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Physicochemical studies of extracellular polysaccharides of *Erwinia chrysanthemi* spp

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Abstract

The chemical and NMR analysis of the extracellular polysaccharides (EPSs) of several *Erwinia chrysanthemi* spp gave clear indications of two different families each showing similar structural characteristics. Two EPSs from streptomycin-resistant strains of *E. chrysanthemi* Ech6 and Ech9, phytopathogen of potatoes, as well as the EPS from the strain SR80, a phytopathogen of maize, have been isolated and purified. Initial structural aspects of these polysaccharides are reflected in the 600 MHz 1D ^{1}H NMR spectra and the high-pH anion-exchange chromatography with pulsed amperometric detection analysis of the complete and partial acid hydrolyzates, which are presented for comparison. The EPS from the strain SR80 is shown to be similar to that from Ech9, and those of the streptomycin-resistant strains are similar to the EPSs of the parent bacteria. Hydrodynamic examinations showed different viscosities and weight average root mean radius (R_w), but there was a linear relationship of these values and their weight average molecular weight (M_w). Within each family, the proposed flexible coil conformations were supported by physical measurements and the stiffness of the coils was greater for the pyruvated molecules in the EPS from Ech6 family. Like many other examples of similar polysaccharides, their pseudoplasticity was most evident in the EPS of higher M_w and R_w, but did not seem to be related to the stiffness parameters. The molecular weights of the polysaccharides range from 0.3 to 2.1×10^6 and their hydrodynamic properties are those of polydisperse, polyanionic biopolymers with pseudoplastic, non-thixotropic flow characteristics in aqueous solutions. © 2003 Elsevier Science Ltd. All rights reserved.

Keywords: Polysaccharide; *Erwinia chrysanthemi*; Hydrodynamics; Streptomycin-resistant

1. Introduction

Erwinia chrysanthemi are gram-negative bacterial phytopathogens (Chatterjee & Starr, 1980; Dickey, 1979; Dickey, Claflin, & Zumoff, 1987; Dickey, Zumoff, & Uyemoto, 1984) many of which produce an extracellular polysaccharide (EPS) when grown on solid media. The structures of the EPSs produced by *E. chrysanthemi* pv. zeae SR260 (Gray, Brand, Koerner, & Montgomery, 1993; Gray, Koerner, & Montgomery, 1995), a strain pathogenic to corn, and *E. chrysanthemi* strains Ech1 and Ech9 (Yang, Gray, & Montgomery, 1996), strains pathogenic to potato, are similar, but different from the EPS produced by *E. chrysanthemi* Ech6 (Yang, Gray, & Montgomery, 1994), another strain pathogenic to potato. Evidence is also presented here showing that the EPS produced by the potato pathogen, *E. chrysanthemi* SR80, is similar to EPS of the strain Ech9.

Two streptomycin-resistant strains (Ech6S+ and Ech9Sm6) from respective parent strains *E. chrysanthemi* Ech6 and Ech9 have been isolated and found to produce more copious amounts of EPS (Yang, Brand, Gray, & Montgomery, 2001a), but shown in the present study to have very similar structures and properties to those from their parent strains.

2. Methodology

2.1. Analytical and general methods

Methods used for monosaccharide analysis by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and for 600 MHz ^{1}H NMR spectroscopy have been described previously (Yang et al., 1994).

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Nomenclature

EPS	extracellular polysaccharide;
EPS6	EPS from Ech6, etc.;
HPAEC-PAD	high-pH anion-exchange chromatography with pulsed amperometric detection;
TFA	trifluoroacetic acid;
LS	light scattering;
MALS	multi-angle light scattering;
SEC	size exclusive chromatography;
RI	refractive index;
dn/dc	specific refractive index increment;
M	molecular weight;
M_w	weight average molecular weight;
M_n	number average molecular weight;
R_w	weight average root mean square radius;
RMS	root mean square radius;
η	viscosity;
η_{sp}	specific viscosity;
η_{sp}/c	reduced viscosity;
η_r	relative viscosity;
η_0	viscosity at zero shear rate;
$[\eta]$	intrinsic viscosity;
I	ionic strength

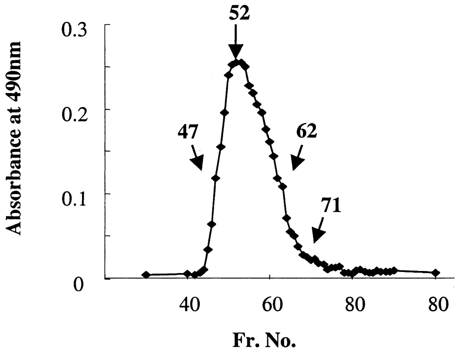
2.2. Production and purification of EPSs

The *E. chrysanthemi* strains were grown on a modified Scott's medium supplemented with 1% glucose and solidified with 1.5% Difco agar (Difco Laboratories, Detroit, USA) as described earlier (Gray et al., 1993; Gray, Linder, Brand, & Mildenhall, 1986). One strain (Ech9Sm6) was also grown in a liquid culture medium as described elsewhere (Yang, Brand, & Montgomery, 2001b).

