JOURNAL OF BACTERIOLOGY, Nov. 1984, p. 510–516
0021-9193/84/110510-07$02.00/0

Vol. 160, No. 2

Bacteriophage-Induced Acidic Heteropolysaccharide Lyases That Convert the Acidic Heteropolysaccharides of *Rhizobium trifolii* into Oligosaccharide Units†

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Received 21 May 1984/Accepted 31 July 1984

Acidic heteropolysaccharide lyases from lysates of phages 4S and BY15 grown on *Rhizobium trifolii* 4S and *R. trifolii* 0403, respectively, were used to analyze the capsular and excreted extracellular acidic polysaccharides of *R. trifolii* 0403. The activities of the enzymes as measured by viscometry were enhanced by the addition of calcium. The oligosaccharide products obtained by depolymerase digestion of the polysaccharides isolated from cells grown on agar plates for 5 days were isolated by gel filtration and had a glycosyl composition of glucose, galactose, glucuronic acid, and α-linked 4-deoxy-L-*threo*-hex-4-enopyranosyluronic acid in an approximate molar ratio of 5:1:1:1. This latter component was identified by ^{1}H-nuclear magnetic resonance spectroscopy and confirmed by UV spectroscopy, ozonolysis, and its reactivity with thiobarbituric acid. The oligosaccharide had glucose as the reducing terminus, 4-deoxy-L-*threo*-hex-4-enopyranosyluronic acid as the enzymatically generated nonreducing terminus, and galactose as the terminus of the branched chain. The noncarbohydrate components of the oligosaccharides were acetate, ketal-linked pyruvate, and ether-linked 3-hydroxybutyrate. The mode of action of the enzymes was by β-elimination from a uronic acid residue with concomitant loss of the glycosyl component substituted at C-4. The 235-nm absorbing properties of the resulting terminal unsaturated sugar were used to study the kinetics of depolymerization of the capsular and excreted extracellular acidic polysaccharides, using the enzyme from phage BY15. The two substrates exhibited different kinetics of depolymerization, and the oligosaccharide products differed in the amount of noncarbohydrate substituents, indicating that the acidic capsular and excreted extracellular polysaccharides from 5-day-old cultures of *R. trifolii* 0403 were different.

Polysaccharide depolymerases have been found in virulent phage lysates of *Rhizobium* species (5, 10) and presumably function to degrade the capsular polysaccharide (CPS) of the bacterial host before the phage adsorbs to its surface receptor. The polysaccharide depolymerase isolated from the phage lysate of *R. trifolii* 4S by phage 4S could hydrolyze extracellular polysaccharides (EPS) of members of the family *Rhizobiaceae* if these polysaccharides contained glucuronic acid as a glycosyl component (10), and this enzyme was used for structural analysis of the EPS of *R. trifolii* 4S (3). This EPS contained glucose, glucuronic acid, acetate, and pyruvate in a molar ratio of 5:2:2:1. Using chemical methods only, Robertson et al. (18) reported that the acidic EPS of *R. trifolii* 0403 was a repeating, branched octasaccharide consisting of glucose, glucuronic acid, galactose, and pyruvate in a molar ratio of 5:2:1:2. In the present study, we use polysaccharide depolymerases from two phage lysates of *R. trifolii* to convert the CPS and EPS of *R. trifolii* 0403 into oligosaccharide units which contain essentially the same structural information as the parent polymers but are more amenable to structural analysis by physical methods which cannot be applied to the polymers. These studies show that the enzymes are acidic heteropolysaccharide lyases and that the CPS and EPS of *R. trifolii* 0403 differ in the amounts of noncarbohydrate substitutions, namely acetate, pyruvate, and the recently discovered (11) ether-linked 3-hydroxybutyrate.

MATERIALS AND METHODS

Microorganisms. *R. trifolii* 0403 was obtained from the Rothamsted Experimental Station, Harpenden, United Kingdom, and maintained on BIII agar medium (7) at 30°C. *R. trifolii* 4S (obtained from S. Higashi, Kagoshima University, Kagoshima, Japan) was maintained on mannitol-yeast extract agar medium (12). Bacteriophage BY15 was isolated from white clover rhizosphere soil after enrichment with *R. trifolii* 0403 as a host (7), and bacteriophage 4S is a virulent phage for *R. trifolii* 4S (10). The plaques formed by both phages on their hosts have large haloes which indicate the presence of soluble polysaccharide depolymerases (15).

Polysaccharide isolation. *R. trifolii* 0403 cells grown on BIII agar plates for 5 days at 30°C were suspended in sterile 10 mM phosphate-buffered saline (PBS), centrifuged (10,000 × *g*) for 15 min at 4°C, and rewashed with PBS and then with BIII broth medium. This procedure removed most of the CPS from the cells. The cells were then resuspended in BIII broth medium and inoculated on BIII agar plates (10-cm diameter) at 10^7 cells per plate. All procedures were performed by aseptic technique. The cells were incubated for 5 days at 30°C, harvested in PBS containing 1 mM $MgSO_4$ and centrifuged (3,000 × *g*) for 30 min at 4°C. The supernatant was recentrifuged (16,000 × *g*) for 60 min at 4°C to remove any remaining cells, and 2 volumes of cold ethanol were added to the supernatant to precipitate EPS. The soft cell pellet from the initial centrifugation step was washed twice in PBS containing 1 mM $MgSO_4$. To extract CPS, the cells

