

STRUCTURAL STUDIES OF GELLAN GUM, AN EXTRACELLULAR POLYSACCHARIDE ELABORATED BY *Pseudomonas elodea*

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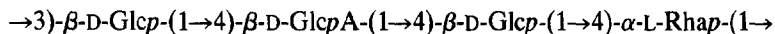
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ABSTRACT

The structure of gellan gum, a polysaccharide of potential commercial usefulness elaborated by *Pseudomonas elodea*, has been investigated. It is concluded that the polysaccharide is composed of tetrasaccharide repeating-units having the following structure.



Of the repeating units, ~25% contain an *O*-acetyl group linked to C-6 of one of the β -D-glucopyranosyl residues.

INTRODUCTION

Several polysaccharides are used industrially as emulsion stabilisers, thickeners, lubricants, or for other purposes. Most of these polysaccharides are produced by plants, but there is an increasing interest in extracellular polysaccharides produced by microorganisms¹. Xanthan gum, elaborated by *Xanthomonas campestris*, has been produced on an industrial scale for several years, and there is a search for other microbial polysaccharides the aqueous solutions of which have physical properties such that they are of interest from an industrial point of view. A polysaccharide of considerable interest in this respect is gellan gum² elaborated by the bacterium *Pseudomonas elodea*³. The native polysaccharide contains *O*-acetyl groups, and the deacetylated polysaccharide gives aqueous solutions which have low viscosities at elevated temperatures but form strong gels on cooling in the presence of cations^{4–6}. We now report structural studies of gellan gum.

RESULTS AND DISCUSSION

Previous reports^{2,4,7} on gellan indicated that the neutral sugars glucose and

TABLE I

METHYLATION ANALYSES OF DIFFERENT GELLAN GUM DERIVATIVES^a

Sugar ^b	T ^c	Mole %		
		A	B	C
2,3-Rha	0.92	28		
1,2,3,5-Rha	—		<i>d</i>	<i>d</i>
2,3,4,6-Glc	1.0			59
2,4,6-Glc	1.82	21		
2,3,6-Glc	2.32	24	100	41
2,3-Glc	4.5	26		

^aKey: A, methylated and borohydride-reduced polysaccharide; B, acidic trisaccharide; C, acidic tetrasaccharide. ^b2,3-Rha = 2,3-di-*O*-methyl-L-rhamnose, etc. ^cRetention time of the derived alditol acetate on an OV-225 column at 170°, relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. ^dNot detected because of its volatility.

rhamnose occur in the molar ratio 3:2. It is well known that it is very difficult to completely hydrolyse complex acidic polysaccharides into their monosaccharide constituents quantitatively and that the data obtained are not necessarily absolute but are dependent upon the hydrolysis conditions used, the derivatives used, and the correction factors applied⁸. With the more optimal hydrolysis conditions used in this report, the sugar analyses (g.l.c. of the alditol acetates) of gellan gum gave D-glucose and L-rhamnose in the molar ratio 1:0.47, close to the actual values of 1:0.5.

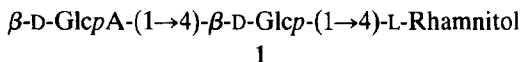
For n.m.r. studies, a sample of deacetylated gellan gum was depolymerised by acid hydrolysis under mild conditions and recovered by dialysis. The ¹H-n.m.r. spectrum showed, *inter alia*, signals for methyl protons of an L-rhamnosyl residue at δ 1.29 ($J_{1,2}$ 6 Hz) and for four anomeric protons at δ 4.53 (1 H, $J_{1,2}$ 7.3 Hz), 4.70 (2 H, J 7.8 Hz), and 5.13 (1 H, not resolved).