The yields of the EPS from agar plates were 1.7 g/l EPS from Ech6S + (EPS6S +), 0.34 g/l EPS from Ech9 (EPS9), 0.85 g/l EPS from Ech9Sm6 (EPS9Sm6) and 0.04 g/l EPS from SR80 (EPS80). In liquid culture, EPS9Sm6 yielded 9.4 g/l.

Crude polysaccharide in phosphate buffered saline (PBS) or 0.86 M NaCl was fractionally precipitated by the dropwise addition of two volumes of ethanol and the polysaccharide fractions were recovered, after standing overnight to ensure complete settling, by low-speed centrifugation.

The resulting precipitate was dissolved and dialyzed extensively against distilled water and freeze-dried. For some experiments, the final precipitate was further purified either by low-pressure size exclusive chromatography (SEC) (1.5 × 98 cm, ToyoPearl HW65F, TosoHaas, Montgomeryville, PA) or anion-exchange chromatography (2.5 × 10 cm, ToyoPearl DEAE-650M), or both, as described previously (Yang, Ding, & Montgomery, 2002; Yang et al., 1994). No neutral oligo- or polysaccharides were present in the EPS preparations.



Fr. No.	Rha	Glc	Man	GlcA
47	2.5	1.0	0.8	0.7
52	2.5	1.0	0.6	0.5
62	2.5	1.0	0.7	0.6
71	2.5	1.0	0.8	0.7

Fig. 1. The test for purity of EPS9Sm6 by SEC. The table is the composition analysis of the fractions across the peak.

2.3. Purity of EPSs

EPS (2 mg) was chromatographed as described earlier. Several fractions from the chromatographic peak were analyzed for their monosaccharide composition (2 M trifluoroacetic acid (TFA), 120 °C, 1 h). In such a hydrolysis condition, aldobiuronic acid is always present in small amounts due to incomplete hydrolysis of the hexuronosyl residue, but the EPS preparation was considered homogeneous by its constant composition across the carbohydrate peak of column chromatography (Fig. 1).

The EPSs of Ech6 family had a composition of Fuc, Gal, Glc, and GlcA in the ratio, 2:2:1:1, and the EPSs of Ech9 family and EPS80 had a composition of Rha, Glc, Man, and GlcA in the ratio, 3:1:1:1.

2.4. Preparation of partial acid hydrolyzates of EPSs

EPS (10 μg) was dissolved in 200 mM TFA (200 μl) and heated at 100 °C for 1 h. The cooled solutions were lyophilized. The products were analyzed by HPAEC-PAD using the gradient (0–150 mM NaOAc in the presence of 40 mM NaOH for 90 min) described previously for oligosaccharide analysis (Yang et al., 1994).

2.5. 1D ^{1}H-NMR analysis

The purified EPSs were exchanged twice by lyophilization from D_2O (99.9 at.% D, Aldrich, Milwaukee, WI) in the presence of 1% NaCl and finally dissolved in D_2O (100 at.% D, 1 ml) containing a trace of acetone as internal standard (resonance set equal to 2.225 ppm). All spectra were recorded at 600 MHz using a Bruker AMX 600 Spectrometer. The relative area of anomeric protons in the spectrum of EPS confirms the repeating unit of monosaccharide residues analyzed by HPAEC-PAD of acid hydrolyzates.

2.6. Analysis by light scattering

EPS (around 100 mg) was dried in a vacuum desiccator to a constant dry weight (80 °C, 24 h) in order to correct for moisture in subsequent weighing. EPS, without previous heating, was dissolved in 0.15 M Na_2SO_4 containing 0.015 M EDTA, and dialyzed against the same solvent for 3 days. It was then filtered through a Millex-HA 0.45 μm filter unit (Millipore Company, Bedford, MA) prior to light scattering (LS) measurements. The carbohydrate concentration was determined by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) using a standard with the monosaccharide composition of each EPS.

2.6.1. Batch mode of analysis

A series of different concentrations (0.1–0.5 mg/ml) of EPS solution in 0.15 M Na_2SO_4 containing 0.015 M EDTA was analyzed by a LS batch mode using a syringe pump to push the solution through the LS cell (internal diameter 1.25 mm) at a constant rate (0.5 ml/min). Different flow rates (0–0.5 ml/min, corresponding to shear rates of 0–29 s^{-1} (Reed, 1995)) had been used to test the impact of the shear rate on the shape of the EPS molecules. The LS detector (with a 5 mW linearly polarized He–Ne 632.8 nm laser, Dawn DSP Laser Photometer, Wyatt Technology Company, Santa Barbara, CA) measures the laser light scattered from the flowing sample at 18 positions, 14 of which were used in this study that ranged from 35 to 153°. Data were acquired and analyzed using Wyatt Technology's ASTRA V4.73.04 software to obtain the molecular weight (M_w) and the root mean square radius (RMS) using Zimm-plot (Zimm, 1948). RMS was obtained after extrapolation to zero polymer concentration, thus in the region of Newtonian flow at zero shear rate, so that no relationship between RMS and shear rate was present.

