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OCCURRENCE OF TWO EXTRACELLULAR ACIDIC POLYSACCHARIDES AS PRODUCTS OF *RHIZOBIUM MELILOTI* 201

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ABSTRACT

The extracellular polysaccharide of Rhizobium meliloti *201 consists of two acidic polysaccharides, APS-I and APS-II. APS-I is composed of D-glucose, D-mannose and D-glucuronic acid in a molar ratio of 3:3:2, whereas APS-II is composed of D-glucose, D-galactose, D-mannose and pyruvic acid in a molar ratio of 4:3:2:1.*

APS-II was separated from the extracellular polysaccharide preparation by hydrolysing APS-I to its octasaccharide repeating unit with a specific enzyme. APS-I and APS-II were also separated by treatment with cetylpyridinium chloride and by paper electrophoresis of the depyruvylated polysaccharide.

1. INTRODUCTION

The extracellular polysaccharides of *Rhizobium* strains are of interest because they are thought to have an important role in the interaction between the bacteria and their host legume plants. There have been many studies (Bailey *et al.*, 1971; Zevenhuizen, 1971; Sømme, 1974; Ghai *et al.*, 1981) on the chemical compositions of extracellular polysaccharides, and the structures of several polysaccharides from strains of *R. meliloti* (Jannson *et al.*, 1977; Amemura *et al.*, 1981; Ghai *et al.*, 1981), *R. trifolii* (Jansson *et al.*, 1979) and *R. japonicum* (Dudman, 1978) have been clarified. Various attempts have been made to relate the extracellular polysaccharide composition of *Rhizobium* to host specificity. Most strains of *R. meliloti* produce polysaccharides of identical structure (Ghai *et al.*, 1981), containing D-glucose, D-galactose and pyruvic acid in a molar ratio of 7 : 1 : 1 with some succinic acid or acetic acid. However,

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Amemura *et al.* (1981) have found that a strain of *R. meliloti* (IFO 13336) produces a different polysaccharide containing D-riburonic acid and D-glucuronic acid. Another example of a different polysaccharide is that produced by *R. meliloti* 201; this polysaccharide is composed of D-glucose, D-galactose, D-mannose, D-glucuronic acid and pyruvic acid in a molar ratio of 4:2:3:1:1 (Ghai *et al.*, 1981). Due to its high viscosity the polysaccharide of *R. meliloti* 201 may have potential applications in food and many other industries. During studies on the structure of this polysaccharide we found that it is a mixture of two acidic polysaccharides. We now report findings on the two acidic polysaccharides, and the separation and component analysis of the two compounds.

2. EXPERIMENTAL

2.1. *Strain*

Rhizobium meliloti 201 was obtained from Professor M. Yatazawa, Nagoya University, Japan.

2.2. *Preparation of the Polysaccharide*

Extracellular polysaccharide was prepared from the culture broth of *R. meliloti* 201 by the method described previously (Misaki *et al.*, 1969) involving precipitation with acetone and cetylpyridinium chloride. The polysaccharide thus obtained was dissolved in water, treated with Amberlite IR-120 (H^+ resin) and freeze dried. The H-form polysaccharide was used for analyses.

2.3. *Quantitative Analysis of Sugars*

The polysaccharide (2 mg) was hydrolysed in a sealed tube first with 1 ml of 90% formic acid for 6 h at 100°C and then with 0·5 ml of 4 M trifluoroacetic acid for 6 h at 100°C. The hydrolysate was converted to a mixture of alditol acetates and analysed (Björndal *et al.*, 1967) in a Shimadzu GC7A gas chromatograph fitted with a flame-ionisation detector and a column (3 mm x 2 m) of 3% ECNSS-M on Gas-chrom Q at 180°C.

Glucuronic acid was determined by the modified carbazole reaction described by Galambos (1967).

2.4. *Quantitative Analysis of Pyruvic Acid*

Pyruvic acid was assayed by the method of Koepsell and Sharpe (1952).

2.5. *Isolation of an Organism Capable of Using the Polysaccharide of* R. meliloti *201 as the Sole Carbon Source*

To study the structure of the polysaccharide of *R. meliloti* 201, we isolated a bacterium (Y3) capable of growing with the polysaccharide as the sole carbon source

from soil by enrichment culture techniques as described previously (Harada *et al.*, 1972).

