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The structure of a novel polysaccharide produced by *Bradyrhizobium* species within soybean nodules

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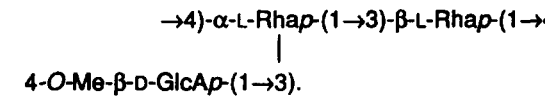
Abstract

Certain strains of *Bradyrhizobium japonicum* and *B. elkanii* produce a polysaccharide within the root nodules of their legume host, soybean. These nodule polysaccharides (NPSs) were isolated and characterized. The NPS produced by *B. elkanii* strains proved to be identical in glycosyl composition and linkages to the extracellular polysaccharide (EPS) of this species indicating that the NPS and EPS for *B. elkanii* have identical structures (W.F. Dudman, *Carbohydr. Res.*, 66 (1978) 9–23),



However, the structure of the NPS from *B. japonicum* proved to be quite different from that of its EPS. Methylation analysis of this NPS showed that it consists of 3-linked Gal, 3-linked Rha, 2,4-linked Rha, 4-linked Rha, and terminal 2-*O*-methyl GlcA in a 1:1:1:1:1 ratio. Stereochemical configurations of the glycosyl residues were determined by the preparation and analysis of trimethylsilyl (Me_3Si) (−)-2-butyl glycosides. NMR spectroscopy (both ^1H and ^{13}C) showed that the Gal residue is α-linked, while all the other glycosyl residues are β-linked. Oligosaccharides produced by periodate oxidation–Smith degradation were purified, as were oligosaccharides

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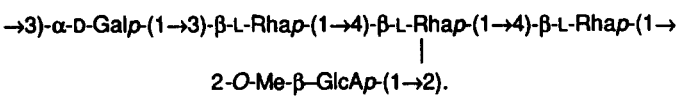
produced by partial acid hydrolysis. Characterization of the Smith degradation products by methylation analysis, NMR spectroscopy, electrospray-mass spectrometry, and characterization of the partial acid hydrolysate oligosaccharides showed that the repeating oligosaccharide unit of the NPS has the structure,



Keywords: *Bradyrhizobium japonicum*; *Bradyrhizobium elkanii*; Nodules; Polysaccharide

1. Introduction

Bradyrhizobium japonicum and *B. elkanii* (previously called type II *B. japonicum*) are Gram-negative bacteria which form symbiotic nitrogen-fixing associations with soybean, *Glycine max* (L.) Merr. Certain strains of these bacteria produce large quantities of polysaccharide in the nodules formed on legume roots [1], i.e., nodule polysaccharide (NPS). Strains of bacteria which produce NPS are most common within serogroups known to be dominant for nodule formation in the field suggesting that NPS may provide some advantage to nodule occupants [1]. However, a function for NPS has not been determined.

For *B. japonicum*, the composition of NPS is very different from that of its extracellular polysaccharide (EPS) produced in culture [1]. Namely, EPS contains glucose mannose, galactose, galacturonic acid, and 4-*O*-methylgalactose [2,3], whereas the NPS contains galactose, rhamnose, and 2-*O*-methylglucuronic acid [1]. Thus, this organism, in the special environment provided by the root nodule, expresses a unique set of genes required for NPS synthesis.

In the case of *B. elkanii*, the composition of the NPS is identical to that of its EPS; namely, rhamnose, and 4-*O*-methylglucuronic acid in a 3:1 ratio [1,4]. Thus, both *B. japonicum* and *B. elkanii* produce NPSs that are rich in rhamnose, and contain a methylated glucuronosyl residue.

In this report we describe the structures of the NPS produced by both *B. japonicum* and *B. elkanii*.

2. Experimental

Source of NPS. Bacterial strains.—Bradyrhizobium japonicum USDA123 and *B. elkanii* USDA31 were obtained from Peter van Berkum at the Nitrogen Fixation and Soybean Genetics Laboratory, National Agricultural Research Center, Beltsville, MD, USA. The bacteria were grown, and the plants were inoculated as previously described [1].

Isolation of NPS.—The NPS was purified as previously described [1] with some modification. Nodules were ground in 50% EtOH (2 vol per nodule fresh weight). The homogenate was placed in a centrifuge tube. The mortar and pestle were rinsed with 36% EtOH, the rinses added to the homogenate and centrifuged. The supernatant was saved, and the pellet suspended in 36% EtOH and centrifuged. The supernatants from both centrifugations were combined, 95% EtOH was added to a final concentration of 50% EtOH, stirred for 15 min, and centrifuged. The supernatant was saved, made 87% EtOH by the addition of 95% EtOH, allowed to stand for > 2 h at 2°C, and centrifuged at 22 000 *g* for 15 min. The pellet was dried under air, dissolved in a small amount of water, and freeze-dried. Approximately 500 mg of the lyophilizate was dissolved in 30 mL of 10 mM NaOAc (pH 5.0) containing 100 mM NaCl. One mg of DNase (2500 units from Sigma Chemical Co.) and 10 mg of RNase (100 units, Sigma Chemical Co.) were added, and the was solution incubated with shaking at 37°C for 1 h. The pH was adjusted to 7.5 using 200 mM Na_2HPO_4, 10 mg of proteinase K (200 units, Sigma Chemical Co.) were added, and the solution was incubated at 37°C for 2 h. EtOH (200 mL) was added, the solution was allowed to stand at 2°C overnight, and then it was centrifuged at 22 000 *g* for 15 min. The pellet was dissolved in deionized water, Dowex 50 (H^+ form) resin was added, and the resulting mixture was stirred for 5 min. The Dowex 50, was removed and the acidified NPS was dialyzed against deionized water and freeze-dried. Approximately 300 mg of sample were dissolved in 40 mL of 5 mM PIPES buffer (pH 7.0) and centrifuged to remove insoluble debris (denatured protein). The supernatant was filtered through a 0.45-mm membrane and loaded onto a 2.6×45 cm column of DEAE Bio-Gel A (Bio-Rad Laboratories). The column was washed with buffer for 30 min at 1 mL/min, then eluted with a 0 to 0.4 M NaCl gradient prepared in the PIPES buffer. Fractions were assayed for uronic acid using the *m*-hydroxybiphenyl method [5], and those giving a positive color reaction were combined, and concentrated to 30 to 40 mL. Ethanol was added (ca. 3 vol) to precipitate the NPS. The precipitated NPS was collected by centrifugation, dissolved in deionized water, dialyzed, and freeze-dried. Further purification was achieved by a second passage (100 mg batches) through a 1.6×45 cm DEAE Bio-Gel A column.