Calcium chloride, solid or in solution, was added to EPS9Sm6 and EPS6 + solutions in 0.86 M NaCl and the size of the molecules was measured by a LS batch mode. The presence of Ca^{2+} did not change the dn/dc to any significant extent.

2.6.2. Size exclusion chromatography/light scattering

A sample (1 mg/ml, 100 μl) of each EPS was analyzed on a TSK6000 SEC column (TosoHaas, Montgomeryville, PA). The flow rate was 0.5 ml/min. The elution was followed sequentially by a DAWN DSP LS and an ERC-7517 RI detector (ERC Inc., Tokyo, Japan). The data from the LS photometers were processed together with the signal from the refractive index (RI) detector by Wyatt Technology's ASTRA V4.73.04 software. The software cuts the elution peak into around 1000 slices, and then calculates the concentration of each slice by specific refractive index increment (dn/dc) combined with a detector constant for the concentration detector. The M_w, R_w, polydispersity (M_w/M_n) and the relation between RMS and M across the entire elution peak are then calculated. Flory–Fox theory was used to calculate the intrinsic viscosity from the RMS and M_w data, using a phi value of 2.87×10^{23} (Flory & Fox, 1951; Ptitsyn & Eizner, 1959).

The dn/dc of EPS was determined at room temperature from the plot of the RI at 632.8 nm against concentration of five different concentrations, ranging from 1 to 5 mg/ml, for each EPS. The value of dn/dc was 0.151 ml/g for the EPS6 and 0.157 ml/g for the EPS9Sm6 as listed in Table 2.

2.7. Determination of viscosity

For qualitative comparison, the intrinsic viscosity $[\eta]$ of each EPS solution in 0.86 M NaCl was measured at 25 °C using Ostwald capillary viscometers and determined by the Huggins and Kraemer plots of relative viscosity and specific viscosity against concentrations 0.5–5.0 mg/ml (Huggins, 1942; Kraemer, 1938).

The viscosity of each EPS solution at different shear rates was determined at 25 °C with a plate-and-cone viscometer (DV-III V3.3 RV, Brookfield Engineering Laboratories Inc., Middleboro, MA). The viscosity at zero shear rate (η_0) was obtained by fitting of the Cross-equation (Cross, 1965; Grassi, Lapasin, & Pricl, 1996; Manca, Lapasin, Partal, & Gallegos, 2001; Morris et al., 2001; Rayment, Ross-Murphy, & Ellis, 1998; Rodd, Dunstan, & Boger, 2000; Wang, Ellis, Ross-Murphy, & Burchard, 1997), which is written as:

$$\eta = \eta_\infty + (\eta_0 - \eta_\infty)/(1 + K\gamma^n) \qquad (1)$$

where η_0 and η_∞ are limiting viscosities at zero and infinite shear rate, K is a constant, γ is the shear rate, and n is a power law exponent. The Cross-equation is used to calculate the η_0 by employing a non-linear least squares fit. The intrinsic viscosity is then calculated from the Huggins and Kraemer plots from a set of η_0 data at different concentrations (Huggins, 1942; Kraemer, 1938).

The effect of ionic strength (I) on the viscosity of the polysaccharide solution was determined by adding a saturated sodium chloride aqueous solution to the polysaccharide solution. The viscosity of the polysaccharide solutions remained unchanged after the salt concentration was above 0.2 M. The effects of pH or Ca^{2+} were therefore determined in 0.86 M NaCl. Hydrochloric acid or sodium hydroxide was added to change the pH of the polysaccharide

Table 1
Molar ratio of monosaccharide composition of EPSs by HPAEC-PAD

Sample	Fuc	Rha	Gal	Glc	Man	GlcA
EPS6	1.8		1.7	1.0		0.7
EPS6S +	1.9		1.6	1.0		0.6
EPS9		2.6		1.0	0.5	0.4
EPS9Sm6[a]		2.3		1.0	0.7	0.5
EPS9Sm6		2.5		1.0	0.8	0.5
EPS80		2.7		1.0	0.6	0.4

[a] From liquid culture.

solution in 0.86 M NaCl and the viscosity was determined at each pH. Calcium chloride, solid or in solution, was added to the polysaccharide solution in 0.86 M NaCl to investigate the effect of the calcium ion concentration on the viscosity.

3. Results

3.1. Preparation of EPSs

The *E. chrysanthemi* spp used in these studies produce EPSs from a variety of sugars, including glucose, galactose, mannose, fructose, and sucrose. The yields of the crude polysaccharides from glucose range from 0.04 g (EPS80) to 9.4 g (EPS9Sm6) per liter of medium. It is of interest to note the increase in yield of EPS that resulted from the isolation of strains that were resistant to streptomycin. In liquid culture, Ech6 produced an average of 2 g/l of EPS compared to 3.5 g/l from Ech6S + , and Ech9 produced a negligible amount of EPS compared to 9.4 g/l from Ech9Sm6. The EPS produced from liquid culture has the same structure as that from agar medium.

