

Studies on an Extracellular Polysaccharide from *Rhizobium meliloti*

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An extracellular acidic polysaccharide produced by *Rhizobium meliloti* has been found to consist of D-glucose, D-galactose, pyruvic acid residues, and O-acetyl groups in the relative proportions 7:1:1:1. By methylation analysis of the native polysaccharide and of chemically modified materials, the linkages in this polysaccharide have been determined.

Bacteria belonging to the genus *Rhizobium* play an important role in nitrogen fixation. They infect the roots of leguminous plants and form nodules. The nitrogen fixed in these nodules is then transferred to the plant. The different species of *Rhizobium* show a high specificity with respect to host plant species, and it has recently been suggested that this specificity is determined by the capsular polysaccharides formed by the bacteria.^{1,2} The structure of these polysaccharides is therefore a matter of considerable interest, and the present paper reports structural studies on an acidic, capsular polysaccharide produced by *R. meliloti*, a species specific to lucerne plants.

Isolation of the polysaccharide. The cell-free solution obtained on centrifugation of the culture medium was poured into ethanol, and the precipitated polysaccharide was purified by precipitation with cetyltrimethylammonium bromide. Some neutral polysaccharide material was not precipitated. This material, which on acid hydrolysis yielded essentially mannose, was not investigated. The acidic polysaccharide, which constituted the main component, on acid hydrolysis yielded D-glucose and D-galactose in the relative proportions 7:1. No acidic sugars were detected, but pyruvic acid (5.0 %) was released during the hydrolysis. The polysaccharide also contained O-acetyl groups (3.7 %). The sodium salt of the polysaccharide showed $[\alpha]_{578}^{20} - 4.0^\circ$. The analytical values are in good agreement with those reported by Dudman and Heidelberger,³ who investigated an acidic polysaccharide from the same species of organism, and show that the approximate proportions of D-glucose, D-galactose, pyruvic acid residues, and O-acetyl groups are 7:1:1:1.

Methylation analysis. The polysaccharide was methylated by treatment with methylsulphonyl sodium and methyl iodide in methyl sulphoxide, as devised by Hakomori.⁴ The fully methylated polysaccharide was hydrolysed and the resulting methylated sugars transferred into their alditol acetates, and analysed by GLC⁵–MS.⁶ Typical chromatograms, on an ECNSS-M and on an OS-138 column, are given in Fig. 1, and the results in Table 1, column A.

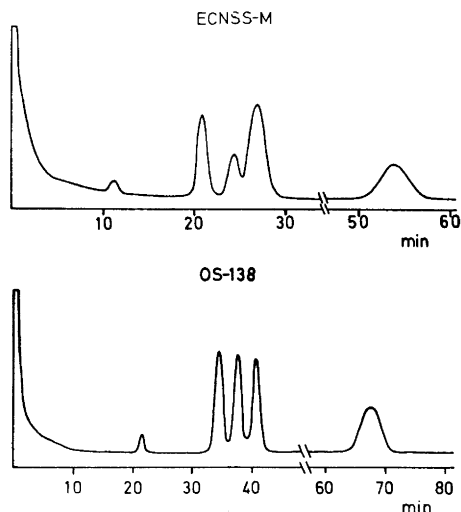


Fig. 1. Chromatograms obtained in the methylation analysis of the extracellular polysaccharide from *R. meliloti* on the ECNSS-M and OS-138 columns, respectively.

Table 1. Methyl ethers obtained on methylation analysis of the polysaccharide (A) and the partially hydrolysed polysaccharide (B).