Chemical analysis.—The NPS samples were methanolyzed in methanolic 1 M HCl at 80°C for 18 h, per-*O*-trimethylsilyl (Me_3Si) deriivatives of the methyl glycosides were prepared and analyzed by gas–liquid chromatography (GLC) [6]. The GLC analysis was performed using a DB-1 column (0.25 mm × 30 m, 0.25 μm film) from J&W Scientific. Glycosyl linkages were determined by methylation analysis. The sample (100 to 500 μg) was dissolved in dimethyl sulfoxide (Me_2SO), butyllithium reagent (Aldrich) was added, followed by iodomethane as previously described [6]. In the case of oligosaccharides, the samples were pre-reduced with NaB^2H_4 in M NH_4OH, prior to methylation. The methylated sample was purified using a SepPak C18 cartridge [7], and the carboxymethyl groups of the uronosyl residues reduced with lithium triethylborodeuteride (Superdeuteride from Aldrich Chemical Co.) [6]. Partially methylated alditol acetates were then prepared [6] and analyzed by combined GLC–mass spectrometry (GLC–MS). Stereochemical configurations were determined by the preparation and GLC analysis of the per-*O*-Me_3Si (−)-2-butyl glycosides as previously described [8].

Smith degradation.—The NPS (10 mg) was oxidized in the dark in 50 mM $NaIO_4$ (4

mL) at 4°C for 4 days. The excess periodate was decomposed by the addition of 50 μL of ethylene glycol, and the solution was allowed to stand at room temperature for 2 h. The oxidized NPS was reduced with 50 mg of NaB^2H_4 at room temperature for 6 h. The excess NaB^2H_4 was decomposed by the dropwise addition of glacial acetic acid (HOAc). The resulting product was dialyzed against deionized water using 1000 molecular-weight-cutoff dialysis tubing for 2 days, and then freeze-dried. The final product was hydrolyzed with 0.5 M CF_3CO_2H (2 mL) at 60°C for 1 h and dried under a stream of N_2 with the addition of 5 drops of 2-propanol. The product was dissolved in 1 mL of deionized water and applied to a Bio-Gel P-2 column (Bio-Rad Laboratories) (1.2 × 150 cm). The column was eluted with H_2O, and 1.0 mL fractions were collected and assayed colorimetrically using the phenol–H_2SO_4 procedure [6]. Fractions yielding positive color reactions were collected and freeze-dried.

Partial acid hydrolysis of the NPS.—A 3 mg/mL solution of NPS in 2 M CF_3CO_2H (1 mL) was prepared and heated at 80°C for 40 min. The hydrolysate was dried with a stream of N_2, and the residual acid was removed by the addition of 2-propanol and drying again with N_2. The acidic oligosaccharides were dissolved in 1 mL of H_2O and applied to a 1 × 5 cm DEAE Sephadex A-25 column (in the $HCOO^-$ form). The column was washed with water to remove the neutral sugars, and then with 10% formic acid to remove the acidic carbohydrate. The acidic fraction was concentrated to dryness under reduced pressure, redissolved in H_2O and freeze-dried. The lyophilizate was then reduced with NaB^2H_4 in M NH_4OH (10 mg/mL), neutralized with glacial HOAc, and repeatedly dried under N_2 after the addition of 1:9 HOAc–MeOH (3 times), and MeOH (3 times).

Purification of NPS-derived oligosaccharides by HPAEC.—The reduced acidic oligosaccharides obtained from the partial acid hydrolysis of NPS were purified by using a CarboPac PA1 column (4 mm × 25 cm) from Dionex, and detected with a pulsed electrochemical detector equipped with a gold working electrode (also from Dionex). The column was eluted at 1 mL/min with a gradient of NaOAc in 100 mM NaOH as follows: 100 mM NaOH (0–5 min), 0–200 mM NaOAc (5–45 min), and 200–700 mM NaOAc (45–50 min). After each run the column was re-equilibrated with 100 mM NaOH for 15 min. Fractions were collected manually, neutralized with acetic acid, desalted with Dowex 50 (H^+ form), and freeze-dried.

