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Structural studies of extracellular polysaccharide produced by *Rhizobium fredii* Tu6, a polysaccharide with nonasaccharide repeating units

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Abstract. Polysaccharide secreted by *Rhizobium fredii* Tu6 was investigated with composition analysis, linkage analysis, Svensson degradations, ^{1}H-NMR, and desorption chemical ionization/mass spectrometry. The polysaccharide is composed of a nonasaccharide repeating unit (seven glucose, two galactose) with five sugar residues in the side chain and four sugar residues in the back bone. The terminal residue of the side chain is a galactose residue. The second residue from the terminal end is a 3-linked glucose substituted by a pyruvate group at the 4, 6-positions. Monosaccharide sequence was determined by Svensson degradations followed by linkage analysis. After removal of a pyruvate group from the methylated polysaccharide, the sugar residues in the side chain were removed sequentially by Svensson degradations. The reaction product of each degradation was then analyzed by methylation analysis. The polysaccharide secreted at pH 5 medium has a structure very similar to the polysaccharide secreted in the medium of pH 7 except that about 30% of the terminal galactose residues are also substituted at the 4, 6-positions by a second pyruvate group.

Keywords: Desorption chemical ionization/mass spectrometry (DCI/MS); Extracellular polysaccharide; *Rhizobium fredii* Tu6; Svensson degradation.

Introduction

Rhizobium species can infect leguminous plants and form nodules. The exopolysaccharides (EPS) excreted by the strains of *Rhizobium* play an important role in the infection of legumes and the *Rhizobium* / legume symbiosis (Phillip-Hollingsworth et al., 1989). *Rhizobium fredii* Tu6, a fast growing bacterium under neutral (pH 7) and acidic (pH 5) conditions, was isolated at Ping-Tong, Taiwan (Cheng and Lin, 1984). We have found a difference in morphological exhibition between neutral and acidic culture environments (unpublished data). We assume that EPS plays not only an important role in infection processes, but also a protective role in environmental adaptation. We now describe an investigation of the structure of the polysaccharide excreted by this bacterium. It is hoped that the investigation of the structure of EPS can provide some clues to the mechanism of infection and/or protection.

Materials and Methods

Isolation of the Polysaccharide

One and half day old (for pH 7) and two day old (for pH 5) cultures of *R. fredii* Tu6 were grown in B III medium (Bishop et al., 1976) and centrifuged. The polysaccharide was precipitated with cetyltrimethylammonium bromide. The precipitate was washed with ethanol, dialyzed against water, and lyophilized. The resulting crude exopolysaccharide was purified by a DEAE-sepharose column. Fractions containing exopolysaccharide were identified with a phenol-sulfuric acid test.

Proton NMR Spectroscopy

Samples for n.m.r. spectroscopy were lyophilized several times with D_2O, and dissolved in D_2O at a concentration of 1–3 mg/ml. Spectra were obtained with a Bruker 300-MHz instrument, with the probe heated to 60°C. The signal at δ 1.47 was assigned to the methyl protons of pyruvic acid residues; the signal at δ 2.13 was assigned to the methyl protons of O-acetyl groups; and the signals at δ 2.57 and δ 2.68 were assigned to the methylene protons of O-succinyl groups (Figure 1). The signal at δ 1.17 remains to be assigned.

Desorption Chemical Ionization Mass Spectrometry

DCI/MS experiments were performed on a Finnigan TSQ-46C triplequadrupole mass spectrometer (Finnigan MAT, CA). Ammonia (from San-Fu Co., Hsinchu, Taiwan) was used as the reagent gas. The sample was air-dried on a platinum or rhenium emitter; the emitter was heated by a separated power supply at a heating rate of 50 mA/sec until the maximum current of 1.3 A was reached.

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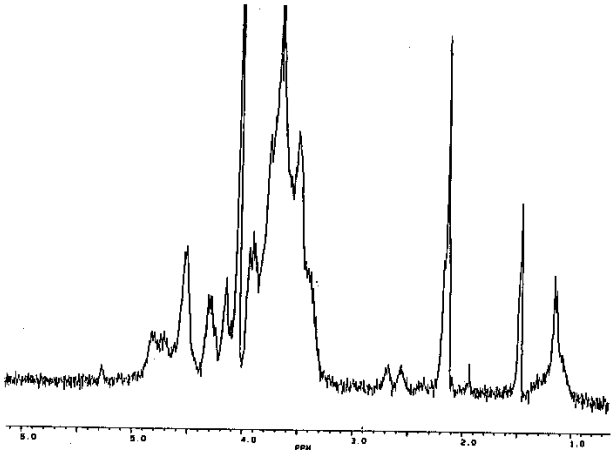


Figure 1. The NMR spectrum of the extracellular polysaccharide secreted by *Rhizobium fredii* Tu6.