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were suspended in 10 mM phosphate buffer (pH 7.0) containing 1 mM $MgSO_4$ and 0.5 M NaCl, stirred vigorously for 1 h at room temperature, and then centrifuged (16,000 × *g*) for 60 min at 4°C. The extracted CPS in the supernatant was precipitated in 2 volumes of cold ethanol. The precipitated polysaccharides were pelleted by centrifugation (16,000 × *g*) for 30 min at 4°C, dissolved in and dialyzed against deionized, distilled water for 3 days at 10°C, and then lyophilized. Deacetylated CPS was prepared by treating a sample of the polysaccharide with 10 mM sodium hydroxide solution under nitrogen at room temperature for 3 h. The solution was then dialyzed against deionized, distilled water and lyophilized. Average yields of CPS and EPS were 2.5 and 6 mg (dry weight) per plate, respectively.

Isolation of polysaccharide depolymerases. One of the depolymerases (designated PD-I) was precipitated from the clarified phage lysate of strain 4S with $(NH_4)_2SO_4$ (70% of saturation) as previously described (10). A second enzyme (PD-II) was obtained from phage BY15 by lysing *R. trifolii* 0403 grown in shaken broth cultures of BIII medium (7) supplemented with 700 μM $CaCl_2$. Phage BY15 was added to the culture in early exponential phase at a multiplicity of infection of 0.1 and shaken gently (100 rpm) for 6 to 7 h at 30°C. PD-II was isolated from the phage lysate as previously described for PD-I (10). The proteins precipitated by $(NH_4)_2SO_4$ were dissolved in 10 mM Tris-hydrochloride buffer (pH 7.2) and dialyzed at 4°C against the same buffer overnight. Both PD-I and PD-II were then further purified by DEAE-cellulose (Whatman, Inc., Clifton, N.J.) column chromatography at 10°C. The column (1.5 by 30 cm) was equilibrated with 20 mM Tris-hydrochloride buffer (pH 7.2) and eluted with linear gradient of 0.05 to 0.8 M NaCl in 20 mM Tris-hydrochloride buffer, pH 7.2. The column effluent was monitored by UV absorbance at 280 nm with an ISCO UA-5 monitor (ISCO Co.). The enzyme activity of the fractions was measured by the decrease in viscosity of a solution of CPS from 5-day-old cultures by using an Ostwald-type viscometer (0.015 centistokes per s). The enzyme fractions were added to the polysaccharide solution (0.25 mg/ml in 25 mM Tris-hydrochloride buffer [pH 7.2] containing 1 mM $CaCl_2$ which was pre-equilibrated to 30°C. Viscosity was measured after the reaction mixture was incubated at 30°C for 30 min and compared to the viscosity of the polysaccharide solution alone (0.69 ± 0.04 centistokes). One unit of enzyme activity was defined as the amount of protein which caused a 50% decrease in relative viscosity after 30 min at 30°C. The active fractions from the ion exchange column were concentrated and desalted by ultrafiltration with an Amicon PM-10 membrane (Amicon Corp., Lexington, Mass.) at 4°C.

Characterization of depolymerase PD-II. The properties of PD-II activity were compared with those already established for PD-I (10). The molecular weight of PD-II was determined by gel permeation chromatography, using a 0.9-by-60-cm column of Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). The sample and molecular weight (MW) standards (aldolase, MW 158,000; catalase, MW 232,000; ferritin, MW 440,000; and thyroglobulin, MW 669,000 [Pharmacia]) were eluted in 100 mM Tris-hydrochloride buffer (pH 7.2) containing 0.5 M NaCl at 10°C. The elution profiles of the standards were obtained by UV monitoring at 280 nm and fractions containing depolymerase activity were detected by viscometry as described above.

The optimum temperature for depolymerase activity was determined by viscometry with the CPS as the substrate in 25 mM Tris-hydrochloride buffer containing 1 mM $CaCl_2$ at pH 7.2. The substrate solution was pre-equilibrated at the test temperature for 15 min, and 1 unit of enzyme was added. The viscosity of the substrate solution was measured before enzyme addition and after 30 min of incubation with the enzyme.

The pH optimum within the range of pH 5.0 to 7.8 was also measured by viscometry. The substrate solution was prepared in 25 mM Tris-malate buffer containing 2.5 mM $CaCl_2$. The reaction mixture was incubated at 37°C, and the viscosity was measured as described above.

The divalent cation requirement for depolymerase activity was studied by examining the effects of Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Hg^{2+} (a general inhibitor of thiol-containing enzymes), and EDTA. Each cation as the chloride salt at 1 mM or EDTA (0.1 mM) was mixed with the CPS solution in 25 mM Tris-hydrochloride buffer at pH 7.2. The mixture was preincubated for 15 min at 30°C, and then 1 unit of PD-II was added. The viscosity was measured after 30 min of incubation.

The relative rates of depolymerization of CPS and EPS by PD-II were examined by UV spectroscopy. Solutions of CPS and EPS (ca. 0.3 mg/ml) in 25 mM Tris-hydrochloride buffer (pH 7.2) containing 2 mM $CaCl_2$ were adjusted to the same concentration as determined by total carbohydrate assay (8). A 1-ml sample of each solution was transferred to separate matched quartz cuvettes, and 25 μl (0.63 U) of a solution of PD-II in the same buffer was added to each cuvette. The increase in absorbance (235 nm) with time was measured for each mixture with a Beckman DU spectrophotometer with Gilford update electronics.