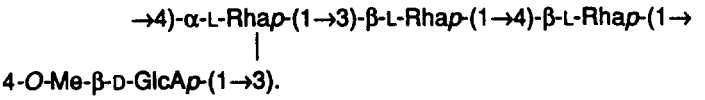
Electrospray-mass (ESMS) spectrometric analysis.—ESMS was performed with an API III Biomolecular Mass Analyzer (PE-SCIEX, Thornhill, Canada) interfaced to a MacIntosh IIfx data station. The mass spectrometer was operated in the positive-ion mode with an ion-spray voltage of 5 kV and an orifice potential of 35 V. Solutions (1 μg/μL) of carbohydrate in aq 30% MeOH were introduced into the instrument at 2 μL/min using a Harvard 22 syringe infusion pump. The mass-to-charge (m/z) range scanned was m/z 300–1200. The scans were collected and averaged.

Nuclear magnetic resonance spectroscopy.—^1H NMR spectroscopy was performed using a Bruker AM 500 instrument. The sample was exchanged several times in ^2H_2O, dissolved in ^2H_2O, and analyzed. Chemical shifts were measured relative to acetone (δ 2.224), which was added as an internal standard. ^{13}C NMR spectroscopy was performed using a Bruker AM 250 instrument. Again, the chemical shifts were measured relative to acetone (δ 31.07), which was added as an internal standard. The 2D NMR. experiments

were performed on a Bruker AMX 600 instrument at 37°C. The samples were prepared as described above. The HMQC [9] experiment was run in the phase-sensitive mode using the TPPI method [10], with a GARP [11] sequence for ^{13}C decoupling. Low power presaturation was applied to the residual HDO signal. The proton spectral width was 6024 Hz (10 ppm), and the carbon spectral width was 9055 Hz (60 ppm) with the carbon frequency set at 81.6 ppm.

3. Results

Analysis of the B. elkanii *NPS.*—Glycosyl composition analysis verified the previously reported results [1] and showed that this NPS consisted of rhamnose and 4-*O*-methylglucuronic acid in a ratio of 3:1. This composition is identical to that for the EPS from *B. elkanii*. The NPS was then subjected to methylation with carboxymethyl reduction using lithium triethylborodeuteride. The results indicated a 1:1:1 ratio of 3,4-linked, 3-linked, 4-linked rhamnosyl residues. The dideuterated PMAA derived from the terminal 4-*O*-methylglucuronosyl residue was also detected, but in a lower than expected proportion due to the fact that this residue is susceptible to elimination during the initial methylation procedure. These results for the NPS are consistent with the structure reported for the EPS [4]; namely,



The fact that the ^{1}H NMR spectrum of the NPS matched that of the EPS (data not shown) supports the conclusion that these two polysaccharides have the same structure.

Analysis of the intact B. japonicum *NPS.*—Composition analysis verified the previously published data [1], and showed that the NPS consists of 2-*O*-methyl-

Table 1
Glycosyl-linkage analysis of the Bio-Gel P2 fractions (F1–F4) produced by Smith degradation of the NPS [a]

Glycosyl linkage	NPS	F1	F2	F3	F4
Terminal Rha	nd [b]	4	2	nd	nd
2-linked Rha	nd	4	nd	nd	nd
3-linked Rha	16	32	36	34	45
4-linked Rha	23	9	12	30	12
2,4-linked Rha	20	23	2	nd	nd
2,3,4-linked Rha	3	2	19	nd	nd
Terminal Gal	nd	24	29	36	43
3-linked Gal	21	1	nd	nd	nd
Terminal 2-*O*-MeGlcA	17	nd	nd	nd	nd

[a] The values represent relative GLC peak area percents.
[b] nd, None detected.