2.6. *Media*

The medium used for isolation of the organism and production of enzyme from the isolated organism (Y3) had a pH of 7 and the following composition (per 100 ml): $(NH_4)_2HPO_4$, 0·15 g; KH_2PO_4, 0·1 g; $MgSO_4 . 7H_2O$, 0·05 g; NaCl, 1 mg; $MnCl_2 . 4H_2O$, 1 mg; polysaccharide of *R. meliloti* 201, 0·3 g.

2.7. *Preparation of Extracellular Enzyme of Strain Y3*

Conical flasks (500 ml) containing 100 ml of medium were inoculated with 2 ml of a seed culture of strain Y3 grown in the same medium. The culture was shaken reciprocally at 120 strokes per min at 30°C for 2 days, then centrifuged at 17 000 × g for 40 min at 4°C. To the clear supernatant, ammonium sulphate was added to 80% saturation, and the resulting precipitate was collected by centrifugation. The precipitate was washed three times with an ammonium sulphate solution (80% saturation), to remove any polysaccharide, and dissolved in 10 ml of 0·04 M acetate buffer (pH 5·8) and dialysed against the same buffer for 2 days. The dialysate (12 ml) was used as crude enzyme. This preparation was free of polysaccharide which had remained in the culture broth without being used by the organism.

2.8. *Digestion of the Polysaccharide with the Enzyme Preparation and Separation of the Hydrolysis Products*

A sample of polysaccharide (200 mg) was dissolved in 100 ml of 0·04 M acetate buffer (pH 5·8) and digested extensively with 10 ml of the enzyme preparation for 2 days at 30°C. Two drops of toluene were added to the reaction mixture to prevent bacterial growth, then the enzyme was inactivated by heating. The mixture was cooled, inactivated proteins were removed by centrifugation and two volumes of acetone were added to the supernatant to precipitate the undigested polysaccharide (termed remaining polysaccharide). The precipitate was removed by centrifugation and dried with acetone (yield: 85 mg). The supernatant was concentrated, passed through a Sephadex G-10 column to remove salts, concentrated by evaporation and freeze dried (yield of oligosaccharide fraction: 83 mg).

2.9. *Separation of Two Acidic Polysaccharides (APS-I and APS-II) with Cetylpyridinium Chloride (CPC)*

The extracellular polysaccharide (300 mg) was dissolved in 300 ml of water and the solution was adjusted to pH 2 with hydrochloric acid. The acidic solution was heated for 2 h at 100°C to remove pyruvic acid, then dialysed overnight against water. The dialysed solution was adjusted to pH 12 with sodium hydroxide, a solution of CPC (12 g/g polysaccharide) was added, and the resulting precipitate (APS-I–CPC complex) was separated by centrifugation. To the clear supernatant, two volumes of acetone

were added to precipitate neutral polysaccharide (depyruvylated (DP) APS-II). The precipitate was washed with a mixture of acetone and water (2:1), dissolved in water, dialysed against water and freeze dried (yield: 114 mg). The APS-I-CPC complex was washed with water, then dissolved in 5% sodium chloride. To the resulting solution, two volumes of acetone were added to precipitate APS-I, and this was dissolved in water, dialysed against water for 2 days, concentrated by evaporation and freeze dried (yield: 133 mg). To remove small amounts of APS-II from the APS-I preparation, the sample was dissolved in water and the above procedure repeated.

2.10. *Paper Chromatography and Electrophoresis*

Paper chromatography was carried out on Toyo filter paper No. 50 with a solvent system of 1-butanol–pyridine–water (6:4:3). Paper electrophoresis was carried out on Toyo glass fibre filter paper GC50 in 0·05 M sodium borate for 15 min at 2000 V.

2.11. *Ultracentrifugation*

Sedimentation analysis was performed at 52 000 rpm in a Spinco model E ultracentrifuge.