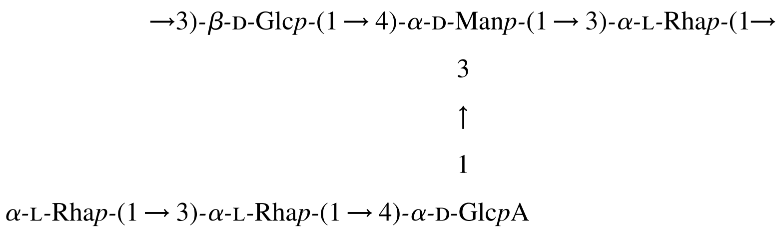
3.2. Structures of EPSs

It has been clearly demonstrated from earlier studies of the EPSs from *E chrysanthemi* spp that the primary repeating unit within each family is characterized to be identical by the monosaccharide composition, 1D ^{1}H NMR spectrum and the HPAEC-PAD analyses of the oligosaccharides produced by identical partial hydrolysis conditions (Yang et al., 1996). These characteristics of the EPS are similar to that of a mixed melting point for crystalline compounds for the demonstration of identity.

HPAEC-PAD analyses of the monosaccharide composition of the EPSs in the present study indicate there are two different groups of microorganisms from the standpoint of the polysaccharides produced (Table 1 and Fig. 2): one group is EPS9, EPS9Sm6, and EPS80, the other EPS6 and EPS6S + . These observations parallel those of the ribotyping data (Gray, Yang, & Montgomery, 2000), which have been extended to include the streptomycin-resistant strains that are essentially identical to the parent strains (Yang et al., 2001a). Structural resemblances of the EPS within each group are also reflected in the HPAEC-PAD analyses of the oligosaccharides produced upon partial acid hydrolysis (Fig. 2) and in the 1D ^{1}H NMR spectra of the EPSs (Fig. 3). Slight differences may be noted in these results; nonetheless, the similarity in the spectra or the chromatograms within each group is striking, indicating the identity of their repeating units.

The 1D ^{1}H NMR spectrum of EPS9Sm6 shows six anomeric protons, of which five are present in the α-configuration and one in the β-configuration, based on the coupling constants and chemical shifts: δ 5.19 (1H, $J_{1,2} = 4.2$ Hz); δ 5.17 (1H, $J_{1,2}$ unresolved); δ 5.05 (1H, $J_{1,2}$ unresolved), δ 5.01 (1H, $J_{1,2}$ unresolved), δ 4.79 (1H, $J_{1,2}$ unresolved), δ 4.55 (1H, $J_{1,2} = 8.4$ Hz). The methyl protons of the rhamnose residues in the EPS resonate at about 1.3 ppm (9H, $J_{1,2} = 6$ Hz). These data indicate the presence of a hexasaccharide repeating unit in the EPS9Sm6 and are essentially identical to those measured for EPS9 (Yang et al., 1996), demonstrating the similarity of EPS from the streptomycin-resistant strain to the EPS from its parent strain Ech9.

From the similarities as noted in HPAEC-PAD and ^{1}H NMR analyses, the EPSs elaborated by *E. chrysanthemi* strains Ech9Sm6 and SR80 have a similar structure to those from *E. chrysanthemi* pv zeae strain SR260, *E. chrysanthemi* strains Ech1 and Ech9, which have been previously determined to have the following hexasaccharide repeating unit (Gray et al., 1993; Gray et al., 1995; Yang et al., 1996):



$$\begin{array}{c} \rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow \\ 3 \\ \uparrow \\ 1 \\ \alpha\text{-L-Rha}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-D-Glc}p\text{A} \end{array}$$

Thus, Ech9Sm6 and SR80 can also be included in the same chemical family of strain SR260, Ech1, and Ech9.

EPSs of *E. chrysanthemi* Ech6 and Ech6S + have a completely different structure from the strains described earlier. By similar evidence, the structure of the EPS from the streptomycin-resistant strain Ech6S + is similar to that of the parent strain