Sugars	T^a		Mol %	
	ECNSS-M	OS-138	A	B
2,3,4,6-Tetra- <i>O</i> -Me-G	1.00	1.00	2.1	15.9
2,3,4,6-Tetra- <i>O</i> -Me-Gal	1.25	1.16	—	1.4
2,4,6-Tri- <i>O</i> -Me-G	1.95	1.63	22.3	15.1
2,4,6-Tri- <i>O</i> -Me-Gal	2.28	1.88	12.8	10.0
2,3,4-Tri- <i>O</i> -Me-G	2.49	1.88	11.8	15.7
2,3,6-Tri- <i>O</i> -Me-G	2.50	1.75	23.7	31.4
2,3-Di- <i>O</i> -Me-G	5.40	3.0	27.3	10.5

^a Retention times of the corresponding alditol acetates relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

The first peak ($T=1.00$) from the ECNSS-M column was identified as the 2,3,4,6-tetra-*O*-methyl-D-glucose derivative. The materials in the following two peaks both gave the mass spectra for 2,4,6-tri-*O*-methyl-D-hexose derivative and were identified as the D-glucose ($T=1.95$) and D-galactose ($T=2.28$) derivatives. As the total D-galactose content of the polysaccharide is accounted for as 2,4,6-tri-*O*-methyl-D-galactose, the material in the other peaks must be derived from D-glucose. The next peak, from its mass spectrum, contained

a mixture of the 2,3,4- and 2,3,6-tri-*O*-methyl-*D*-glucose derivatives ($T = 2.49$ and 2.50 , respectively). The last peak was derived from 2,3-di-*O*-methyl-*D*-glucose. The 2,3,4- and 2,3,6-tri-*O*-methyl-*D*-glucose derivatives were resolved on the OS-138 column, although the former was no longer separated from the 2,4,6-tri-*O*-methyl-*D*-galactose derivative. By combining the results of the two analyses, the relative proportions of all components could be determined.

Location of the pyruvic acid residues. Ketals of pyruvic acid, linked to the 4- and 6-positions of *D*-glucose or *D*-galactose residues, have been found in several polysaccharides, and the configuration at the ketal carbon atom has been determined.⁷

It seemed reasonable to assume that part of the 2,3-di-*O*-methyl-*D*-glucose found in the methylation analysis was derived from terminal 4,6-*O*-(1-carboxyethylidene)-*D*-glucose residues, and an equivalent part was derived from the *D*-glucose branch units, substituted in the 4- and 6-positions by other sugar residues. Part of the polysaccharide was therefore treated with acid under conditions producing essentially only cleavage of the pyruvic acid residues. The resulting polysaccharide material was thereafter subjected to methylation analysis. The results (Table 1, column B) demonstrate a considerable increase in 2,3,4,6-tetra-*O*-methyl-*D*-glucose and a corresponding decrease in 2,3-di-*O*-methyl-*D*-glucose, in agreement with expectations.

Location of the O-acetyl groups. In order to locate the *O*-acetyl groups, which are lost during Hakomori methylation, a methylation analysis was performed on a polysaccharide material, in which all free hydroxyls had been protected by treatment with methyl vinyl ether, as devised by de Belder and Norrman.⁸ In addition to *D*-glucose and *D*-galactose, a new component was obtained, and was identified by its mass spectrum and T -value (5.62) as derived from 6-*O*-methyl-*D*-glucose. The *O*-acetyl groups are therefore linked to *D*-glucose in the 6-position, although the experiment does not distinguish between the 3-linked and 4-linked chain *D*-glucose residues.

The acetalisation of the polysaccharide was therefore repeated and the product was methylated with trideuteromethyl iodide. The material was then hydrolysed under mild conditions, so that the acetal and the ketal linkages, but not the glycosidic linkages, were cleaved. The recovered polysaccharide material was methylated with methyl iodide, hydrolysed, and the resulting methylated sugars transferred into alditol acetates and analysed.

The GLC was in most details similar to that obtained in the methylation analysis of the native polysaccharide. MS demonstrated, however, that both the 2,4,6- and the 2,3,6-tri-*O*-methyl-*D*-glucose contained trideuteromethyl groups in the 6-position, giving, *inter alia*, the typical fragment m/e 48 ($D_3CO \pm CH_2$).

The experiment therefore demonstrates that the *O*-acetyl groups do not occupy a unique position in the polysaccharide, but may be found in the 6-position of both the (1→3)- and (1→4)-linked chain *D*-glucose residues.

DISCUSSION

It has not been unequivocally established that this acidic polysaccharide from *R. meliloti* is homogeneous. Chromatography on a DEAE-Sephadex col-

umn proved impractical, as the polysaccharide formed highly viscous solutions. Different preparations of the polysaccharide have shown consistent sugar analyses and optical rotations, which may indicate homogeneity.

