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Influence of acetyl substituent on oligoglucuronans separation by anion exchange chromatography

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Abstract

The *S. meliloti* M5N1CS mutant strain produces during fermentation a polyglucuronan and oligoglucuronans β-(1 → 4) linked and partially acetylated. The oligosaccharides formed were the result of polysaccharide degradation by a glucuronan lyase. The oligoglucuronan fraction is composed of a mixture of molecules which are very heterogeneous with respect to both, the degree of polymerization and the degree of substitution by acetate. The different oligomers can be separated by chromatography on a DEAE anion-exchange column. In this work, it is shown that the degree of acetyl substitution influences the association between the anionic oligosaccharide and DEAE-Sepharose.

Keywords: *Sinorhizobium meliloti*; Oligosaccharide; Substituent; Chromatography

1. Introduction

Under specific conditions, *Sinorhizobium meliloti* strains produce an exopolysaccharide: the succinoglycan, and oligosaccharides which generally have an octosaccharide repeating-unit as an intermediate for the synthesis of the extracellular polysaccharide (Amemura et al., 1983). These oligosuccinoglycans play a role in the plant-bacteria symbiosis (Battisti et al., 1992). The *S. meliloti* M5N1CS mutant strain (NCIMB 40472) (Courtois et al., 1992; Heyraud et al., 1993) deficient in the production of succinoglycan and oligosuccinoglycans is able to infect alfalfa (Gonzales et al., 1996). The mutant strain produces during fermentation a β-(1 → 4) glucuronan, partially acetylated on the C2 and/or the C3 position (Heyraud et al., 1993; Courtois et al., 1993). High molecular weight glucuronans ($1.5 \times 10^4 <$ Mw $< 3 \times 10^5$) (Dantas et al., 1994a), and low molecular weight osidic components: cyclic β-(1 → 2)-D-glucans and partially acetylated oligoglucuronans containing a 4-5 unsatured residue, at the non-reducing terminal unit, were detected in the fermentation medium (Michaud et al., 1995). These oligomers formed, by action of a glucuronan lyase on the polymer (Michaud et al., 1997), constitute a mixture of oligoglucuronans with a wide variability in the degree of polymerization (d.p.) and the degree of substitution (d.s.).

In order to identify the anionic oligosaccharides produced by enzymic degradation of the polymer, preparative quantities were necessary. For this reason the potential of anion exchange chromatography for separating different oligosaccharides was tested. In this paper, we report the influence of acetate residues present on oligoglucuronans, on their purification by anion exchange chromatography.

2. Materials and methods

2.1. Fermentation

The *S. meliloti* M5N1CS strain (NCIMB 40472) was cultivated in a 2 L fermentor (from Setric) containing 1.5 L of *Rhizobium* complete (RC) medium (Courtois et al., 1983) supplemented with sucrose (1% w/v) (RCS). The inoculum was 150 mL of a *S. meliloti* M5N1CS culture in RCS medium first incubated 20 h on a rotary shaker (100 rev min^{-1}) at 30°C, the cell density in the inoculum was 1.8×10^9 CFU mL^{-1}.

The pH in the fermentor was maintained at 7.2 by

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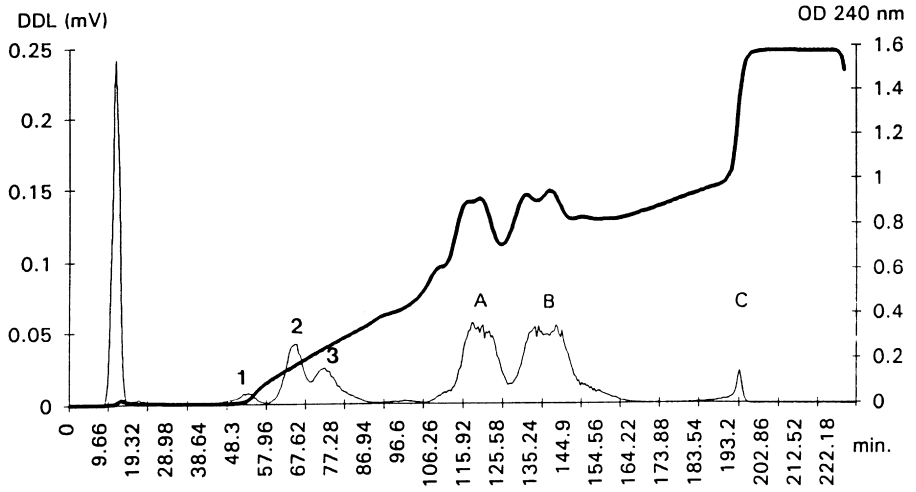