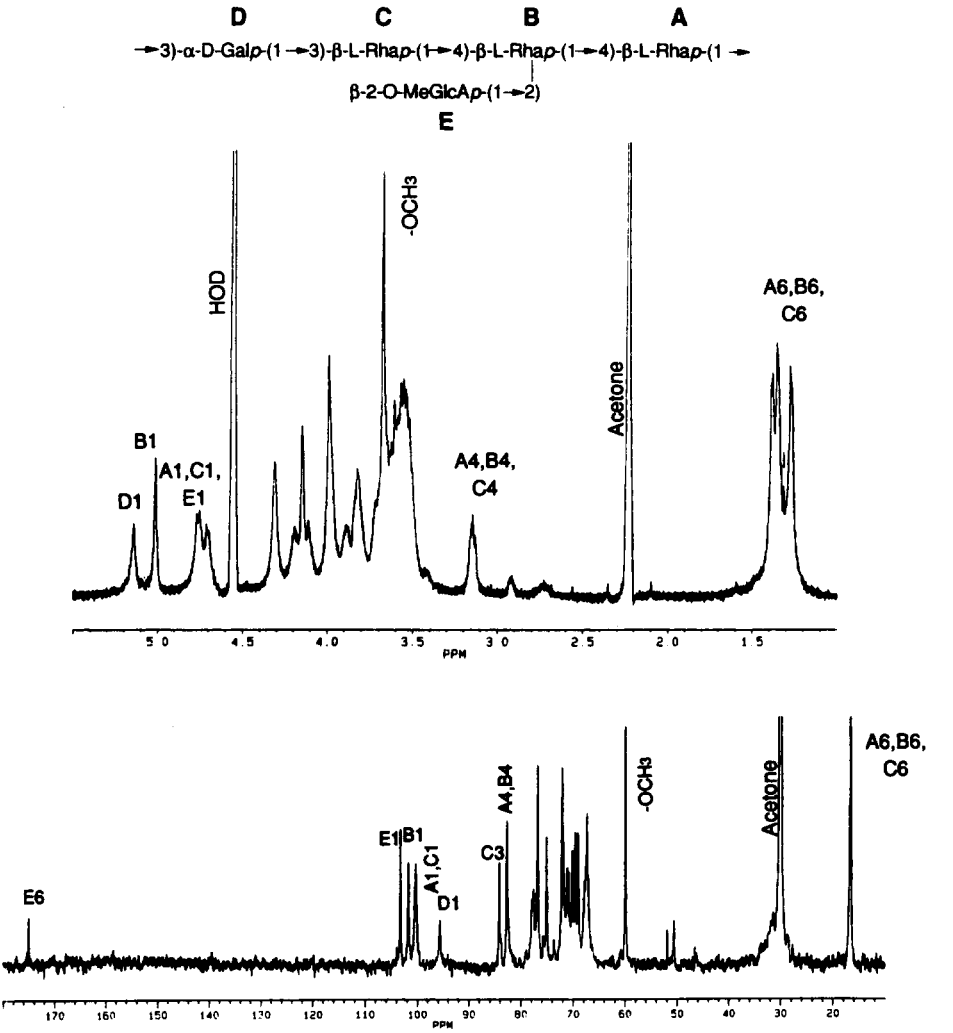


Fig. 1. ^{1}H (A) and ^{13}C (B) NMR spectra of the NPS produced by *B. japonicum*. Some of the proton and carbon resonances could be assigned as indicated. All chemical shifts were measured relative to acetone, which was added as an internal standard. There were no resonances in the "acetone" region of the spectrum.

glucuronic acid, galactose, and rhamnose in a 1:1:3 ratio. Methylation analysis (see Table 1) showed the presence of 3-linked galactose, 3-linked rhamnose, 4-linked rhamnose, 2,4-linked rhamnose, and terminal 2-*O*-methylglucuronic acid in a 1:1:1:1:1 ratio. Both ^{1}H and ^{13}C NMR analyses gave spectra that are consistent with these composition and linkage data (Fig. 1). The proton spectrum shows anomeric proton resonances at δ 5.15, 5.01, and several between δ 4.78 and 4.65. Also, the characteristic rhamnosyl methyl protons at δ 1.2–1.4 and the methoxyl protons at δ 3.62 are observed. In addition, the ^{13}C NMR spectrum also shows anomeric resonances at δ

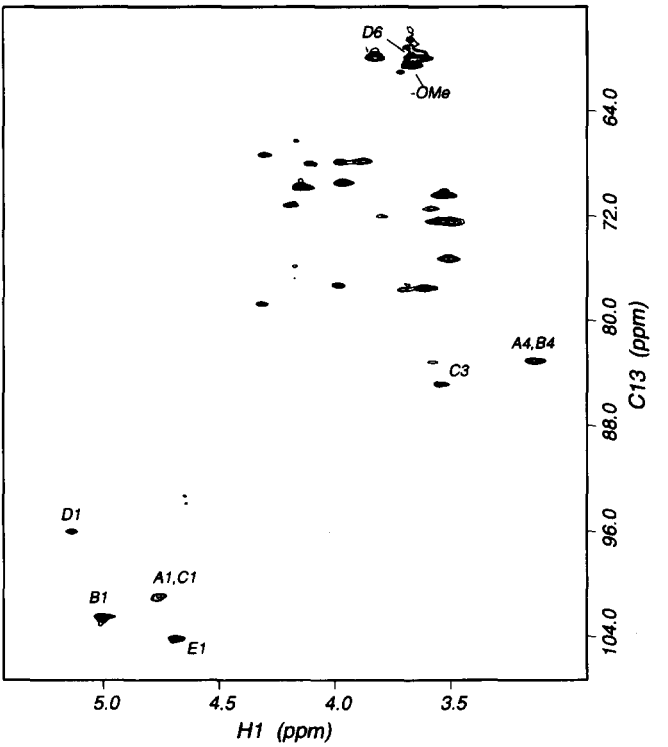


Fig. 2. A two-dimensional HMQC spectrum of *B. japonicum*. Various assignments are indicated and are described in the text.

103.0, 101.5, 100.1, and 95.4, the carboxyl carbonyl resonance of the uronosyl residue at δ 174.8, and the rhamnosyl methyl carbons at δ 16–17. The methoxyl and C-6 carbons of the 2-*O*-methylglucuronosyl and galactosyl residues, respectively, can be assigned to the resonance at δ 60. The chemical shifts and integration of the anomeric resonances showed that there are five different glycosyl residues in this NPS. Two of the anomeric carbon resonances were not resolved, i.e., those at $\delta \sim 100$. An HMQC experiment (Fig. 2) shows the following C–H couplings: δ_C 95.4–δ_H 5.15 ($J_{C,H}$ 169 Hz), δ_C 100–101–δ_H 4.74–4.77 ($J_{C,H}$ 159 Hz, integration of this region indicates two carbons), δ_C 101.5–δ_H 5.01 ($J_{C,H}$ 164 Hz), and δ_C 103.0–δ_H 4.71 (J_{CH} 166 Hz). The chemical shifts and $J_{C,H}$ values support the conclusion that the residue whose C-1–H-1 resonances are δ_C 95.4–δ_H 5.15 is α-linked, while the remaining four residues are β-linked. The narrow peak width of the H-1 resonance at δ 5.01 is indicative of a small $J_{1,2}$ coupling constant and, therefore, this resonance can be assigned to the H-1 of one of the three rhamnosyl residues. The broader resonance at δ 5.15 indicates a larger coupling constant and, therefore, can be assigned to H-1 of the galactosyl or 2-*O*-methylglucuronosyl residue. Analysis of the Smith degradation product (described below) showed that this latter resonance was due to the galactosyl residue. The resonances between δ 3.1–3.2 are likely due to the H-4 protons of the rhamnosyl residues since