Isolation of oligosaccharide fragments. The polysaccharide substrates (10 ml at 1.0 mg/ml of distilled water) were mixed with 10 ml of buffer (25 mM Tris-hydrochloride [pH 7.2] containing 2 mM $CaCl_2$), 200 μl of enzyme (0.5 U of PD-I per mg of substrate [dry weight] or 2 U of PD-II per mg of substrate [dry weight], and 10 μl of toluene. The reaction mixture (20.21-ml total) was incubated at 30°C for 20 h with gentle shaking. After incubation, the solution was concentrated by flash evaporation under reduced pressure at 40°C, applied to a Bio-Gel P10 column (1.5 by 80 cm; Bio-Rad Laboratories, Richmond, Calif.), and eluted with 20 mM Tris-hydrochloride buffer (pH 7.2) at room temperature. Carbohydrates in the collected fractions were determined by the phenol-H_2SO_4 reaction (8). The fractions containing oligosaccharides were pooled, concentrated by flash evaporation and desalted by gel filtration through Bio-Gel P2.

^{1}H-NMR analysis of oligosaccharides. For ^{1}H-nuclear magnetic resonance (NMR) analysis of oligosaccharides, samples (1 mg) were dissolved in 500 μl of deuterium oxide (99.7% D obtained from Aldrich Chem. Co.) and the samples were then lyophilized. The deuterium-exchanged samples were then dissolved in 300 μl of 99.97% deuterium oxide (Aldrich Chemical Co., Milwaukee, Wis.), and the spectra were recorded on a Bruker WM-250 instrument. The chemical shifts were measured relative to an external tetramethylsilane standard.

Ozonolysis of oligosaccharide from phage 4S-cleaved CPS. The oligosaccharide sample (1 mg) was dissolved in 100 μl of water, and the solution was cooled to 10°C. A slow, steady stream of ozone from a Welsbach automatic ozonator (Welsbach Corp.) was passed through the solution for 10 min. The excess ozone was removed by passing a rapid stream of nitrogen through the solution for 4 min. A 10-μl sample was withdrawn, and the UV spectrum was measured after dilution with water. UV spectra were recorded on a Varian Cary-219 spectrophotometer. The rest of the reaction mix-

ture was lyophilized, and the ^{1}H-NMR spectrum was measured after equilibration in D_2O.

Preparation of deuterium-labeled, carboxyl-reduced oligosaccharides. The carboxyl groups were methylated and then reduced with sodium borodeuteride. Oligosaccharide (0.5 mg) was dissolved in 200 μl of 0.05% anhydrous methanolic hydrogen chloride in a Teflon-lined, screw-capped vial. Trimethylorthoformate (100 μl) was added, and the solution allowed to stand at 45°C for 2 h and then evaporated to dryness under a stream of nitrogen at room temperature. The residue was dissolved in 75% aqueous ethanol (1.5 ml), and 2 mg of sodium borodeuteride (98% D; Aldrich Chemical Co.) was added. The mixture was allowed to stand at room temperature for 8 h, and the excess borohydride was destroyed by drop-wise addition of 20% acetic acid in methanol until effervescence ceased. The solution was then evaporated to dryness.

Glycosyl composition analysis. The deuterium-labeled, carboxyl-reduced oligosaccharide was hydrolyzed with trifluoroacetic acid and then reduced with sodium borohydride as described previously (2). The hydrolysate was peracetylated with acetic anhydride and pyridine (200 μl of a 1:1 mixture) at 100°C for 30 min, and the mixture was evaporated to dryness, redissolved in chloroform, and analyzed by gas liquid chromatography (GLC) and GLC-mass spectrometry (MS). GLC analyses were performed on a Varian 3700 instrument equipped with a flame ionization detector and a Hewlett-Packard 3390A digital integrator. GLC-MS analyses were carried out on a Hewlett-Packard 5985 mass spectrometer interfaced with a Hewlett-Packard 5840 gas chromatograph. Glucuronic acid was determined by selective ion monitoring for the fragments with *m/z* 219 (from deuterium-labeled glucitol hexa-acetate) and *m/z* 217 (from both the labeled and unlabeled glucitol hexa-acetate), followed by reconstruction of the specific ion chromatograms for these two fragments. The ratio of glucose to glucuronic acid was then calculated from the areas of the two peaks. All GLC analyses were done on glass-packed columns (2 m by 0.3 cm) of 10% SP 2330 on 100 to 120 mesh chromosorb WAW.

Terminal residue analysis. Oligosaccharide (50 μg) was reduced with sodium borohydride, and the product was hydrolyzed and peracetylated as before. The mixture was then analyzed by GLC-MS. The thiobarbituric acid test (17) was used to confirm the presence of the terminal unsaturated sugar. Methylation analysis was also performed (6, 9).

