Partially methylated alditol acetates were separated on an SP-1000 fused-silica capillary column at 200°. G.l.c.–m.s. was performed on a Varian MAT 311 instrument, using the latter phase. All identifications of mass spectra were unambiguous and will not be discussed. Optical rotations were measured at 22° with a Perkin–Elmer 241 polarimeter.

The glucuronic acid was identified by using the method of Clamp *et al.*[5]. Absolute configurations of the sugars were determined by the methods devised by Gerwig *et al.*[6] and by Leontein *et al.*[7].

Methylation analyses were performed essentially as previously described[13]. Methylated polymers were recovered by dialysis against water, followed by freeze-drying. Low-molecular-weight products were recovered by reversed phase chromatography on Sep-Pak C_{18} cartridges[14]. The sample was diluted with an equal volume of water and applied to the column. This was washed with water and acetonitrile–water (15:85), and the sample was eluted with acetonitrile.

N.m.r. spectra of solutions in deuterium oxide were determined at 70° (^{13}C) or 85° (^{1}H) with a JEOL FX-100 or GX-400 instrument. Chemical shifts are reported in p.p.m. downfield from external tetramethylsilane (^{13}C) and internal acetone (δ 2.22 downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate; ^{1}H).

Carboxyl-reduction of methylated S-130. — The methylated polysaccharide (1.5 mg) was dissolved in freshly distilled tetrahydrofuran (2 mL). Lithium borohydride (10 mg) was added and the solution boiled under reflux for 2 h. Excess of lithium borohydride was decomposed with M acetic acid, chloroform (5 mL) was added, and the solution was washed several times with water, dried, and concentrated.

Uronic acid-degradation of S-130. — To a solution of the methylated polysaccharide (1.5 mg) in dimethyl sulfoxide (1.5 mL) were added a trace of toluene-*p*-sulfonic acid and 2,2-dimethoxypropane (0.1 mL) in order to eliminate any water present. Sodium methylsulfinylmethanide in dimethyl sulfoxide (2M, 1 mL) was added, and the mixture was agitated in an ultrasonic bath for 30 min and kept at room temperature for 15 h. Trideuteriomethyl iodide (0.5 mL) was added with external cooling and the mixture 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 added to a Sep-Pak C_{18} cartridge. The material was

recovered as described above. The product was hydrolysed with 2M trifluoroacetic acid for 15 h at 100° and the mixture of methylated products was analysed (Table I, column C).

Partial hydrolysis of S-130. — Deacetylated S-130 (150 mg) was dissolved in water (190 mL) at 100°, and trifluoroacetic acid was added to a concentration of 0.1M. The solution was kept at 100° for 3 h, cooled, and freeze-dried. Part of the product (40 mg) was fractionated on a Sephadex G-25 column (3 × 80 cm) irrigated with mM aqueous formic acid, a differential refractometer being used for monitoring the effluents. The material eluted in the tetrasaccharide region was divided into fractions, which were worked-up and analysed separately. One of the fractions, eluted fairly late, consisted of a pure tetrasaccharide, as evident by methylation analysis (Table I, column D) and the ^{1}H-n.m.r. spectrum of the derived alditol formed by reduction with sodium borohydride. Another fraction, eluted earlier, contained this tetrasaccharide and also a pentasaccharide, with L-mannose as one component, according to methylation analysis (Table I, column E) and the ^{1}H-n.m.r. spectrum of the derived alditol.

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