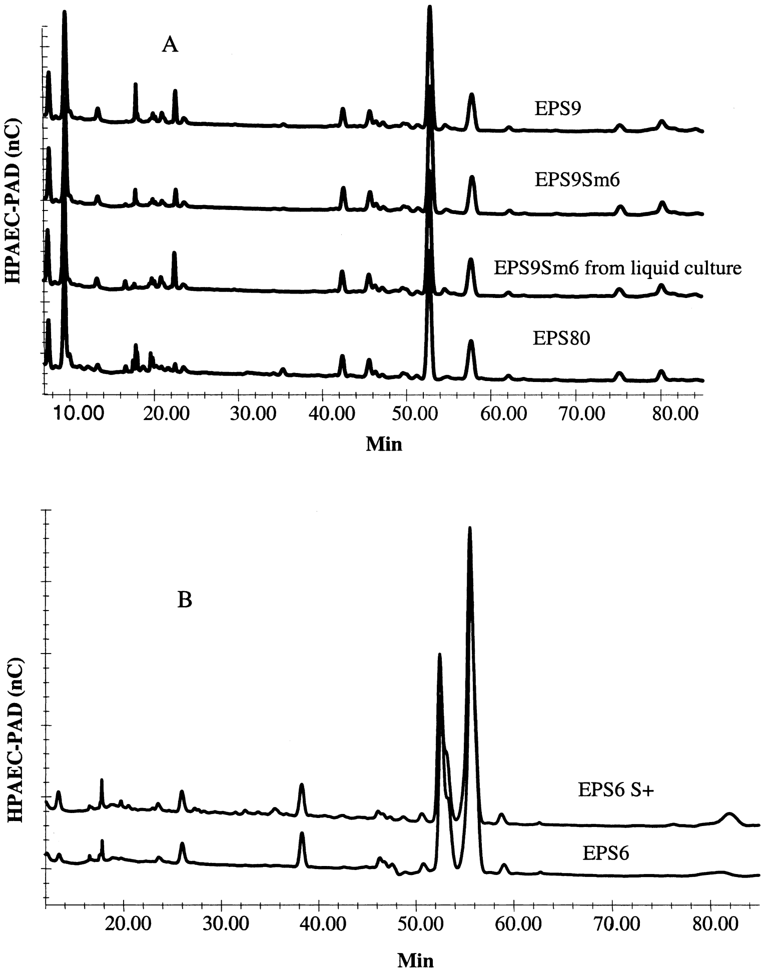


Fig. 2. HPAEC-PAD analyses of partial acid hydrolysates (200 mM TFA, 100 °C, 1 h) of EPSs from *E. chrysanthemi* spp. (A) EPS9 (from parent strain), EPS9Sm6 (from streptomycin-resistant strain, plate culture and liquid culture) and EPS80; (B) EPS6 (from parent strain) and EPS6S + (from streptomycin-resistant strain). The products were analyzed by HPAEC-PAD using the gradient (0–150 mM NaOAc in the presence of 40 mM NaOH for 90 min) described previously for oligosaccharide analysis (Yang et al., 1994). Monosaccharides resulting from the hydrolyses are not shown.

Table 2
Molecular parameters, intrinsic viscosities $[\eta]$ and B values of EPSs in 0.15 M Na_2SO_4 containing 0.015 M EDTA at 25 °C by LS and viscometry

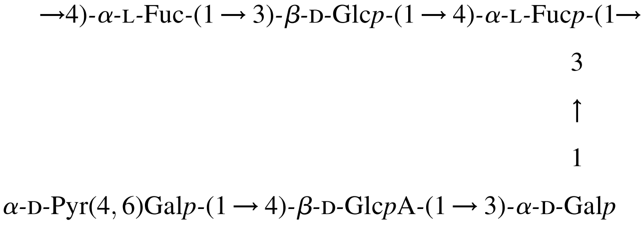
Sample	dn/dc	LS batch mode		SEC/LS					$[\eta]_0$ (ml/g)	B
		M_w (× 10^6)	RMS (nm)	M_w (× 10^6)	R_w (nm)	Slope of RMS versus M	Polydispersity (M_w/M_n)	$[\eta]$[a] (ml/g)		
EPS6	0.151	0.62 ± 0.02	118.1 ± 1.9	0.66 ± 0.01	70.8 ± 1.6	0.67 ± 0.05	1.4	960	978[b]	0.013
EPS6S +	0.147	0.77 ± 0.02	93.1 ± 1.9	0.62 ± 0.01	52.3 ± 1.6	0.57 ± 0.08	1.4	1294	1136[b]	0.019
EPS9	0.154	1.22 ± 0.07	121.8 ± 2.4	1.18 ± 0.05	91.5 ± 1.4	0.42 ± 0.06	1.6	779	860[b]	0.091
EPS9Sm6	0.157	2.09 ± 0.12	142.6 ± 3.0	2.08 ± 0.08	128.0 ± 1.9	0.56 ± 0.03	1.9	1855	1673[b]	0.050
EPS80	0.157	0.71 ± 0.01	68.4 ± 0.5	0.53 ± 0.01	44.8 ± 1.3	0.41 ± 0.08	1.9	513	593[c]	0.11
TPK-1	0.157	0.59 ± 0.01	54.2 ± 0.4	0.66 ± 0.01	50.1 ± 1.5	0.51 ± 0.03	1.5	361	334[c]	0.14
TPK-2	0.151	0.28 ± 0.00	40.8 ± 0.6	0.31 ± 0.00	25.3 ± 2.6	0.49 ± 0.04	1.9	167	173[c]	0.10

[a] The intrinsic viscosity calculated by Astra software using Flory–Fox equation.

[b] The viscosity at zero shear rate fitted by the Cross-equation.

[c] The viscosity at lowest readable shear rate.

Ech6. The structure is (Yang et al., 2001b; Yang et al., 1994):



$$\begin{array}{r}
\rightarrow 4)\text{-}\alpha\text{-L-Fuc-}(1\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-L-Fuc}p\text{-}(1\rightarrow \\
3\quad \\
\uparrow\quad \\
1\quad \\
\alpha\text{-D-Pyr}(4,6)\text{Gal}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{A-}(1\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\qquad
\end{array}$$

The NMR spectrum of EPS6 was difficult to analyze perhaps due to the viscosity of its solution causing the broadening of the peaks (Yang et al., 1994). Attempts to record the spectra at higher temperatures (95 °C) resulted in significant loss of pyruvate with no significant improvement in the peak shape.