3. RESULTS AND DISCUSSION

3.1. *Enzymic Digestion of the Polysaccharide*

To study the structure of the polysaccharide of *R. meliloti* 201, we isolated a bacterium (Y3) capable of growing with the polysaccharide as the sole source of carbon and tried to hydrolyse the polysaccharide with a crude enzyme preparation obtained from the culture filtrate of the organism. The viscosity of the reaction mixture decreased very rapidly during the digestion. An aliquot of the mixture was applied to paper and the hydrolysis products were examined by paper chromatography. Only one spot of oligosaccharide was detected near the origin as a reducing compound, while polysaccharide (termed remaining polysaccharide) remained in the reaction mixture even after extensive digestion.

3.2. *Components of the Oligosaccharide and Remaining Polysaccharide*

The components of the oligosaccharide and the remaining polysaccharide are shown in Table 1. The oligosaccharide was composed of D-glucose, D-mannose and D-glucuronic acid in a molar ratio of 3:3:2, while the remaining polysaccharide was composed of D-glucose, D-galactose, D-mannose and pyruvic acid in a molar ratio of 4:3:2:1.

The degree of polymerisation of the oligosaccharide was determined as follows (Hisamatsu *et al.*, 1980). The carboxyl groups and reducing end sugars of the oligosaccharide were reduced by the method of Taylor & Conrad (1972), the reduced sample was hydrolysed completely and the hydrolysate was analysed as acetyl

TABLE 1
Components of Oligosaccharide and Remaining Polysaccharide Produced by Enzyme Digestion

	Molar ratio				
	D-Glucose	*D-Galactose*	*D-Mannose*	*D-Glucuronic acid*	*Pyruvic acid*
Oligosaccharide	3·2	0·0	3	1·9	0·0
Remaining polysaccharide	4·3	3·4	2	0·0	1·1

derivatives by gas–liquid chromatography on a column of Silicone OV-17. In the resulting chromatogram the ratio of D-glucose pentaacetate and D-mannose pentaacetate to hexitol hexaacetate (this hexitol hexaacetate was determined as D-glucitol hexaacetate on a column of ECNSS-M) was 7. Thus the degree of polymerisation of the oligosaccharide was 8.

A polysaccharide remained in the culture supernatant which had not been utilised by the organism. This polysaccharide was isolated by acetone precipitation and the components were assayed. The polysaccharide was composed of D-glucose, D-galactose, D-mannose and pyruvic acid in a molar ratio of 4 : 3 : 2 : 1. This ratio is the same as that of the remaining polysaccharide obtained by enzymic digestion.

3.3. *Separation of Polysaccharide with CPC*

One possible explanation for the presence of the remaining polysaccharide is that an extracellular enzyme of strain Y3 might cleave the regular units of the native polysaccharide, such as branches, and that the remaining polysaccharide might be the backbone of the polysaccharide. An alternative explanation is that the polysaccharide preparation was a mixture of two acidic polysaccharides and that only one of these was hydrolysed to repeating units by the enzyme. If the latter explanation is true, these polysaccharides should be separated by treatment with CPC after removing pyruvic acid, because the polysaccharide containing pyruvic acid should be converted to a neutral polysaccharide by removing pyruvic acid.

When CPC was added to a solution of depyruvylated (DP) polysaccharide, the polysaccharide–CPC complex was precipitated, and the sugar components of the precipitated polysaccharide were identical with those of the native polysaccharide. No neutral fraction was obtained from the supernatant. When the solution of the depyruvylated polysaccharide was adjusted to pH 12, and then CPC was added, an acidic polysaccharide (APS-I) was separated from a neutral polysaccharide (DP-APS-II). Alkaline conditions seemed to be favourable for the separation for some reason.

APS-I was composed of D-glucose, D-mannose and D-glucuronic acid in a molar ratio of 3 : 3 : 2, while DP-APS-II was composed of D-glucose, D-galactose and D-mannose in a molar ratio of 4 : 3 : 2 (Table 2). The ratios of the components of these compounds were the same as those of the oligosaccharide and the remaining poly-

TABLE 2
Components of APS-I and DP-APS-II Obtained by Treatment with CPC

	Molar ratio				
	D-Glucose	*D-Galactose*	*D-Mannose*	*D-Glucuronic acid*	*Pyruvic acid*
APS-I	3·3	0·0	3	2·0	0
DP-APS-II	3·8	3·0	2	0·0	0

saccharide, respectively, obtained by enzymic hydrolysis. APS-I was also hydrolysed by the extracellular enzyme of strain Y3 to oligosaccharide of DP*n* 8.