RESULTS

Properties of the polysaccharide depolymerases. Isolated PD-I and PD-II had specific activities of 70 and 250 U/mg of protein, respectively, using the CPS of *R. trifolii* 0403 grown for 5 days on BIII plates as substrate. The average yield of PD-I and PD-II activity was ca. 1 U/ml of broth culture lysate. PD-II had an approximate MW of 540,000 (determined by gel filtration under nondenaturing conditions), an optimum temperature of 40°C, and an optimum pH of 5.4 when measured by viscometry. However, when the products of depolymerization were examined by gel filtration, depolymerization to the lowest-MW carbohydrate product was optimal at pH 7.2 at 30°C. PD-I had a similar optimum temperature (32 to 40°C) but a higher optimal pH (6.8) when measured by viscometry and a lower approximate MW (440,000). PD-II activity was stimulated approximately fivefold by 1 mM Ca^{2+}, twofold by 1 mM Zn^{2+}, and slightly by Mn^{2+}, but it was inhibited by 1 mM Mg^{2+}, 1 mM Hg^{2+}, and 100 μM EDTA. The inhibition by Mg^{2+} and EDTA was reversed by the addition of 1 mM Ca^{2+} (data not shown).

Isolation of oligosaccharides. The oligosaccharide products of depolymerase digestion of the CPS and EPS of *R. trifolii* 0403 were isolated by gel filtration chromatography through Bio-Gel P10. The elution profile of the digestion products from the CPS when treated by PD-I and PD-II are shown in Fig. 1A and B, respectively. The lowest-MW products made by both enzymes eluted in a symmetrical peak and had the same elution volumes. A larger intermediate eluting between the voided substrate and the smallest product was found in the cleavage by PD-II when lower levels of enzyme were used (data not shown). Similar elution profiles were obtained with the EPS isolated from cells of the same culture. Yields of the lowest-MW products from 100 mg of substrate were 70 to 75 mg with PD-I and 30 to 35 mg with PD-II. Unlike PD-I, PD-II was unable to depolymerize the polysaccharides completely (ca. 35% depolymerization) unless they were first deacetylated (Fig. 1C).

Oligosaccharide composition. Gas chromatographic and combined GLC-MS analysis of the oligosaccharides indicated that they contained galactose, glucose, and glucuronic acid in the approximate molar proportion of 1:5:1. The ^{1}H-NMR spectra of the oligomers displayed a downfield multiplet at δ 5.8 to 5.9 (Fig. 2A) which sharpened into a well-defined doublet of doublets after mild base hydrolysis (Fig. 2B). The chemical shift of this proton indicated that an unsaturated sugar was also present. This sugar was identified as 4-deoxy-L-*threo*-hex-4-enopyranosyluronic acid (Fig. 3), which was the nonreducing terminus of the oligosaccharide formed in an enzymatic elimination reaction from a glucuronic acid residue. The presence of this unsaturated sugar was confirmed by the thiobarbituric acid assay, UV spectroscopy of the oligomers (Fig. 4), and NMR (Fig. 5) and UV analysis of the ozonolysis product of the EPS oligomer.

Methylation analysis indicated that the only terminal reducing sugar was glucose and the only other nonreducing terminal sugar (at the end of the branch) was galactose. 2,3-Di-*O*-methyl galactitol was identified by methylation analysis of the deuterium-labeled, carboxyl-reduced oligosaccharide using GLC-MS. 2,3,4,6-Tetra-*O*-methyl galactitol was also detected. This latter component increased and the former component disappeared when the oligosaccharides were depyruvylated before methylation. This indicated that the terminal galactose residue was pyruvylated.

The ^{1}H-NMR spectra also showed the presence of ketal-linked pyruvate, acetate and 3-hydroxybutyrate in the oligosaccharides (Fig. 2A). These noncarbohydrate substitutions were quantitated by comparing the integrals of the signals for the respective groups to the area of the signal from the C-4 proton of the single unsaturated terminal sugar in each oligosaccharide. PD-I and PD-II depolymerized the EPS into oligosaccharide products which contained the same amounts of pyruvate and 3-hydroxybutyrate substitutions (Table 1). However, the amounts of acetate, pyruvate, and 3-hydroxybutyrate were higher in oligosaccharides of CPS obtained by PD-II than in those obtained by PD-I (Table 1). Oligosaccharides from the PD-I depolymerization of CPS and EPS had the same levels of pyruvate and 3-hydroxybutyrate, but the acetate levels were higher in the CPS. In contrast, PD-II depolymerization of CPS resulted in oligosaccharides with higher levels of all three substitutions than did oligosaccharides derived from EPS.

The UV kinetic study of the comparative rates of depolymerization of CPS and EPS by PD-II indicated that the EPS was cleaved at a much faster initial rate than the CPS (Fig. 6). A linear regression analysis during the first 4 h indicated

that the rates of depolymerization of EPS and CPS by PD-II had slopes of 0.116 and 0.080 (linear correlation coefficients of 0.999 and 0.997), respectively. Thus, PD-II depolymerized EPS 1.45-fold faster than CPS from the same culture (different at the 99.9% confidence level). The profile of the EPS reaction showed an initial rapid accumulation of product, followed by a gradual decrease in rate. In contrast, the CPS reaction showed a slower initial rate with a much less pronounced slower phase (Fig. 6). Eventually, the two rates became similar (Fig. 6).

DISCUSSION

Polysaccharide depolymerases from phage lysates of rhizobia have been used to cleave rhizobial CPS (3, 5, 10). This study identifies the mode of cleavage of two such enzymes as being uronic acid lyases (β-eliminases), which remove the C-5 proton and the C-4 alkoxy group of 4-substituted uronic acids to give an unsaturated linkage between C-4 and C-5. This agrees with previous observations that the enzymes do not cleave polysaccharides which lack uronic acids (10). The increased activity in the presence of calcium ions is also characteristic of enzymes which are known to function by a similar mechanism (13).