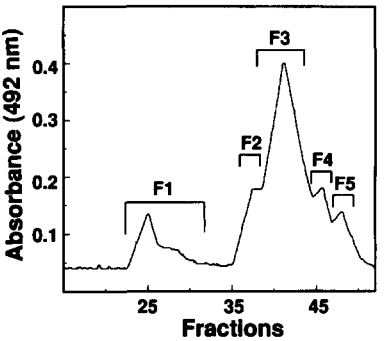


Fig. 3. A Bio-Gel P2 chromatography elution profile of the oligosaccharides derived from Smith degradation of *B. japonicum* NPS. The column had a bed volume of ~ 50 mL, 1-mL fractions were collected and portions (30 μL) were measured colorimetrically (492 nm) for sugars using the phenol–sulfuric acid reagent. The indicated peaks (F1–F5) were collected and analyzed as described in the text.

these protons are reported to resonate furthest upfield (i.e., δ 3.26) for 6-deoxy-β-D-mannosyl residues [12]. In addition, the HMQC experiment (Fig. 2) shows that these protons are coupled to carbons at δ 82, which can be assigned, based on published data [13], to the C-4 carbons of the 4- and 2,4-linked rhamnosyl residues. Similarly, the coupled C–H resonances at δ_C 84.8–δ_H 3.59 can be assigned to C-3–H-3 of the 3-linked rhamnosyl residue. These methylation and NMR results are consistent with the NPS having a five-sugar repeating unit consisting of one α-galactosyl, three β-rhamnosyl, and one 2-*O*-methyl-β-D-glucuronosyl residues. There was no indication of noncarbohydrate substituent groups, e.g., acetyl, pyruvyl, etc.

The stereochemical configurations were determined from GLC analysis of the per-*O*-Me_3Si (−)-2-butyl glycosides. The results showed that the NPS glycosyl residues consist of L-rhamnose and D-galactose. No standard was available for the 2-*O*-methyl-glucuronosyl residue.

From the results described above, two of the five sugars in the repeating oligosaccharides should be sensitive to Smith degradation, namely, the terminal 2-*O*-methyl-glucuronsyl and 4-linked rhamnosyl residues. Therefore, the NPS was Smith degraded as described in the Experimental section, and the products were purified by gel-filtration chromatography using Bio-Gel P-2. A column profile is shown in Fig. 3. Five fractions, F1–F5, were obtained, of which F3 was the major fraction comprising approximately 60% of the total carbohydrate. These fractions, except for F5, of which there was insufficient material, were further characterized. All of these fractions consisted of low molecular weight oligosaccharides indicating that the 4-linked rhamnosyl residue must reside in the backbone of the NPS repeating oligosaccharide.

Analysis of Fraction F3 produced by Smith degradation of the B. japonicum *NPS.*—Analysis of F3 by ESMS showed two major ions, m/z 562 and 584. These masses are consistent with the $[M + H]^+$ and $[M + Na]^+$ ions of an oligomer containing one galactosyl, and two rhamnosyl residues plus an additional component with a mass of 90 amu. This additional component is due to a deoxytetritol (presumably 4-deoxy-L-threitol) residue containing one deuterium atom, a fragment resulting from the Smith

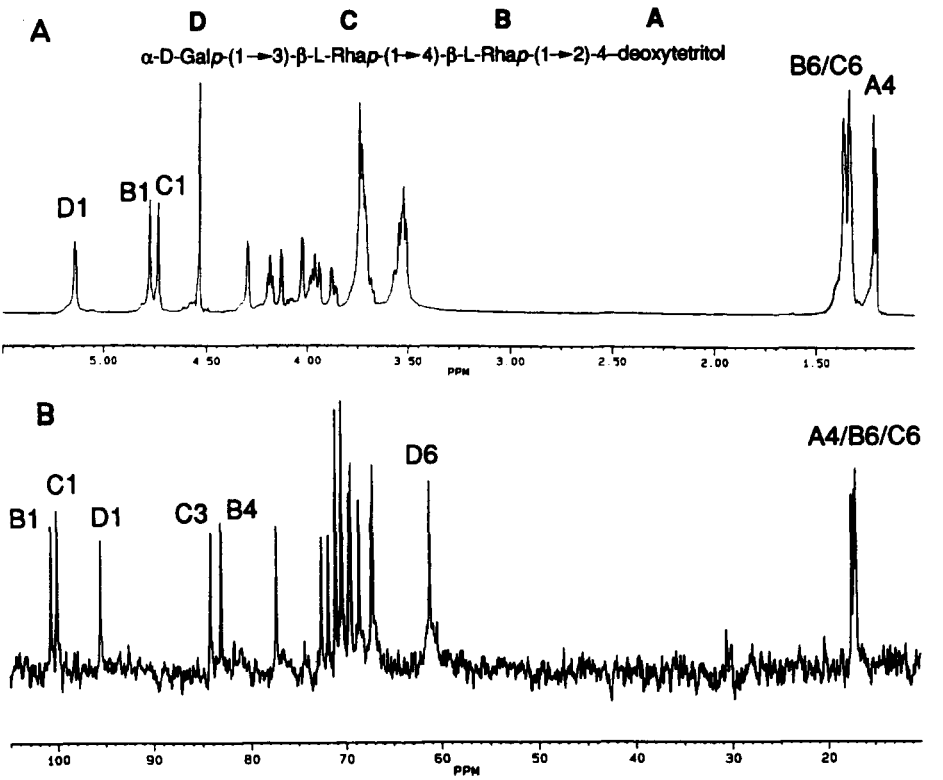


Fig. 4. ^{1}H (A) and ^{13}C (B) NMR spectra of fraction F3 obtained from Smith degradation of *B. japonicum* NPS. Assignments of the various resonances are as indicated.