Composition Analysis

The glycosyl composition of the purified polysaccharide was determined by hydrolysis, reduction and acetylation followed by gas-liquid chromatography analysis of the alditol acetates (Biermann and McGinnis, 1989).

Linkage Analysis

Partially methylated alditol acetates were prepared (Biermann and McGinnis, 1989) and analyzed with a Hewlett Packard 5890 series 2 GC (Hewlett-Packard, PA, USA) and a VG-MD800 GC/MS instrument (Fisons, Manchester, UK). For GC separation, a DB-5 capillary column (30-m) was used. The column was temperature programmed by holding for 1 min at 75°C, increasing to 150°C at a rate of 20°C/min, and finally to 300°C at a rate of 2°C/min. Identification of alditol acetates was achieved by comparison of GC relative retention times and electron impact mass spectra of standard compounds.

Svensson Degradation (Jansson et al., 1977)

The EPS was permethylated, reduced by $LiAlH_4$, depyruvylated (with 50% acetic acid at 100°C for 2 h) and then oxidized by DMSO activated by either oxalyl chloride (Mancuso et al., 1978) or P_2O_5 (Taber et al., 1987). After the oxidation, triethylamine (1 ml) was then added and stirred for 30 min. The solution was warmed to room temperature, dialyzed, and concentrated to dryness under vacuum. The sample was redissolved in 1 ml methylene chloride, treated with sodium ethoxide in ethanol (1 M, 1 ml), stored at room temperature for 1.5 h, neutralized with diluted hydrochloric acid, mixed with 1.5 ml 50% acetic acid, and heated to 100°C for 3 h. The solution was dried under vacuum and an aliquot of the sample was used for linkage analysis.

PA (2-Aminopyridine) Labeled Trisaccharide

The reductive amination procedure developed by Hase et al. (1979) was adopted for the preparation of PA labeled trisaccharide.

Results and Discussion

The results of the composition analysis suggested repeating units of nine sugars consisting of seven glucose and two galactose. Proton NMR obtained at 60°C showed that the EPS was substituted with pyruvate, acetyl, succinyl, and one unidentified group (Figure 1).

Desorption Chemical Ionization (DCI) mass spectrometry has proved to be a powerful technique for the determination of polysaccharide sequences (Her et al., 1990). Although no molecular ion can be detected, useful structural information may be obtained by DCI/MS with the detection of pyrolytic fragments. The formation of fragments along the backbone requires the cleavage of two bonds whereas a single bond cleavage is needed for the formation of a fragment in the side chain. Consequently, fragment ions from the side chain should be more abundant than backbone ions. The ammonia DCI mass spectrum obtained with permethylated and depyruvylated EPS (Figure 2) suggested that the side chain could consist of five sugar residues because the intensities of the signals decreased sharply after the fifth sugar residue.

Glycosidic linkages of the polysaccharide were determined by GC and GC/MS after permethylation, hydrolysis, reduction, and peracetylation (Harris and Henery, 1984). A total of nine sugar residues, including one nonreducing terminal galactose, two 3-substituted glucoses, two 4-substituted glucoses, one 3-substituted galactose, one 6-substituted glucose, one 4,6-disubstituted glucose,

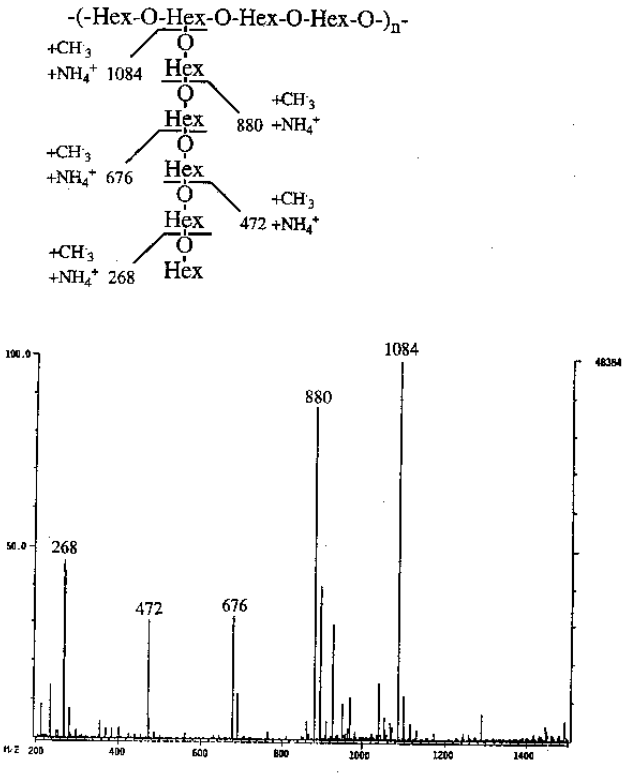


Figure 2. The ammonia DCI mass spectrum obtained with permethylated and depyruvylated EPS.