Both enzymes, PD-I and PD-II, could convert the CPS and EPS of *R. trifolii* 0403 to oligosaccharide fragments. Only PD-I could completely depolymerize these polysaccharide substrates. PD-II could completely depolymerize only deacetylated substrate, suggesting that its action might be regulated by acetate substituents. Similar inhibitory effects of acetate substituents on depolymerization of rhizobial polysaccharides by certain fungal enzymes have been reported (4). PD-I and PD-II also differed in optimal pH, aggregated MW, metal requirements, and location of substrate cleavage. The absence of intermediate molecular weight products in the gel filtration profile of the PD-I products indicates that this enzyme cleaves the substrate at the site closest to the terminus of the chain. In contrast, the profile for PD-II shows the presence of intermediate MW products, indicating random endo-cleavage.

Based on a combination of GLC, GLC-MS, and UV and ^{1}H-NMR spectroscopy, the glycosyl composition of the oligosaccharide products obtained by depolymerization of the CPS and EPS of 5-day-old cultures of *R. trifolii* 0403 was determined. The oligosaccharides contained glucose, galactose, glucuronic acid, and 4-deoxy-L-*threo*-hex-4-enopyranosyluronic acid in the approximate molar ratio of 5:1:1:1. Evidence for purity of the smallest oligosaccharide product is based on the elution of a symmetrical peak in gel filtration within the included volume and a methylation analysis of the terminal sugars which indicate that cleavage occurred at only one site in the repeating oligosaccharide. Additional evidence for purity of the oligosaccharides is provided by the ^{1}H-NMR spectra of the deacetylated samples. Single spin systems were found for the readily assignable groups, indicating that they are in a pure molecular environment and not attached randomly within the oligosaccharide as would occur if the products were generated by random, indiscriminate glycosidic cleavage. The β-eliminase activity was established by the unequivocal identification of the unsaturated residue at the newly created nonreducing terminus. A characteristic feature of all of the ^{1}H-NMR spectra of the oligosaccharides was a multiplet which appeared considerably downfield of all the other signals and had a chemical shift

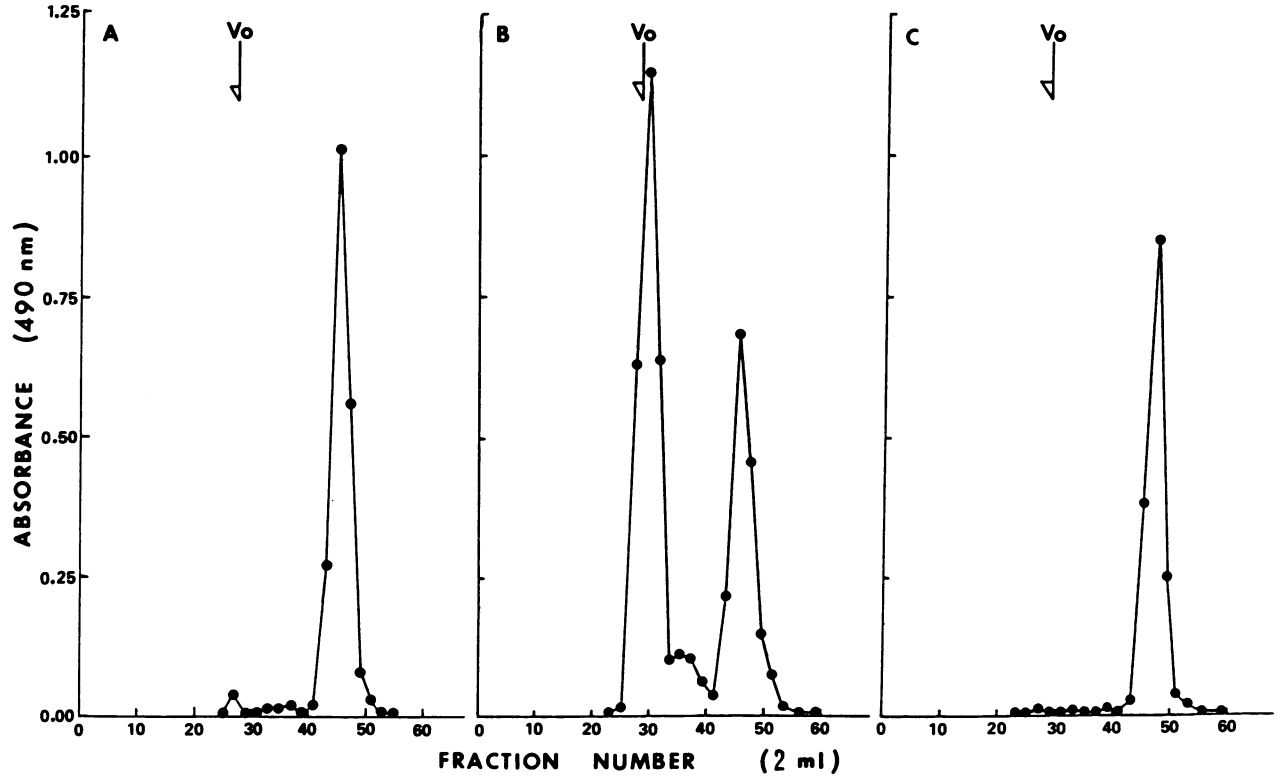

FIG. 1. Elution profile of carbohydrates separated by gel filtration chromatography (Bio-Gel P10) of CPS from *R. trifolii* 0403 after enzymatic depolymerization. (A) PD-I enzyme, (B) PD-II enzyme, (C) PD-II with deacetylated CPS as the substrate. V_o, the voided volume, is indicated by an arrow. Total included volume was ca. 141 ml.