3.3. Hydrodynamic properties of EPSs

3.3.1. Molecular weight (M_w) of EPSs

The molecular weights of the EPSs were examined by batch and SEC techniques, using multi-angle light scattering (MALS) and RI detectors. The initial solvent system was 0.1 M NaCl, containing 0.05 % (w/v) NaN_3 but with several different chromatographic columns the recovery of EPSs was found to be low (50–70%). The problem was corrected by using 0.15 M Na_2SO_4 containing 0.015 M EDTA when the recoveries were essentially 100%. The dn/dc was different for each EPS family and it was critical to establish the volatile (moisture) content of the samples to be certain of the concentration of the EPS in the series of solutions. The M_w, R_w, and polydispersities are summarized in Table 2 for both the batch and SEC/LS modes of determination. The results of the batch mode were analyzed by the Zimm procedure (first-order fit) (Zimm, 1948). These results are compared with that from the SEC/LS experiments, where the M_w calculated from the total elution peak includes the small amount of polymer at the beginning, which may be due to aggregation or at the limit of the column resolution near the void volume, and the portion at the trailing segment, where the concentration of polymer is low and the signal to noise ratio in the LS detection is low. This is illustrated in the log RMS versus log M plots (Fig. 4) where the linear portion does not extend across the whole peak. These analyses are summarized in Table 2; The EPSs range in molecular weight from 0.5 to 1.6 MDa.

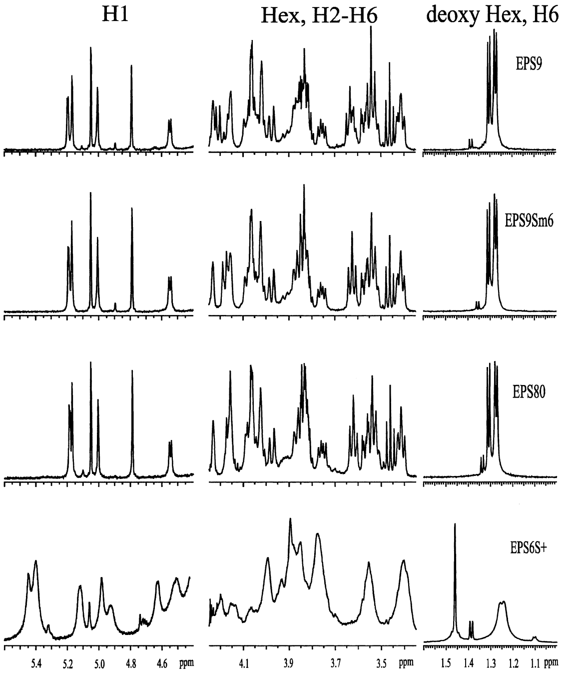


Fig. 3. The 1D ^1H NMR spectra of EPS9, EPS9Sm6, EPS80, and EPS6S + obtained at 600 MHz in 1% NaCl in D_2O at 338 K.

For the most EPSs, the two sets of M_w results from the batch and the SEC/LS mode give similar results. The size parameter, RMS, is a z-average from the Zimm-plot (Zimm, 1948), which is different from the R_w obtained as a weight average size over all the slices of the peak obtained in the SEC/LS. The RMS and R_w are not comparable.

3.3.2. Viscometric behavior of EPSs

The EPSs demonstrate pseudoplastic flow characteristics (shear-thinning), where the viscosity η decreases with the increase of shear rate (Tanner & Walter, 1998), similar to that of xanthan (Cottrell, 1980) (Fig. 5). It was clear that the EPSs are not thixotropic by the phenomena that increasing or decreasing shear rates gave the same curve with very slight deviation noted for EPS9Sm6 and EPS80 (Fig. 5). The relative decrease in viscosity is more pronounced in the shear rate range of 0–20 s^{-1} than at values larger than 200 s^{-1}. This is also shown in a series of viscosity determinations made with capillary viscometers of different capillary size and thus different shear rates. The average shear rates for the capillary viscometers are 400–3316 s^{-1} and the maximum shear rates are 603–4974 s^{-1} (Reed, 1995). In the cases of viscous polysaccharides, such as EPS6, EPS6S +, EPS9, and EPS9Sm6, the viscosity at these ranges of shear rate is changing, which makes direct comparisons of the two methods difficult. The use of

Table 3
The effect of Ca^{2+} on the molecular parameters of EPS6S + and EPS9Sm6 in 0.86 M NaCl at 25 °C by LS batch mode

Sample	Ca^{2+} concentration (M)	M_w ($\times 10^6$)	RMS (nm)
EPS6S +	0	0.52 ± 0.08	112.3 ± 2.8
	0.09	0.51 ± 0.07	123.3 ± 9.6
	0.45	0.51 ± 0.01	108.3 ± 1.6
	0.90	0.57 ± 0.02	102.8 ± 1.6
	1.80	0.61 ± 0.05	107.9 ± 4.2
EPS9Sm6	0	1.64 ± 0.05	138.8 ± 1.2
	0.09	1.75 ± 0.18	143.7 ± 3.7
	0.45	1.87 ± 0.06	136.1 ± 1.8
	0.90	3.00 ± 0.09	131.7 ± 1.9