Fig. 1. Fractionation on a DEAE-Sepharose column (10.5 × 1 cm) eluted by AcOH/$AcONH_4$ (from 5×10^{-2} to 2 M) of oligoglucuronans obtained by fermentation of the *S. meliloti* M5N1CS strain; detection with an UV detector at 240 nm (—) and with a light scattering detector (—).

addition of KOH (2 M) and the pO_2 was first stabilized at 80% (using a mass flow meter) during the exponential growth phase (20 h), and then at 50% for 71 h. The temperature was maintained at 30°C.

2.2. EPS isolation and purification

After 91 h of fermentation, the medium was centrifuged (34,000 g for 30 min) and the supernatant containing the polysaccharides and oligosaccharides was collected. Partial purifications were performed by successive ultrafiltrations: the high molecular weight (HMW) EPS fraction was concentrated by ultrafiltration on a 100,000 cut off NMWCO membrane from Sartorius. The medium molecular weight (MMW) EPS fraction was extracted from the previous retentate by ultrafiltration on a 20,000 membrane, the low molecular weight (LMW) EPS fraction contained in the previous retentate fraction was then purified by ultrafiltration on a 5,000 NMWCO membrane, the retentate was diluted with one volume of distilled water and purified by ultrafiltration with the same membrane as previously, this step was repeated six times, the LMW EPS fraction (between 20,000 and 5,000 daltons) was dried by lyophilization.

2.3. Chromatography of the LMW fraction on DEAE-sepharose

500 l of the LMW fraction (5 g L^{-1}) in AcOH/$AcONH_4$ (5×10^{-2} M, pH = 4.8) were applied to a column (10.5 × 1 cm) of DEAE-Sepharose CL6B equilibrated with AcOH/$AcONH_4$ (5×10^{-2} M, pH = 4.8), the flow rate was 0.5 mL min^{-1}. The eluent was first 12.5 mL of AcOH/$AcONH_4$ (5×10^{-2} M), then 75 mL of a linear gradient of AcOH/$AcONH_4$ from 5×10^{-2} to 1 M, at last, the elution was completed with 12.5 mL of the 2 M buffer. The column was connected to an ultraviolet detector (240 nm) and an evaporative light scattering detector (from Alltech).

The collected fractions were pooled, desalted by chromatography on a Sephadex G-10 (Mw 700) column (1.6 × 50 cm) and dried as previously.

2.4. Fractionation of oligosaccharides on a Bio-Gel P6 column

The oligoglucuronans were fractionated by gel permeation chromatography on a Biogel P6 (Biorad) (1,000 < Mw < 6,000) column (100 × 2.5 cm) eluted by $NaNO_3$ (50 mM) at a flow rate of 94 mL h^{-1}. The detection system consisted of an Iota refractometer. Fractions collected with an autosampler corresponding to each separate peak were gathered, desalted by gel permeation chromatography on a HW 40F/50F column (50 × 2.5 cm) from Interchim and dried as previously.

2.5. NMR studies

^1H NMR analyses were performed at 85°C with an AC-300 Bruker Fourier transform spectrometer according conditions described previously (Dantas et al., 1994b).

2.6. Deacetylation of oligoglucuronans

The LMW fraction was completely deacetylated after incubation for 5.5 h at 30°C at pH 10 (with NaOH 1M). The deacetylated oligoglucuronan solution was desalted by chromatography on a Sephadex G-10 column and dried as previously.