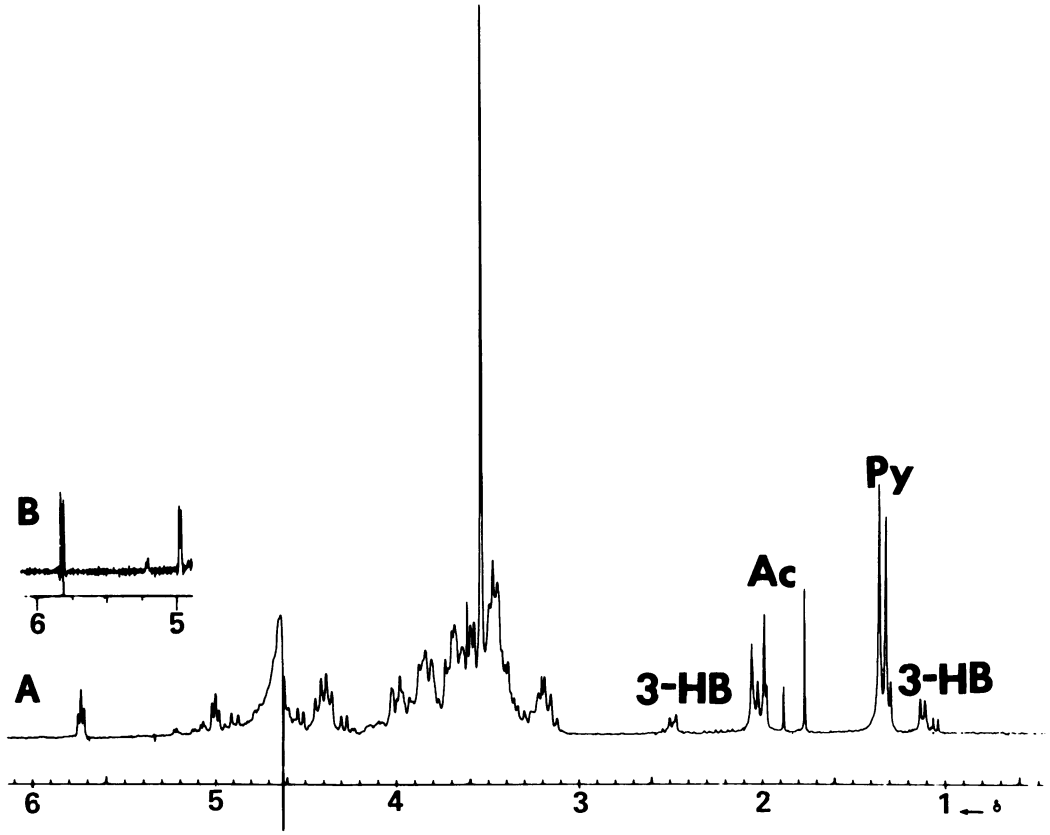

FIG. 2. (A) ^{1}H-NMR spectrum of the oligosaccharides produced by depolymerization of CPS from *R. trifolii* 0403 with PD-II enzyme showing acetate (Ac), pyruvate (Py), and the methylene (δ 2.45 to 2.55) and methyl (δ 1.0 to 1.20) groups of 3-hydroxybutanoate (3HB) substituents. The multiplet at δ 5.80 was assigned to the C-4 proton of the terminal unsaturated sugar. (B) Partial ^{1}H-NMR spectrum of the same oligosaccharide after deacetylation. Note that the signal at δ 5.80 is now a well-defined doublet on which is superimposed a smaller long-range coupling. The doublet at δ 4.98 was assigned to the anomeric proton of the unsaturated sugar.

of δ 5.8 to 5.9 (Fig. 2A). On mild base catalyzed deacetylation of the oligosaccharides, the signal was not shifted, indicating that it could not possibly be due to a proton attached to a carbon atom bearing an acetate group. The signal did, however, become resolved into a well-defined doublet of doublets (J = 3.65 Hz and 0.85 Hz; Fig. 2B). The larger splitting was assigned to vicinal coupling, and the smaller splitting was assigned to long-ranged coupling. Although the long-range effect was not noted earlier, a similar signal was reported in the ^{1}H-NMR spectrum of oligosaccharides obtained from depolymerization of the *R. trifolii* 4S acidic extracellular polysaccharide (3). These authors assigned the signal to H-5 of a glucuronic acid residue since it disappeared on carboxyl reduction. However, the chemical shift of H-5 glucuronic acid derivatives is typically no more than δ 4.4 in the α-anomer and 4.1 in the β-anomer (13). In addition, the coupling constant between H-5 and H-4 in glucuronic acid is about 10 Hz (13), as is expected for a pair of vicinal protons which are antiperiplanar to each other. There is also no appreciable long-range coupling involving H-5 of glucuronic acid since H-5 and H-3 are in a 1,3-diaxial orientation.