degradation of the 4-linked rhamnosyl residue. Methylation analysis of F3 (Table 1) showed that it consists of terminal galactose, 3-linked rhamnose, and 4-linked rhamnose in a 1:1:1 ratio. The 4-linked rhamnosyl residue in F3 is due to removal, via Smith degradation, of the 2-*O*-methylglucuronsyl residue from O-2 of the 2,4-linked rhamnosyl residue in the NPS.

The F3 fraction was also analyzed by and ^{1}H and ^{13}C NMR spectroscopy; the spectra are shown in Fig. 4. Comparison of these spectra with those of the intact NPS (Fig. 1) reveals that F3 has three rather than five anomeric resonances due to Smith degradation of the 4-linked rhamnosyl and terminal 2-*O*-methylglucuronosyl residues in the NPS. Two of the three anomeric resonances in F3 are the same as those in the NPS spectra; namely those at δ_H 5.15 ($J_{1,2}$ 3.5 Hz) and δ_C 95.4 (J_{CH} 169 Hz), and δ_H 4.75 ($J_{1,2} < 2$ Hz) and δ_C 100.1 (J_{CH} 159 Hz). The former H-1–C-1 resonances can be assigned to the α-linked galactosyl residue due to their chemical shifts and $J_{1,2}$ and J_{CH} coupling constants, and the latter H-1–C-1 resonances to the 3-linked β-rhamnosyl residue because of the small $J_{1,2}$ and $J_{C,H}$ coupling constants and since this residue is present in both F3 and the intact NPS. The remaining H–C anomeric resonance at δ_H 4.78 ($J_{1,2} < 2$ Hz) and δ_C 100.7 ($J_{C,H}$ 164 Hz) can, therefore, be assigned to the

4-linked β-rhamnosyl residue that was derived by removal of terminal 2-*O*-methylglucuronic acid from O-2 of the 2,4-linked rhamnosyl residue in the intact NPS. The change in chemical shift of this anomeric resonance from δ_H 5.01 and δ_C 101.5 in the intact NPS to δ_H 4.78 and δ_C 100.7 in F3 is also due to the removal of the terminal 2-*O*-methylglucuronosyl residue. The chemical shifts (and $J_{C,H}$ values) of the two rhamnosyl residues in fraction F3 and in the intact NPS are consistent with β-linked [4], and not α-linked rhamnosyl residues whose H-1 resonances are reported to occur further downfield [14]. The two anomeric resonances present in the spectra of the intact NPS but missing in the spectra of fraction F3 are δ_H 4.71 and δ_C 103.0, and δ_H 4.77 and δ_C 100; the former set can be assigned to the β-terminal 2-*O*-methylglucuronosyl residue, and the latter set to the β-4-linked rhamnosyl residue, both of which are destroyed by Smith degradation.

Some of the remaining resonances in the F3 spectra can be assigned based on published data for 3- and 4-linked rhamnosyl residues [13]. The resonances at δ 84 and 83 can be assigned to the C-3 and C-4 carbons of these respective rhamnosyl residues in F3. The resonance at δ 61 can be assigned to C-6 of the terminal galactosyl residue, and the resonances at δ_C 17–20 and δ_H 1.2–1.5 can be assigned to the methyl C-6–H-6 atoms of the two rhamnosyl and one 4-deoxytetritol residues. These results, together with the methylation results of the intact NPS described above, support one of the two following structures for F3:

α-D-Gal-(1→3)-β-L-Rha-(1→4)-β-L-Rha-(1→2)-(4-deoxy)tetritol,

1

or

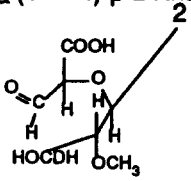
α-D-Gal-(1→4)-β-L-Rha-(1→3)-β-L-Rha-(1→2)-(4-deoxy)tetritol.

2

Analysis of Fraction F1.—Analysis of this fraction supported the possible structures for F3, i.e., **1** or **2**, just described, and also supported the above result that the terminal 2-*O*-methylglucuronosyl residue is located at O-2 of the branching 2,4-rhamnosyl residue. Methylation analysis of F1 (Table 1) showed that this fraction consists principally of terminal galactose, 2,4-linked rhamnose, and 3-linked rhamnose in a 1:1:1.3 ratio. Lesser amounts of terminal rhamnose, 2-linked rhamnose, 4-linked rhamnose, etc., are present and indicate that this fraction is somewhat heterogeneous (also indicated by the P-2 elution profile, Fig. 3), a result that may be due to partial acid hydrolysis of the NPS during the Smith degradation procedure. Analysis of F1 by ESMS (spectrum not shown) showed two principal ions at m/z 753 and 775, $[M + H]^+$ and $[M + Na]^+$, respectively. Both the methylation and the MS results are consistent with F1 having the same backbone structure of F3 with an additional component of 191 amu presumably linked to O-2 of the 2,4-linked rhamnosyl residue. This component is most