In the ¹³C-n.m.r. spectrum, a signal at δ 18.7 was assigned to the methyl group of the L-rhamnosyl residue, signals at δ ~104.0 (3 C) and 102.3 (C) to four anomeric carbons, and a signal at δ 176.1 to the carboxyl group of a D-glucosyluronic acid residue. In addition, four signals at δ 84.1, 83.1, 82.4, and 80.8 were assigned to glycosyloxyated carbon atoms, and a 2-carbon signal at δ ~62.8 to hydroxymethyl groups. The $J_{C,H}$ value of the signal at δ 102.3 was 170 Hz, and those of the three other signals for anomeric protons were ~160 Hz, indicating one α - and three β -pyranosidic residues in the repeating unit. This conclusion accords with the ¹H-n.m.r. data which further indicate that the L-rhamnopyranosidic residue is α -linked and the three other residues are β -linked. These assignments are also consistent with the $[\alpha]_{578}$ value [-43° (water)] of deacetylated gellan gum.

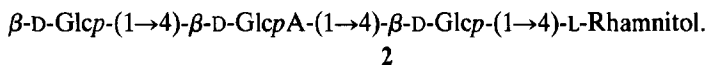
Methylation analysis⁹ of gellan gum, with carboxyl-reduction of the fully methylated polysaccharide, gave the sugars listed in Table I, column A. The 2,3-di-*O*-methyl-D-glucose was formed from the β -D-glucopyranosyluronic acid residue,

which should be linked through O-4. The result confirms that the polysaccharide is composed of tetrasaccharide repeating-units. The three other residues in this repeating unit are two β -D-glucopyranosyl residues, linked through O-3 and O-4, respectively, and an α -L-rhamnopyranosyl residue, linked through O-4.

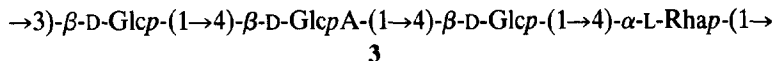
In order to determine the sugar sequence, deacetylated gellan gum was treated with acid under conditions such that tri- and tetra-saccharides were produced. The hydrolysate was fractionated by chromatography on Sephadex G-15, and two acidic oligosaccharides were obtained. One of the oligosaccharides was an aldetriuronic acid which gave $^1\text{H-n.m.r.}$ signals for a methyl group at δ 1.33 ($J_{5,6}$ 6 Hz) and for anomeric protons at δ 4.53 ($J_{1,2}$ 7 Hz), 4.74 ($J_{1,2}$ 7 Hz), 4.85 (0.5 H, $J_{1,2}$ small), and 5.09 (0.5 H, $J_{1,2}$ small). Methylation analysis of the derived alditol (Table I, column B) revealed one D-glucopyranosyl residue linked through O-4. The L-rhamnitol residue could not be detected because of its volatility, but the reducing sugar, from n.m.r. evidence, has to be L-rhamnose. From these results, it is evident that the aldetriuronic acid has structure 1.



The second oligosaccharide was indicated by its $^1\text{H-n.m.r.}$ spectrum to be an aldotetrauronic acid. Signals for anomeric protons at δ 4.50 ($J_{1,2}$ 7 Hz), 4.57 ($J_{1,2}$ 7 Hz), 4.69 ($J_{1,2}$ 7 Hz), 4.81 (0.5 H, $J_{1,2}$ small), and 5.07 (0.5 H, $J_{1,2}$ small), and for a methyl group at δ 1.30 ($J_{5,6}$ 6 Hz), were observed. Methylation analysis of the derived alditol (Table I, column C) revealed one terminal D-glucopyranosyl group and one 4-linked D-glucopyranosyl residue. It can be inferred that one 4-linked L-rhamnitol residue is present, but it is not demonstrated by the methylation analysis (Table I). As the tetrasaccharide-alditol should contain 1 as a structural element, it consequently has structure 2.



The structure of the tetrasaccharide repeating-units of gellan gum is thereby defined as 3.



The structure 3 was confirmed by degradation of the uronic acid of the fully methylated polysaccharide with a strong base¹⁰. On this treatment, both sugars linked to the uronic acid residue should be released, one as a non-reducing D-glucopyranosyl group, and the other as a reducing D-glucopyranose residue. The latter is then subjected to a second β -elimination, with exposure of an L-rhamnopyranose

residue, which, in turn, is subjected to a third β -elimination; the only sugar surviving is the 4-substituted D-glucopyranosyl residue linked to O-4 of the L-rhamnopyranosyl residue. In agreement with this sequence, tetra-O-methyl-D-glucose was the only methylated sugar observed on methylation analysis of the degraded product. Only the sequence of sugar residues given in **3** accounts for this result.