The assignment of the signal at δ 5.82 to that of a proton attached to the β-carbon of an α,β-unsaturated carboxylic acid was more likely than the original assignment (3). This was confirmed by measuring the UV spectra of the oligomers. All of the spectra showed absorption maxima between 230 and 240 nm. This is characteristic of 4-deoxy-hex-4-enopyranosyluronic acids (13, 14) and definitive proof of carbon-carbon unsaturation. The oligomers also gave positive thiobarbituric acid test reactions, indicating that a 2-keto-3-deoxy sugar acid is either present or can be formed under the test conditions (17). The ^{1}H-NMR data also agreed with that published for similar systems (16). The terminal nonreducing sugar at the site of enzyme cleavage was therefore assigned to an α-linked 4-deoxy-L-*threo*-hex-4-enopyranosyluronic uronic residue (Fig. 3). The *threo*- configuration was inferred from the fact that the coupling constant between H-4 and H-3 was 3.65 Hz, and the long-ranged coupling exhibited by H-4 meant that it was capable

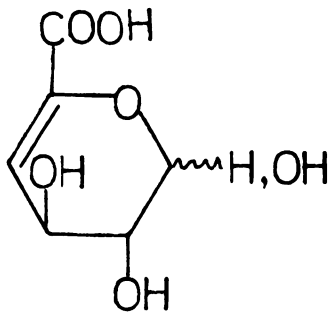

FIG. 3. 4-deoxy-L-*threo*-hex-4-enopyranosyluronic acid.

of existing in a "W" configuration with H-2. This is not possible in the alternative *erythro-* configuration. Since the C-2 and C-3 of the original glucuronic acid molecule was not involved in the elimination, the configuration of the resulting sugar is expected to be *threo-*. The anomeric configuration was designated α- because the signal at δ 4.98 (J = 2.8 Hz, Fig. 2B) also showed signs of long-ranged coupling. This is not observed in glucose or galactose, and so this signal was assigned to the unsaturated sugar. This assignment was reinforced by the earlier observation (16) that long-range coupling involving H-4 of these unsaturated systems is limited to the α-anomer and that the anomeric proton is also coupled to H-3. The connectivity between these two signals and the presence of an unsaturated linkage was confirmed by cleaving the double bond by ozonolysis at 10°C for 15 min and then examining the products by ^{1}H-NMR spectroscopy and by UV spectroscopy. Neither the NMR signals assigned to H-4 and H-1 of the unsaturated acid (Fig. 5B), nor the absorption maximum at 234 nm were present after this treatment.

The ^{1}H-NMR spectra of the oligosaccharides obtained from the CPS and EPS of *R. trifolii* 0403 also indicated the presence of acetate esters, carboxyethylidene groups (ketal-linked pyruvate), and ether-linked 3-hydroxybutanoic acid (Fig. 2B). The molar proportions of these noncarbohydrate substitutions in the oligosaccharide would differ based on which substrate (CPS or EPS) and which enzyme (PD-I or PD-II) were used.

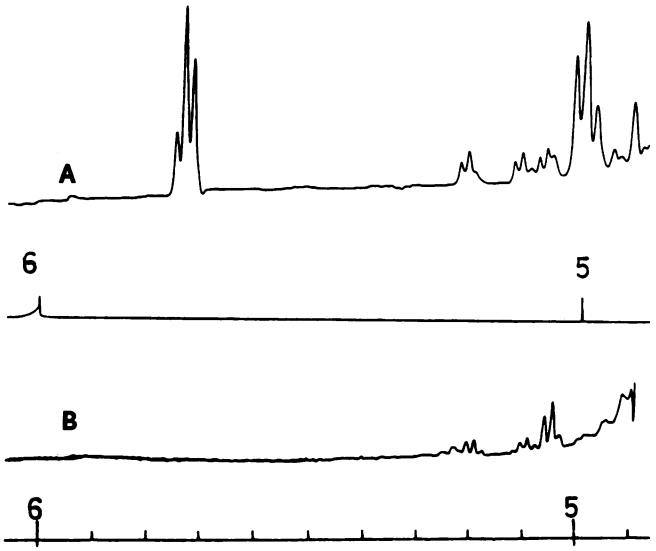

FIG. 5. (A) Partial ^{1}H-NMR spectrum of the oligosaccharides obtained by degrading the CPS of *R. trifolii* 0403 with PD-I. Note the C-1 and C-4 protons of the terminal unsaturated sugar at δ 4.98 and 5.80, respectively. (B) The same region of the spectrum after mild ozonolysis. Note the disappearance of the two signals.