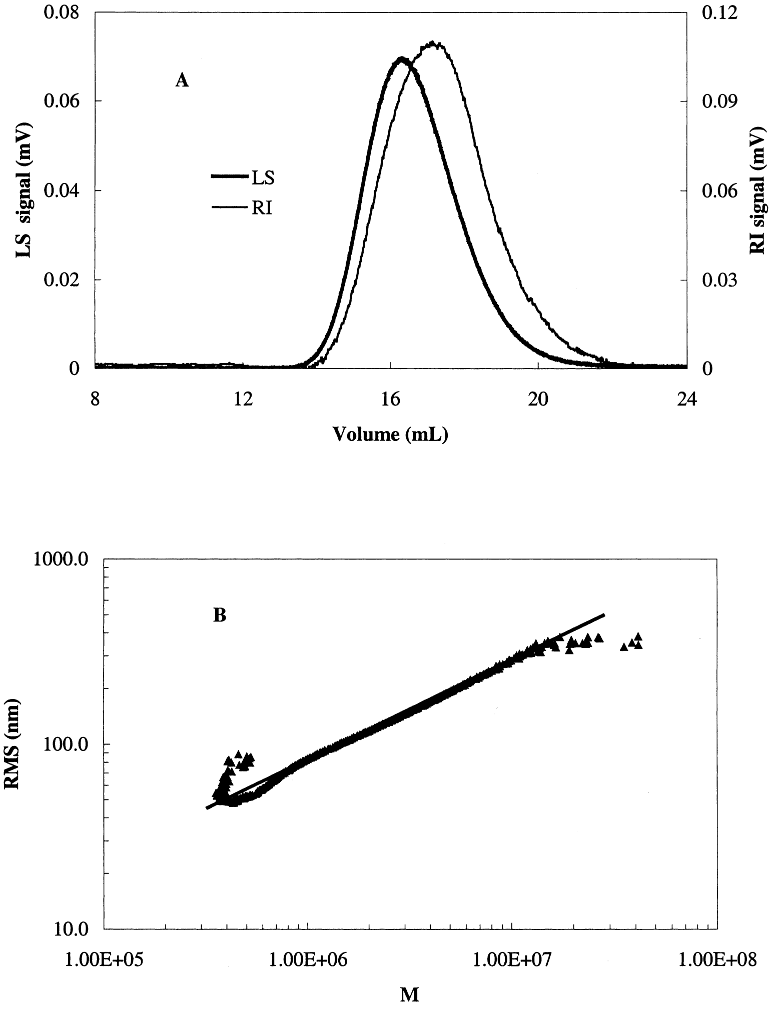


Fig. 4. The SEC/LS chromatogram of EPS9Sm6 in 0.15 M Na_2SO_4 containing 0.015 M EDTA at 25 °C. (A) Elution from TSK 6000 column showing 90° LS and RI detections; (B) RMS ~ M plot with each data point calculated for each elution slice in the SEC/LS chromatogram.

capillary viscometer is used only for qualitative comparisons of the pseudoplastic EPSs. For EPS80 and EPS Teranera fractions PK-1 and PK-2 (see below), the degree of the shear-thinning effect is small so that the viscosity from the capillary viscometers is close to the data from the Brookfield instrument.

Although only a tendency to reach the Newtonian plateau at low shear was observed, the viscosity at zero shear rate (η_0) was obtained by fitting of the Cross-equation (Eq. (1)). For EPS9, EPS9Sm6, EPS6, and EPS6S + , the fitting gives curves close to the measured data points (Fig. 6). For the other less viscous polysaccharides, because of small shear-thinning effects, the fitting was not good.

The reduced viscosity (η_{sp}/c) of the polysaccharide solutions in 0.86 M NaCl increases linearly with increasing EPS concentration. However, η_{sp}/c of the polysaccharides decreases dramatically in the range of zero to 0.86 M NaCl (Fig. 7), but when the sodium chloride concentration reaches 0.2 M the viscosity reaches an asymptote. All these represent the typical viscometric behavior of polyelectrolytes (Lapsin & Pricl, 1995 see Bozzi et al., 1996. Gianni et al., 1999. Oba et al., 1999).

The viscosity $[\eta]$ of EPS6S + and EPS9Sm6 is almost independent of pH between 4 and 9, and decreases over the pH range of 1–4 or 9–13 (Fig. 8).

The addition of Ca^{2+} increases the intrinsic viscosity $[\eta]$ of EPS9Sm6, shown in Fig. 9, but here the properties of the two families of EPSs are different. The $[\eta]$ of the EPS9Sm6 increases significantly with increase in Ca^{2+} concentration commencing at 0.01 M, possibly due to the aggregation of molecules. This association is supported by LS (Table 3), where the measured M_w increased with the addition of Ca^{2+}. This is in contrast with the behavior of EPS6 + where the first additions of Ca^{2+} associates with carboxylate groups on the same chain with little influence on the $[\eta]$ or M_w up to 0.09 M Ca^{2+}.