3. Results and discussion

3.1. Fractionation and characterization of the LMW fraction

The *S. meliloti* M5N1CS was cultivated during 91 h at 30°C in a 2 L fermentor containing RCS medium, then, the

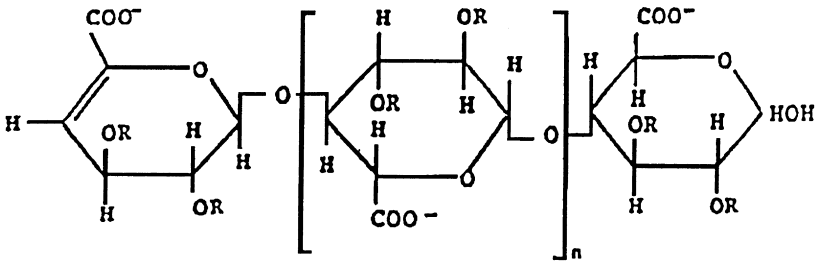

Fig. 2. Schematic representation of glucoronan containing a 4,5-unsaturared glucuronic unit partially acetylated; R = H or CO–CH_3.

bacterial suspension was centrifuged. The supernatant was collected and ultrafiltered successively through 100,000, 20,000, and 5,000 NMWCO membranes. In order to study only LMW polymers, the 5,000 retentate (5,000 < Mw < 20,000) was collected, purified by ultrafiltration and dried; the production was 0.2 g L^{-1} (± 15%) while the whole EPS produced under the conditions used was 2.8 g L^{-1}(± 15%).

The different components contained in 5 mg of LMW EPS dissolved in AcOH/$AcONH_4$ (5.10^{-2} M, pH = 4.8) (0.5 mL) were separated by chromatography on a DEAE anion exchange column. Six fractions (denoted 1, 2, 3, A, B and C) detected with a light scattering detector were fractionated (Fig. 1); absorbance at 240 nm detected for A and B fractions led us to suppose the presence of 4-5 unsatured residues (Fig. 2) in the two fractions.

The six fractions were collected, desalted and dried prior to characterization by ^{1}H NMR.

^{1}H NMR spectra of the fractions denoted 1, 2 and 3 revealed the presence of signals corresponding only to protons of cyclic glucans (data not shown); this result agree with the absence of absorbance at 240 nm of the three fractions.

^{1}H NMR spectra of the complete A and B fractions (data not shown) were similar to spectra obtained from oligoglucuronans. They revealed the presence of doublets at 5.65 ppm characteristic of a H-4 in an unsaturated residue and signals in the 2 ppm region characteristic of protons from *O*-acetyl groups, these results confirmed A and B fractions contained oligoglucuronans. ^{1}H NMR spectra of the C fraction was characteristic of LMW glucuronans; the absence of detection at 240 nm of the C fraction was because of the absorbance at 240 nm of AcOH/$AcONH_4$ (2 M) used as eluent for the chromatography on the DEAE column.

3.2. Determination of the degree of polymerization of oligoglucuronans from A, B and C fractions

In order to estimate the d.p. of the oligomers present in A, B and C fractions, samples were analyzed by size-exclusion chromatography on a Bio-Gel P6 column. The elution profils obtained with the oligoglucuronans from the A and B fractions (Fig. 3) were compared to standards obtained with oligoglucuronates prepared by enzymic hydrolysis of the polymers (Dantas et al., 1994b) (data not shown). The d.p. of oligoglucuronans was confirmed after chromatography on a DEAE column (Fig. 4) of the deacetylated fractions and comparison with standards. The presence of d.p. 1 and d.p. 2 molecules in the deacetylated fraction was the result of degradation (β-elimination) of higher