and one 3,4,6-trisubstituted glucose were detected. Pyruvic acid was linked to the 4,6 positions of the 3,4,6-trisubstituted glucose because linkage analysis of the depyruvylated polysaccharide showed the disappearance of the signal corresponding to 3,4,6-trisubstituted glucose and an increase in the intensity of the 3-substituted glucose. The branching point of the polysaccharide was indicated by the 4,6-disubstituted glucosyl residue.

Monosaccharide sequence was determined mainly by the Svensson degradation method (Figure 3). This method requires selective exposure of the hydroxyl group followed by oxidation of the exposed hydroxyl group, base-catalyzed β-elimination, and mild acid hydrolysis. The residue with the free hydroxyl group is degraded, and the sugars linked to this residue are released as a nonreducing and a reducing moieties. The free hydroxyl group in the reducing moiety provides a point of oxidation for a second Svensson degradation.

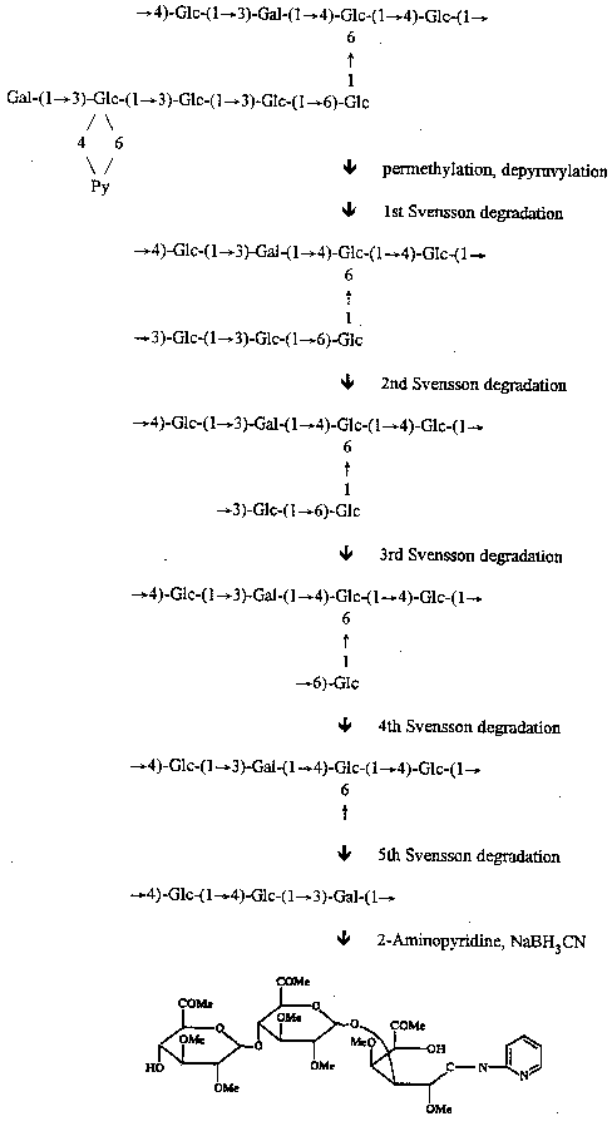


Figure 3. Sequence analysis of the extracellular polysaccharide secreted by *Rhizobium fredii* Tu6.

The pyruvate group in EPS Tu6 was reduced and hydrolyzed (Taylor and Conrad, 1972). After removal of the pyruvate group, the sugar residues in the side chain were removed sequentially by Svensson degradations. Linkage analysis of the first four degradation products are shown in Table 1. Seven sugar residues were detected for the first Svensson degradation indicating the substitution of pyruvate group on the glucose residue next to the terminal galactose (Gal1-3Glc(py)-). The third sugar residue was assigned as a 3 substituted glucose because linkage analysis of the repermethylated first degradation product showed the appearance of a terminal glucose and a decrease of the signal corresponding to 3 substituted glucose (Gal1-3Glc(py)1-3Glc-). This assignment is consistent with the linkage analysis of the second degradation product (see Table 1). Based on this approach, the fourth and fifth sugar residues in the side chain were designated a 3 linked glucose and a 6 linked glucose, respectively (Gal1-3Glc(py)1-3Glc1-3Glc1-6Glc). The side chain was believed to consist of five sugar residues because no signal corresponding to terminal sugar residue was observed in the linkage analysis of the repermethylated fourth degradation product. These data are in agreement with the results obtained in the DCI mass spectrum.