3.4. *Separation of APS-I and APS-II by Paper Electrophoresis*

The above findings strongly suggest that the extracellular enzyme of strain Y3 hydrolysed APS-I to a repeating unit octasaccharide and that APS-II was not hydrolysed. This suggestion was confirmed by paper electrophoresis in 0·05 M borate (pH 9·3). As shown in Fig. 1, the native polysaccharide remained at the origin, probably owing to its high viscosity. The depyruvylated polysaccharide formed two bands: a fast moving band coinciding with DP-APS-II, and a slow moving band coinciding with APS-I. The fast moving band also coincided with that of the remaining depyruvylated polysaccharide. These two bands were also obtained in a solvent system of pyridine–acetic acid–water (5 : 0·2 : 0·95, pH 6·0).

Thus, the polysaccharide of *R. meliloti* 201 consists of two component acidic polysaccharides, APS-I and APS-II. However, the depyruvylated polysaccharide gave

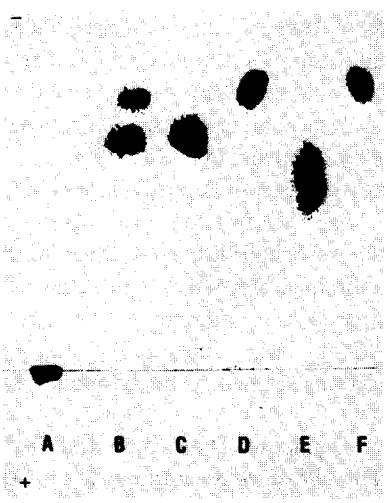
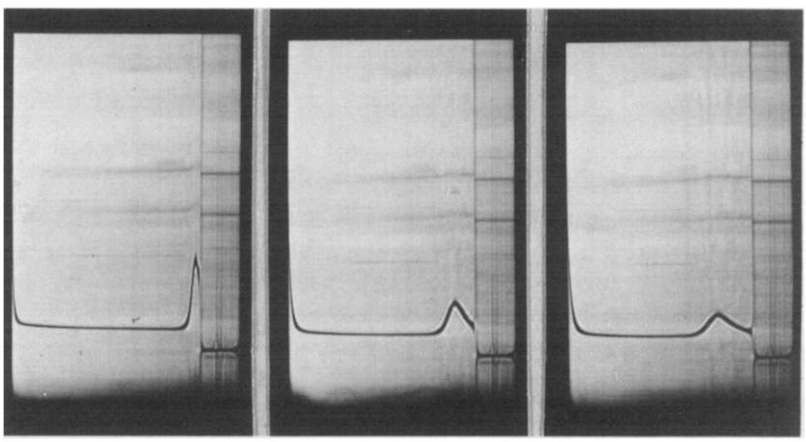


Fig. 1. Paper electrophoresis of native (A) and depyruvylated (B) polysaccharides, APS-I (C), DP-APS-II (D), remaining polysaccharides (E) and remaining depyruvylated polysaccharides (F).

a single peak (S20, w = 1·5 S) on analytical ultracentrifugation (Fig. 2), indicating that it was apparently homogeneous, although the native polysaccharide did not give a clear sedimentation pattern owing to formation of a weak gel even at low concentration. Therefore, the molecular weights of APS-I and APS-II may be quite similar.

The mannose-containing polysaccharide of *R. meliloti* 206 (Ghai *et al.*, 1981) was also not homogeneous and separated into three components on paper electrophoresis after depyruvylation. However, the polysaccharides of *R. japonicum* strains TISTR 121, 135 and 159, which contain D-glucose, D-galactose, D-mannose, D-glucuronic acid and pyruvic acid (Footrakul *et al.*, 1981), were homogeneous.



A B C

Fig. 2. Sedimentation pattern of depyruvylated polysaccharide in 0·1 M sodium chloride. The photographs were taken at 20 (A), 50 (B) and 80 (C) min after reaching full speed (52 000 rpm).

The two acidic polysaccharides of *R. meliloti* 201 are produced by a single organism, not by a mixture of this strain and contaminating organisms, because all eight cultures obtained by repeated single-colony isolation produced two acidic polysaccharides. The physiological significances of these polysaccharides to the host plant are unknown.

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