The ^1H -n.m.r. spectrum of native gellan gum showed, *inter alia*, signals for the methyl group of L-rhamnosyl residues at δ 1.3, and for OAc groups at δ 2.15 (s, 0.75 H), indicating that $\sim 25\%$ of the repeating units contain an O-acetyl group. In the ^{13}C -n.m.r. spectrum of this material, part of the signal at δ 62.5 in the spectrum of deacetylated gellan gum had shifted to δ 64.9, indicating one of the D-glucopyranosyl residues to be partially acetylated at position 6. The β -D-glucopyranosyl residue acetylated was not identified.

Another significant shift was observed for the signal of the anomeric carbon of the L-rhamnosyl residue, from δ 102.3 to 101.2. The possibility that this was due to O-acetylation at position 2 in the α -L-rhamnopyranosyl residue was excluded, as this should result in a shift in the signal for H-2 to $\delta \sim 5.7$; no such shift was observed. The shift in the ^{13}C -n.m.r. spectrum is therefore most probably due to a remote substituent effect.

EXPERIMENTAL

General methods. — Concentrations were performed under diminished pressure at $>40^\circ$ (bath). For g.l.c., Perkin-Elmer 990 and Hewlett-Packard 5830 A instruments fitted with flame-ionisation detectors were used. Separations of alditol acetates were performed on glass columns containing 3% of OV-225 on Gas Chrom Q at 190° , and of partially methylated alditol acetates on the same columns at 170° . G.l.c.-m.s. was performed on a Varian MAT 311 instrument, using the same 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. A differential refractometer was used for monitoring the Sephadex column effluent. Methylations were performed according to the Hakomori procedure¹¹ with sodium methylsulfinylmethanide-methyl iodide in methyl sulfoxide. Carboxyl-reduction of the methylated products was performed with lithium borohydride in tetrahydrofuran under reflux. After neutralisation, the material was dialysed and freeze-dried. For materials of low molecular weight, methyl sulfoxide was removed by freeze-drying, and the product was isolated by partition between chloroform and water. When necessary, it was further purified by chromatography on Sephadex LH-20. The absolute configuration of the rhamnose was determined according to the procedure of Leontein *et al.*¹².

For n.m.r. spectroscopy, a JEOL FX-100 spectrometer was used. Chemical shifts are reported in p.p.m. downfield from that of the signal for external Me_4Si (^{13}C) or internal sodium 1,1,2,2,3,3-hexadeuterio-4,4-dimethyl-4-silapentane-1-sulfonate (^1H). Spectra were recorded at 85° .

P. elodea was grown and the exocellular polysaccharide, gellan gum, was isolated essentially as described earlier³.

Partial hydrolysis of gellan gum. — To a solution of gellan gum (2 g) in water (500 mL) at 100° was added trifluoroacetic acid to a concentration of 0.1M. After 10 min, part of the solution was freeze-dried for use in n.m.r. spectroscopy. The main part was hydrolysed for 4 h and then freeze-dried, and the residue was put onto a column of Dowex 2 (free base). Acidic oligosaccharides were eluted with aqueous 10% formic acid, and the eluate was freeze-dried. The oligosaccharide mixture was fractionated on a column of Sephadex G-15, yielding mainly tetra- and tri-saccharides. These were obtained pure by rechromatography on the same column, and used for n.m.r. spectroscopy and methylation analyses.

Uronic acid degradation. — To a solution of methylated gellan gum (2 mg) in methyl sulfoxide (1 mL) was added sodium methylsulfinylmethanide (0.5 mL). The mixture was agitated in an ultrasonic bath for 30 min and then kept at room temperature overnight. Trideuteriomethyl iodide was added with external cooling and the mixture was agitated in an ultrasonic bath for 10 min. Excess of trideuteriomethyl iodide was distilled off and the mixture was freeze-dried. The product was dissolved in chloroform and the salts were removed by extraction with water. Hydrolysis of the product yielded 2,3,4,6-tetra-*O*-methyl-D-glucose, labeled at O-4 with a trideuteriomethyl group, as the single sugar derivative.

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