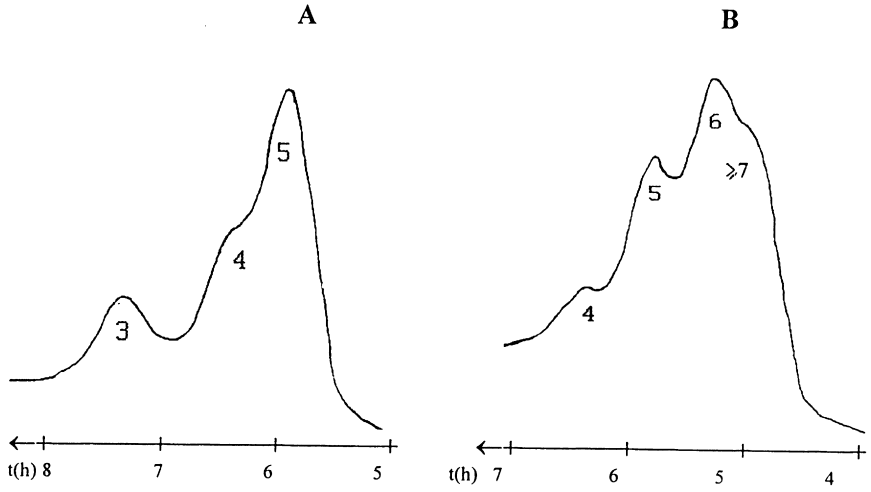

Fig. 3. Fractionation by size exclusion chromatography on a Bio-Gel-P6 column (100 × 2.5 cm) eluted by $NaNO_3$ (50 mM) of acetylated oligoglucuronan fractions A and B obtained by chromatography on an anion exchange column of oligoglucuronans produced by the *S. meliloti* M5N1CS strain. The flow rate was 94 mL h^{-1}, the detector was an Iota refractometer. The degree of polymerization is indicated by the numbers under the peaks.

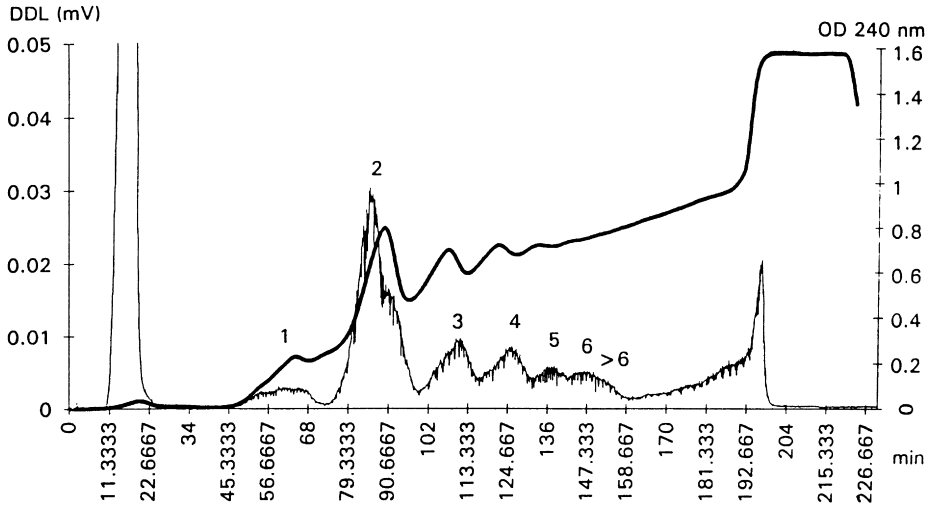

Fig. 4. Elution profile on a DEAE-Sepharose column (10.5 × 1 cm) eluted by AcOH/$AcONH_4$ (from 5×10^{-2} to 2 M) of deacetylated oligoglucuronans from the fraction A; detection with an UV detector at 240 nm (—) and with a light scattering detector (—) (the number above each peak corresponds to the degree of polymerization of the oligomer).

oligoglucuronans. We determined that the concentration of these small components increased during NaOH treatment. Molecules in the C fraction were not separated on the Biogel P6 column (data not shown), this result confirmed the C fraction was composed of LMW glucuronans with d.p. > 10.

We conclued that the A fraction, eluted first after chromatography on the DEAE column contained oligoglucuronans absorbing at 240 nm, the majority of them were d.p. 3 to 5 molecules (noted A3, A4, A5); the concentration of d.p. 5 was predominant in the A fraction. As the previous fraction, the B fraction contained oligoglucuronans absorbing at 240 nm, molecules with d.p. ranging from 4 to 7 were predominant (noted B4, B5, B6, B7); oligoglucuronans with d.p**.** 7 were outnumbered in the B fraction. The significance of these results were that oligoglucuronans containing the same number of glucuronic residues were present in fractions eluted separately after chromatography on DEAE column.