The structural studies have revealed the following features, summarised in Fig. 2.

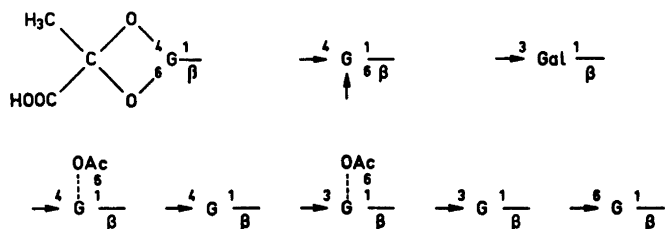


Fig. 2. Structural features demonstrated for the *R. meliloti* polysaccharide. All sugar residues are pyranosidic. A dotted line indicates that only some of the residues carry this substituent.

1. The polysaccharide contains approximately one D-galactose residue, one pyruvic acid residue, and one *O*-acetyl group per 7 D-glucose residues.

2. Methylation analysis, in conjunction with the partial hydrolysis studies, shows that all sugar residues are pyranosidic. The negative optical rotation of the polysaccharide indicates that they are all β -linked.

3. The D-galactose residue is present as a chain residue, substituted at the 3-position. Of the 7 D-glucose residues, one is present as a terminal 4,6-*O*-(1-carboxyethylidene)-D-glucose residue. One represents a branch unit and is linked at the 4- and 6-positions. The others are chain residues, two of which are substituted at the 3-position, two at the 4-position, and one at the 6-position.

4. The *O*-acetyl groups are attached to the 6-position of both the 3- and 4-substituted D-glucose residues.

5. The small percentage of terminal D-glucose residues indicates a minimum DP_n of approximately 50. This value may be considerably higher if all the terminal D-glucose residues in the branches are not substituted with pyruvic acid.

Several bacterial polysaccharides have regular structures and are composed of oligosaccharide repeating units. The present results indicate simple stoichiometric relationships between the different structural elements as required by a regular structure. The repeating unit would thus be composed of 8 sugar residues, which is a rather high value, and the possibility that the polysaccharide has a less regular structure cannot be entirely dismissed.

No information on the mutual order of the sugar residues is obtained from the present results. Attempted fragmentation analysis was not successful, as the polysaccharide was resistant to the cellulase, endo-(1 \rightarrow 3)- β -D-glucanase, and other, less pure, enzyme preparations tried.

EXPERIMENTAL

General methods. Concentrations were carried out at diminished pressure at bath temperatures not exceeding 40°. GLC was carried out on columns (200 cm × 0.3 cm) containing 3 % (w/w) of ECNSS-M on Gas Chrom Q (100–120 mesh) at 170°, and on an OS-138 SCOT-column at 190°. For mass spectrometry, the mixture of partially methylated alditol acetates, dissolved in chloroform, was injected into an ECNSS-M column, mounted in a Perkin-Elmer 270 combined gaschromatograph-mass spectrometer. The mass spectra were recorded at an inlet temperature of 200°, ionisation potential of 60 eV, ionisation current of 80 μ A, and a temperature of the ion source of 80°. Optical rotations were determined with a Perkin-Elmer 141 photoelectric polarimeter.

Isolation of the polysaccharide. *Rhizobium meliloti*, strain U 27 (Rhizobium Culture Collection, Agricultural College, Uppsala), was cultivated at 24°C with shaking in 1 l Erlenmeyer flasks, containing 250 ml Dorn's A, liquid medium.⁹ From a 4-day-old culture, the bacterial cells were separated by centrifugation. The water-soluble extracellular bacterial polysaccharide present in the supernatant liquid was precipitated in 70 % ethanol, washed and dried.⁸

The polysaccharide material (0.46 g) was dissolved in water (250 ml), and 3 % CTA-Br (50 ml) was added. The precipitate formed was separated by centrifugation and washed with water. After dissolving in 10 % NaCl (100 ml), the acidic polysaccharide fraction was precipitated with ethanol-acetone. The polysaccharide (0.23 g) showed $[\alpha]_{578}^{20} = -4.0^\circ$ (c 0.2, water).