probably due to a fragment of the 2-*O*-methylglucuronosyl residue formed by oxidation of the C-2–C-3 bond and reduction of only one of the two resulting carbonyls by NaB^2H_4. NMR analysis of F1 supported the presence of such a fragment in that the resonance for the 2-*O*-methoxyl protons was still present, i.e., a sharp singlet at δ 3.51. In addition, the resonances at δ 5.15 for the galactosyl H-1 and at δ 4.76 for the 3-linked rhamnosyl H-1 (both of which are also observed in the spectrum of F3), as well as a resonance at δ 4.84, presumably due to H-1 of the 2,4-linked rhamnosyl residue, were observed. Possible structures of the major F1 component are the following:

α-D-Gal-(1→3)-β-L-Rha-(1→4)-β-L-Rha-(1→2)-(4-deoxy)tetritol



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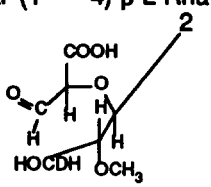
or

α-D-Gal-(1→4)-β-L-Rha-(1→3)-β-L-Rha-(1→2)-(4-deoxy)tetritol

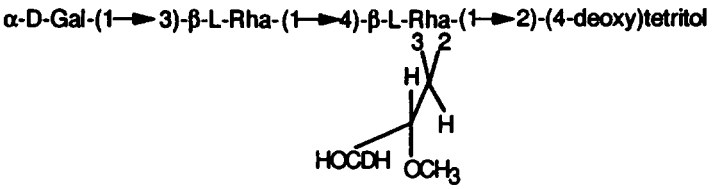


4

In these two structures, it is possible that the carbonyl be either the C-3 or C-4 carbon.

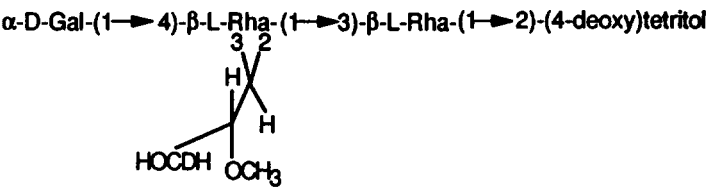
Analysis of Fraction F2.—Fraction F2, which was partially resolved from F3 by P-2 chromatography, was analyzed by ESMS and showed two major ions at m/z 649 and 671, $[M + H]^+$ and $[M + Na]^+$, respectively. The characteristic ions for F3, m/z 562 and 584, were also present in the spectrum of F2 showing that this fraction contains a significant amount of contaminating F3. Methylation analysis of F2 showed the presence of 3-linked rhamnose (36%), 4-linked rhamnose (12%), 2,3,4-linked rhamnose (19%), and terminal galactose (29%). All of the 4-linked rhamnosyl, and part of the 3-linked rhamnosyl and terminal galactosyl residues are due to the presence of contaminating F3. When this is taken into consideration by subtracting the 12% value from the values for the 3-linked rhamnosyl and terminal galactosyl residues, then the glycosyl linkages in F2 are likely to be: 3-linked rhamnose, 2,3,4-linked rhamnose, and terminal galactose in approximately a 1:1:1 molar ratio. These results indicate that F2 has the backbone

structure shown for F3 (described above), but with a component of 87 amu attached to the O-2 and O-3 positions of the 4-linked rhamnosyl residue. It is likely that this component is a fragment of the 2-*O*-methylglucuronosyl residue that results from the Smith degradation procedure. A candidate for this fragment, and mechanism by which it may be formed, is shown in Fig. 5. Thus, the possible structures for F2 are as follows:



5

or



6

These two possible structures are supported by NMR analysis of F2 which, while complicated by the presence of F3, clearly showed the presence of methoxyl protons, i.e., a sharp resonance at δ 3.58. Also, the odd value for $[M + H]^+$ of 649 indicates that this additional component contains a single deuterium atom. Additionally, ESMS analysis of F2 after permethylation resulted in $[M + H]^+$ and $[M + Na]^+$ ions of m/z 775 and 797, respectively, values that are consistent with structures **5** or **6**.

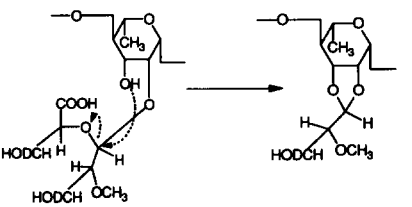


Fig. 5. A possible mechanism by which F2 may be derived during Smith degradation of the NPS. The carboxyl group proton of the oxidized/reduced uronosyl residue may assist this mechanism by protonating its glycosylic ring oxygen. Subsequently the 3-hydroxyl group of the 2,4-linked rhamnosyl residue attacks the uronosyl glycosidic carbon, releasing 2,3-dihydroxypropionic acid and forming a 3-hydroxy-2-methoxypropanal acetal of the 2,4-linked rhamnosyl residue involving the rhamnosyl O-2 and O-3.