3.3. Identification of oligoglucuronans in A and B fractions by ^1H NMR studies

Oligosaccharides present in the A and B fractions contained an unsaturated residue at the non-reducing terminal unit and are partially acetylated. The degree of substitution was determined from integration of the resonances in the downfield, upfield and acetyl region (Courtois et al., 1994). The oligosaccharides contained in the fraction A, eluted first after chromatography on a DEAE column, were more acetylated than the oligosaccharides contained in the fraction B (Table 1). The molar proportion of the different species of residues (2-*O*-acetyl; 3-*O*-acetyl; 2,3-di-*O*-acetyl; and unacetylated) was determined (Table 1). These results indicate that acetate residues influence the fixation of the glucuronan on the DEAE Sepharose, but we were not certain if the fixation is influenced by the localisation of the subsituent at the C2 or C3 position.

In order to confirm the oligoglucuronan composition in A and B fractions, ^1H NMR studies were performed on the oligoglucuronans purified by size exclusion chromatography on Bio-Gel P6. ^1H NMR spectra on A3, A4 (Fig. 5a), A5, B4 (Fig. 5b) and B5 fractions were obtained. By comparing the integral of the H-1 on the reducing end (doublets at 5.1 and 4.5 ppm respectively) with the integral of the H-1 signal of the unacetylated and acetylated residues (4.4 and 4.3 ppm) (Dantas et al., 1994b), the polymerization degree of the oligomers present in the A3, A4, A5, B4 and B5, which were determined by size exclusion chromatography (SEC), was confirmed.

The degree of acetyl substitution determined by comparison of the total H1 resonances from oligoglucuronan (4.3–5.1 ppm) to protons in the acetyl region (1.8–2.1 ppm) was 22%, 38% and 50%.respectively for A3, A4 and A5. The d.s. for B4 and B5 was 10% and 12.5%. respectively. These results indicated that oligoglucuronans with the same d.p. but presenting different d.s. can be separated by chromatography on an anion exchange column. The more acetylated oligoglucuronans were retained less on the DEAE sepharose

Table 1
1: Substitution degree and molar ratio of different species of residues (2-O-Ac-GlcpA, 3-O-Ac-GlcpA, 2,3-O-Ac-GlcpA and unacetylated) of oligoglucuronans fractionated by DEAE anion-exchange column chromatography.

	Peak A	Peak B	Peak C
Average substitution degree	38%	24%	17%
2-*O*-acetylated	6.5%	3.5%	4.5%
3-*O*-acetylated	12%	9%	6.5%
2,3-di-*O*-acetylated	15.5%	9%	3%
Unacetylated	65.5%	79%	86%