For the sugar analysis, the polysaccharide (5 mg) was dissolved in 0.25 M sulphuric acid (5 ml), the solution was kept for 12 h at 100° and then neutralised with barium carbonate, and the monosaccharides were converted into alditol acetates and analysed by GLC.¹⁰ The identity of the sugars was confirmed by mass spectrometry.¹¹

Acetic acid was identified as methylacetate. The polysaccharide (15 mg) was treated with 2 % methanolic hydrogen chloride (0.1 ml) for 2 h at 100°, and part of the solution was analysed by GLC on a Carbowax column at 30°. The acetyl content of the polysaccharide (3.7 %) was determined by a colorimetric procedure, based on the color complex formation of an acyl hydroxamic acid and ferric ions.¹²

Pyruvic acid, isolated by ether extractions of the hydrolysate of the polysaccharide, was identified as the 2,4-dinitrophenyl-hydrazone derivative, m.p. and mixed m.p. 212–214°. The pyruvic acid content (5.0 %) was determined by a colorimetric procedure based on the color complex formation of the 2,4-dinitrophenylhydrazone derivative.¹³

A neutral polysaccharide fraction precipitated when the above supernatant was made alkaline. This fraction, which on acid hydrolysis yielded mainly mannose, was not further investigated.

Methylation of the polysaccharide. The polysaccharide (10 mg), in a 5 ml serum bottle sealed with a rubber cap, was dissolved in dry methyl sulphoxide (2.5 ml). Nitrogen gas was flushed through the bottle, and a 2 M solution of methylsulphonyl sodium in methyl sulphoxide (1.5 ml) was added dropwise, using a syringe. The resulting gelatinous solution was agitated in an ultrasonic bath (40 kc/s) for 1 h and kept at room temperature for 3 h. Methyl iodide (0.5 ml) was then added dropwise, cooling externally with tap-water and the resulting turbid solution agitated for 1 h in the ultrasonic bath, after which it became clear. The solution was then poured into water, dialysed overnight against tap-water, and evaporated to dryness. The methylated polysaccharide was treated with 90 % formic acid (3 ml) at 100° for 4 h, concentrated, and then hydrolysed in 0.25 M sulphuric acid (3 ml) at 100° for 12 h. The hydrolysate was neutralised with barium carbonate, and the sugars converted into alditol acetates and analysed by GLC⁵ (Fig. 1) — mass spectrometry.⁶ The results are summarised in Table 1, column A.

Partial acid hydrolysis of the polysaccharide. The acidic polysaccharide (100 mg) was dissolved in 0.25 M sulphuric acid (50 ml) and kept at 100° for 0.5 h. The hydrolysate was neutralised with barium carbonate, concentrated, and the residual polymeric material (40 mg) was precipitated with ethanol. Part of this product (10 mg) was subjected to methylation analysis. The results are summarised in Table 1, column B.

Location of O-acetyl groups in the polysaccharide. The polysaccharide (20 mg) was dissolved in dry methyl sulphoxide (2 ml). *p*-Toluenesulphonic acid (20 mg) was added, and the solution was thermostated at 15°. With stirring, methyl vinyl ether (2 ml), condensed at –30°, was then added in portions. Shaking was continued for 3.5 h at 15°.

The resulting red reaction mixture was then placed on a Sephadex LH-20 column (27 × 2 cm) and eluted with anhydrous acetone. The optical rotation of the eluate was determined, and the reaction product was collected and concentrated, giving a red syrup. The syrup was methylated, and the methylated product was hydrolysed as described above. The resulting sugars were reduced to alditols, acetylated, and analysed by GLC-mass spectrometry.

Another portion of the polysaccharide (30 mg) was acetalated as above and treated with methylsulphinyl sodium-trideuteromethyl iodide in methyl sulphoxide. The recovered material was treated with 50 % acetic acid for 2 h at 100°, in order to hydrolyse the acetal groups. The partially trideuteromethylated polysaccharide was then remethylated, using methyl iodide as methylation agent, and after hydrolysis analysed as above.

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