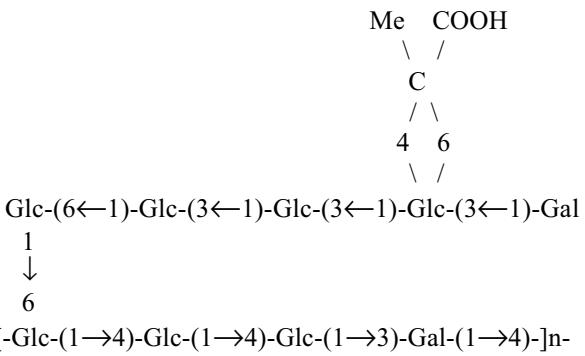
Table 1. Glycosyl linkage composition of the native and Svensson degradation products.

	EPS	1st	1st*	2nd	2nd*	3rd*	4th*
Ter-Glc			1.1		1.1	0.9	
Ter-Gal	1						
3-Glc	1.7	1.7	0.9	0.9			
4-Glc	2.3	2.3	1.9	2.1	2.3	2.4	3.4
3-Gal	1.1	1.0	1.0	1.0	0.9	1	1
6-Glc	1.1	1.0	1.0	1.0	0.7		
4,6-Glc	1.1	0.8	1.0	1.1	0.7	0.9	
3,4,6-Glc	1.1						

*Repermethylated the Svensson degradation products.

Because the side chain consists of five sugar residues (Gal1-3Glc(py)1-3Glc1-3Glc1-6Glc), the repeating unit of the backbone should consist of four sugar residues. These four sugars consist of three 4 substituted glucose (including the 4,6 linked branching sugar) and one 3 substituted galactose. The side chain was cleaved with the first four degradation reactions. The fifth oxidation reaction should oxidize the branching sugar, and a trisaccharide was therefore left after the reaction. It is known that the branching sugar is a 4, 6 disubstituted glucose, so there are three possible arrangements for the three remaining sugars (4Glc1-4Glc1-3Gal-, 4Glc1-3Gal1-4Glc-, 3Gal1-4Glc1-4Glc-). Linkage analysis of the repermethylated trisaccharide could determine the terminal sugar but not the order of the other two sugars. In order to determine the sequence of the trisaccharide, the reducing sugar was labeled with 2-aminopyridine by reductive amination (Hase et al., 1979) before the permethylation and linkage analysis. The result showed that the terminal sugar was a 3 substituted galactose. This data suggested a sequence of 4Glc (branching sugar)-4Glc-4Glc-3Gal- for the backbone.

EPS isolated from pH 5 medium and pH 7 medium have a very similar structure, except that the terminal galactose of the EPS obtained under pH 7 was partially substituted (about 30%) with pyruvic acid. As has been shown in *R. trifolii* (Abe et al., 1984), the non-carbohydrate substitutions of the *R. fredii* EPS change during culture age.



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根瘤菌 *Rhizobium fredii* Tu6 外多醣之結構鑑定

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Rhizobium fredii Tu6 所分泌的細胞外多醣經分離、純化後利用組成分析、鍵結點分析、氧化降解、核磁共振光譜儀、化學揮離法／質譜的方法鑑定其結構。此外多醣具有 9 個單醣的重覆單元：一 4 個單醣的主鏈與一 5 個單醣的支鏈。支鏈末端為半乳糖，末端第二個為 3 鍵結的葡萄糖並且在 4,6 位置上與丙酮酸形成縮酮。將經甲基化的外多醣去除丙酮酸後，支鏈上單醣的順序以 Svensson 氧化降解的方法鑑定。主鏈上的順序則是氧化降解後，再以 2-Aminopyridine 標記還原端的單醣。由酸性與中性的培養液中所分離出的外多醣結構十分類似。唯一的不同是中性條件下分離出的外多醣，其非還原端的半乳糖上約 30% 具有丙酮酸取代基。

關鍵詞：化學揮離法／質譜法；細胞外多醣； *Rhizobium fredii* Tu6 ； Svensson degradation 。