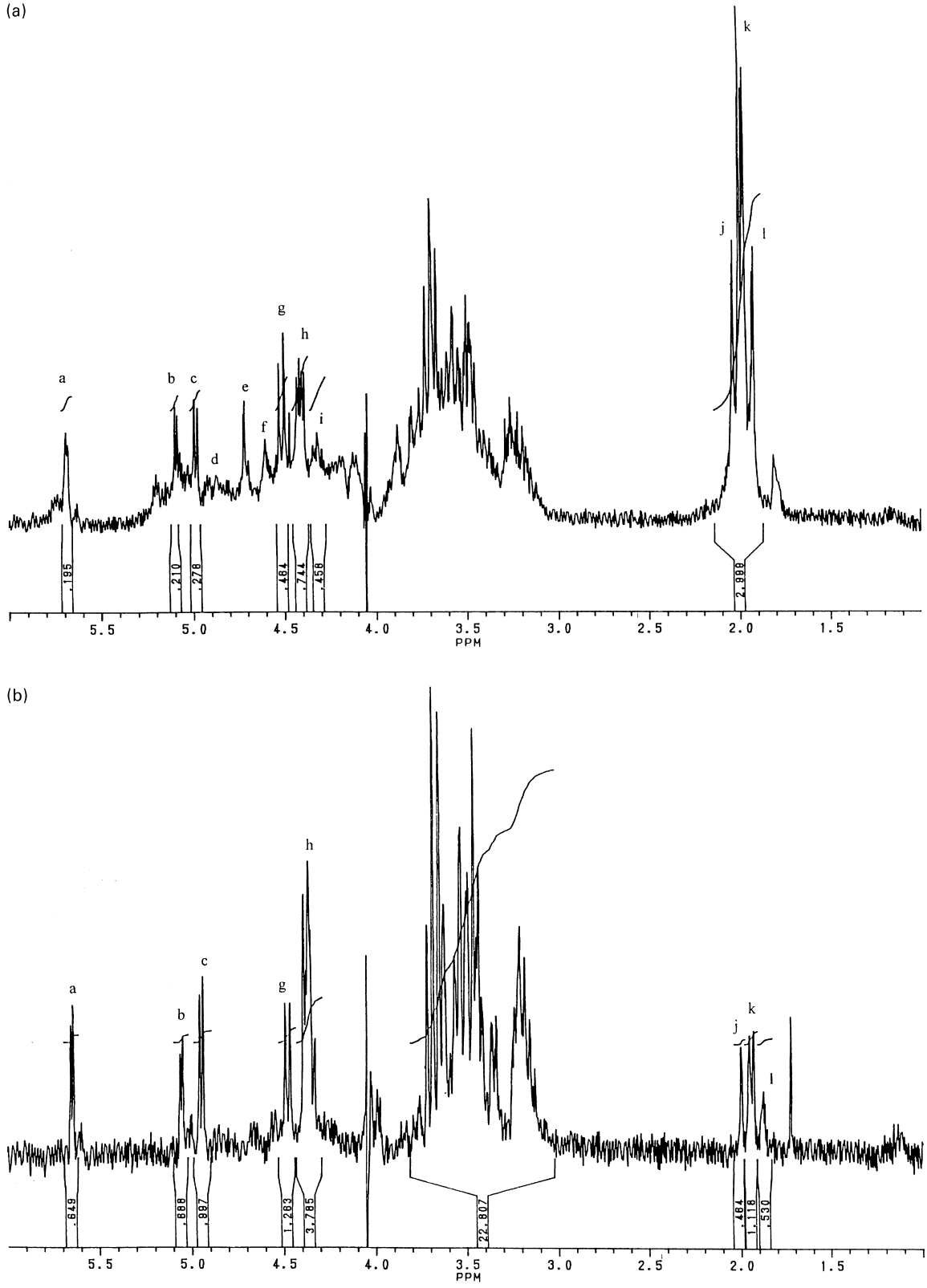

Fig. 5. ^{1}H NMR spectra of acetylated oligoglucuronans A4 (A) and B4 (B) in D_2O (at 300 MHz; *T*, 85°C). (a) H-4 of the unsaturated non-reducing terminus; (b) H-1 of the reducing end unit; (c) H-1 of the unsaturated non-reducing teminus; (d) H-3 of O-acetylated residues; (e) H-1 + H-2 of 2,3-di-O-acetylated residues; (f) H-1 + H-2 of 2-O-acetylated residues; (g) H-1 of the reducing terminus; (h) H-1 of the unacetylated residues; (i) H-1 of unacetylated residues before or after acetylated residues; (j) protons of the acetyl group at C-2 in the 2-O-acetylated residues; (k) protons of the acetyl group at C-3 in the 3-O-acetylated residues; (l) protons of the acetyl group at C-2 in the 2,3 di-O-acetylated residues and at C-3 in the 2,3 di-O-acetylated residues.

anion exchange column than the less acetylated. This result was confirmed by separation of deacetylated oligoglucuronans on the DEAE sepharose column (Fig. 4).

These results indicate that acetyl residues influence the association of oligoglucuronans to DEAE sepharose. It was reported that acetyl groups influence the association of xanthan to plant polysaccharides (Sutherland, 1995). Biological properties were detected for low molecular weight succinoglycans which restored symbiotic properties to *exoA* and exoH *S. meliloti* mutants (Urzainki and Walker, 1992), low molecular weight EPS containing acidic substituants may interact with basic amino groups on proteins (Battisti et al., 1992) and function as specific signals on specific plant receptor; however, heterologous acidic exopolysaccharides are sufficient to enable nodule development with *Rhizobium sp*. strain NGR234 (Gray et al., 1991). The purification of oligoglucuronans with different degree of acetylation will be useful in testing the influence of acetyl groups on oligosaccharides in plant infection by *S. meliloti* exo mutants.

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