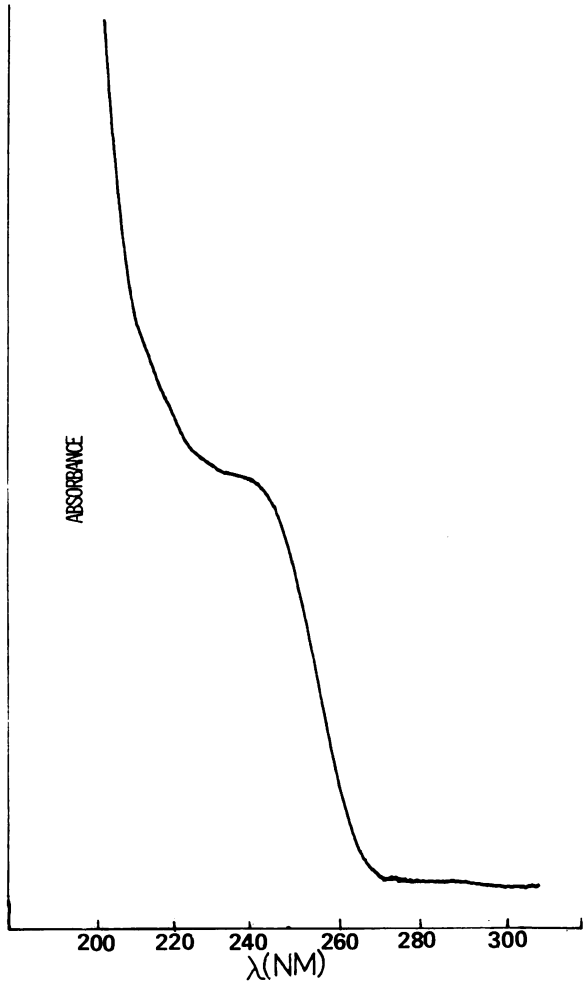

FIG. 4. A UV spectrum of the oligosaccharides obtained by depolymerization of the CPS of *R. trifolii* 0403 with enzyme PD-II.

The UV-absorbing terminal unsaturated sugar in the products provided a convenient and efficient method to follow the conversion of substrate to products. The kinetic study indicated that the CPS was depolymerized much more slowly than the EPS when the two substrates were incubated with PD-II under identical conditions (Fig. 6). This is consistent with the fact that the CPS contains appreciably higher levels of acetate than the EPS in the same culture, and PD-II enzyme is inhibited by acetate groups in the substrate. The differences in kinetics of depolymerization between EPS and CPS of *R. trifolii* 0403 and the quantitative difference in noncarbohydrate composition of the products of depolymerization (Table 1) indicate that the CPS and the EPS obtained as described here are not identical. Perhaps these chemical differences contribute to the greater affinity of CPS for the cell during growth and centrifugation.

In a separate study (1), we have demonstrated biological activities of these oligosaccharides associated with binding to clover lectin, stimulation of root hair infection, and

TABLE 1. Degree of noncarbohydrate substitution in oligosaccharides obtained by depolymerization of CPS and EPS from 5-day-old cultures of *R. trifolii* 0403

Substrate	Enzyme	Molar proportions of components per oligosaccharide[a]		
		Acetate	Pyruvate	3-Hydroxybutyrate
CPS	PD-I	1.70 ± 0.04	1.74 ± 0.04	0.41 ± 0.05
EPS	PD-I	1.45 ± 0.04	1.80 ± 0.04	0.40 ± 0.05
CPS	PD-II	2.03 ± 0.04	2.71 ± 0.04	0.67 ± 0.05
EPS	PD-II	1.33 ± 0.04	1.85 ± 0.04	0.36 ± 0.05

[a] Values were obtained from ^{1}H-NMR spectroscopy by comparing the integrals for the various groups with that of the single H-4 proton of the unsaturated terminal sugar in each oligosaccharide.

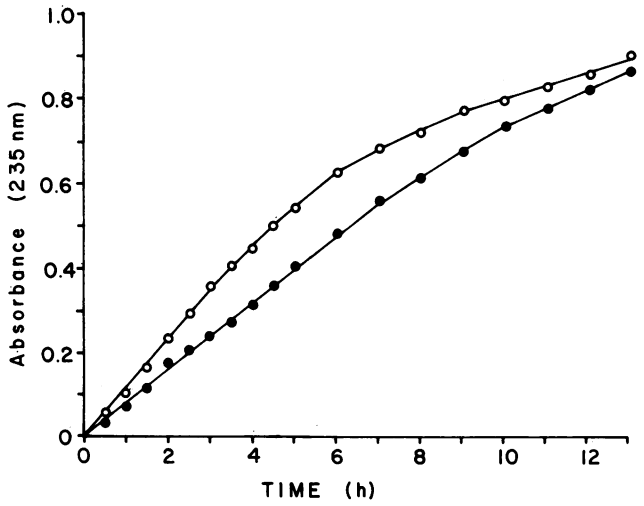

FIG. 6. UV kinetic study of the relative rates of depolymerization of the CPS and EPS from *R. trifolii* 0403 under identical conditions and at the same concentration. PD-II is the enzyme.

compositional changes with culture age. These oligomers will be useful in establishing the complete structures of the acidic heteropolysaccharides produced by *R. trifolii* 0403 and their possible role in the *Rhizobium*-clover symbiosis.

ACKNOWLEDGMENTS

This work was supported by grants PCM 80-21906 and PCM 84-09279 from the National Science Foundation, competitive grant 82-CRCR-1-1040 from the U.S. Department of Agriculture, and the Michigan Agricultural Experiment Station Project 1314H.

We thank Harold Sadoff, Rosetta Reusch, Walter Esselman, Charles Sweeley, Klass Hallenga, Charles Suelter, Gerasimos Karabatsos, Estelle Hrabak, Alicia Gardiol, and Dave Gerhold for their helpful suggestions.

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