Analysis of fraction F4.—ESMS of F4, which was partially resolved from F3 by P-2 chromatography, shows two major ions at m/z 416 and 438. These ions correspond to $[M + H]^+$ and $[M + Na]^+$ of a trimer composed of one galactose, one rhamnose, and one deoxytetritol. Glycosyl linkage analysis (Table 1) showed that F4 comprises 3-linked rhamnose and terminal galactose in a 1:1 ratio. The small amount of 4-linked rhamnose is most probably due to contaminating F3. NMR analysis of F4 shows two anomeric proton resonances at δ 5.17 ($J_{1,2}$ 3.5 Hz) and δ 4.79 ($J_{1,2} < 2$ Hz), which can be assigned to the terminal galactose and 3-linked rhamnose, respectively. These data indicate that F4 has the following structure:

α-D-Gal-(1→3)-β-L-Rha-(1→2)-(4-deoxy)tetritol.

7

This molecule may have been derived from a region of the NPS that contains the following structure:

→3)-α-D-Gal-(1→3)-β-L-Rha-(1→4)-β-L-Rha-(1→4)-β-L-Rha-(1→.

8

Smith degradation of such a structural region would result in structure **7**. In addition, structure **7** shows that the galactosyl residue in the NPS is linked to rhamnose at O-3. Therefore, of the structures described above for F3, F1, and F2; the correct ones are **1**, **3**, and **5**, respectively.

Partial acid hydrolysis of the NPS.—Results from the partial acid hydrolysis (2 M CF_3CO_2H at 80°C for 40 min) of the NPS support a repeating unit structure, in which the galactosyl residue is linked to O-3 of a rhamnosyl residue and not to O-2 of the branching 2,4-linked rhamnose. The acidic oligosaccharides produced by partial acid hydrolysis were reduced with NaB^2H_4 and purified by HPAEC (Fig. 6). Twelve fractions (1–12) were neutralized, desalted, and lyophilized. Analysis by ESMS showed

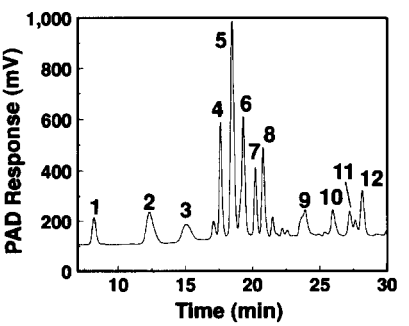


Fig. 6. Separation by HPAEC of the oligosaccharides generated by mild acid hydrolysis of NPS. The various indicated peaks were collected, desalted, and freeze-dried.

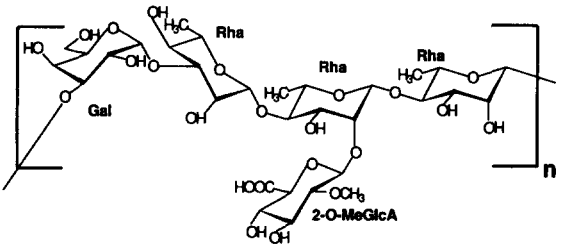


Fig. 7. The structure of the NPS repeating unit.

that the majority of fractions were mixtures of various oligosaccharides. However, ESMS of fraction 1 showed only two ions of m/z 672 and 694. These ions correspond to the $[M + Na]^+$ and $[M + 2Na^+]^+$ ions of a tetrasaccharide containing one 2-*O*-methylglucuronosyl, two rhamnosyl, and one rhamnitol residues. Sufficient amounts of this fraction were available for methylation analysis, which showed the presence of terminal rhamnose, terminal 2-*O*-methylglucuronic acid, and 2,4-linked rhamnose in a 1:1:1 molar ratio. The rhamnitol residue was lost during purification of the partially methylated alditol acetates due to its high volatility. These results, in conjunction with those for the Smith degradation products described above, support the following structure for this fraction:

β-L-Rha-(1→4)-[β-2-*O*-MeGlcA-(1→2)]-β-L-Rha-(1→4)-L-Rha-ol.

9

This structure confirms that the 3-linked rhamnosyl, and not the galactosyl residue, is linked to the branching 2,4-linked rhamnosyl residue.

In summary, the analyses of fractions F1–F3 show that NPS is comprised of the repeating oligosaccharide shown in Fig. 7. The small amount of F4 (ca. 10% of the total products of Smith degradation) indicates that a small percentage of the NPS lacks the terminal 2-*O*-methylglucuronosyl residue that is normally linked to O-2 of the 2,4-linked rhamnosyl residue.

4. Discussion

This report describes the structures of two polysaccharides produced by certain strains of *B. japonicum* and *B. elkanii* within soybean root nodules. The data presented show that the NPS and EPS produced by *B. elkanii* have the same structure, while the NPS from *B. japonicum* is very different in structure from that of the EPS produced in culture. The production of a novel NPS in nodules by *B. japonicum* suggests that genes responsible for this polysaccharide are induced by conditions, or factors, unique to the host root nodule. The production of a second polysaccharide by genes that are "cryptic"

under normal laboratory culture conditions is similar to results for *Rhizobium meliloti*, which has been found to produce a second EPS that is normally repressed [15–17].

While the *B. elkanii* and *B. japonicum* NPSs have different structures, both have in common a rhamnose-rich backbone with a single methylated glucuronosyl branching unit. Thus, it is possible that this general NPS structural motif, i.e., a rhamnose-rich backbone with a single branching methylated glucuronosyl residue, has an important function in nodule physiology. The symbiotic role of NPS is not known, although its presence is correlated with those serogroups that are dominant in the soil [1]. NPS-negative mutants are now available and are being employed in studies to elucidate the function of this polysaccharide.

Acknowledgements

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