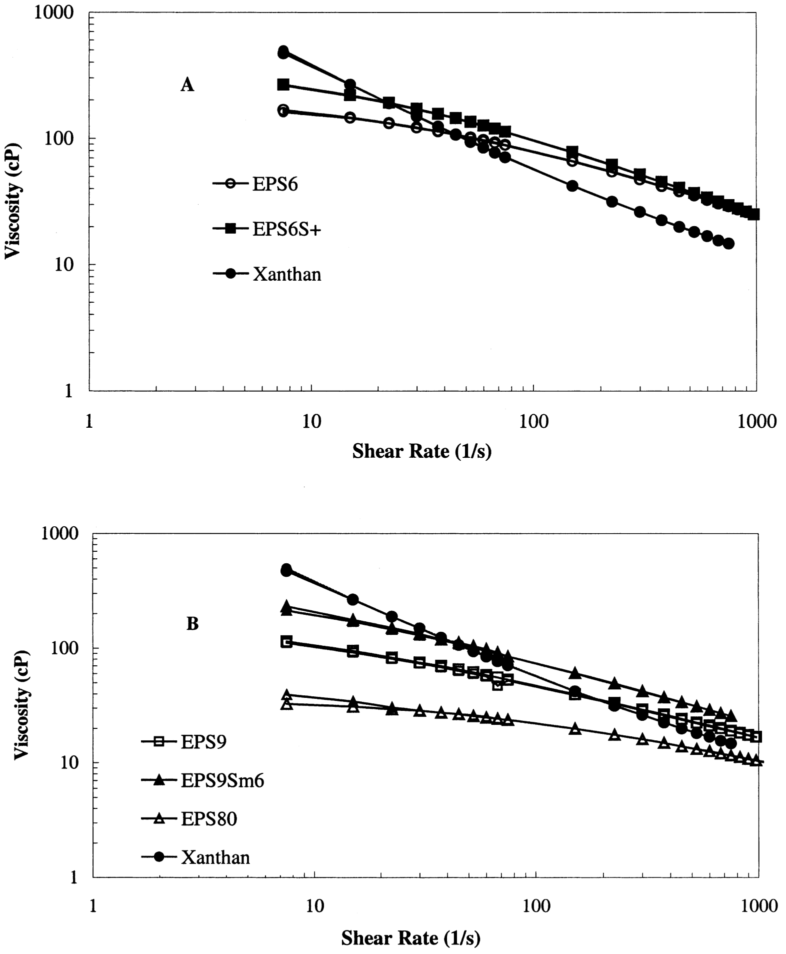
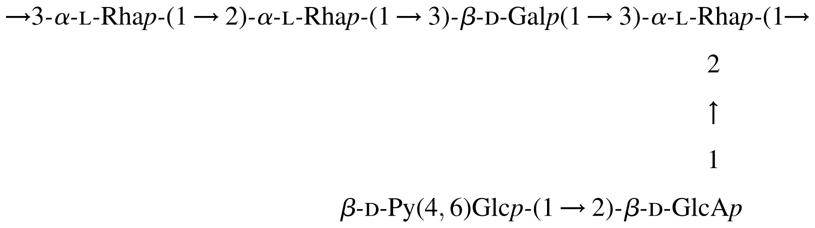


Fig. 5. The viscosity η/shear rate relationship of EPS6 and EPS6S + (A) and EPS9, EPS9Sm6, and EPS80 (B) compared with xanthan using the Brookfield viscometer at 25 °C. All measured at a concentration of 5 mg/ml in water with increasing then decreasing shear rates.

4. Discussions

4.1. Structure of EPSs

The EPSs from the two families of bacteria, Ech6 and Ech9, are branched, high molecular weight and polyanionic biopolymers that are structurally similar in some respects but unique in others. Included in these studies are additional EPSs from *E. teranera*, a bacterium associated with a fungus that causes canker disease in *Eucalyptus* sp. (Yang et al., 2002). One EPS, EPS Teranera PK-1, has a repeating unit identical with EPS9, while the second EPS, EPS Teranera PK-2, has the following structure, which is more similar to EPS6 (Yang et al., 1994):



$$\begin{array}{c} \rightarrow 3\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 2)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Gal}p(1\rightarrow 3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow \\ 2 \\ \uparrow \\ 1 \\ \beta\text{-D-Py}(4,6)\text{Glc}p\text{-}(1\rightarrow 2)\text{-}\beta\text{-D-GlcA}p \end{array}$$

4.2. Conformation of EPSs in aqueous solution

The glycosidic linkages of the backbone constituents in each of these EPSs are similar to several polysaccharides for which X-ray studies have shown that a helix is introduced into the backbone with the side chains branching from it (Harding, 1997; Hember, Richardson, & Morris, 1994; Maeda et al., 2001; Ogawa, Yui, Nakata, Kakuta, & Misaki, 1997; Ogawa et al., 1996; Sutherland, 1994; Yui, Ogawa, Kakuta, & Misaki, 1995). The pitch of such helices in the EPSs studied here would be such that the side chains of adjacent repeating units would not overlay each other so that the charges on the uronic acid residues in the EPS9 family would be similarly removed. This is in contrast to the pyruvate residues at the ends of the three-unit sidechains in

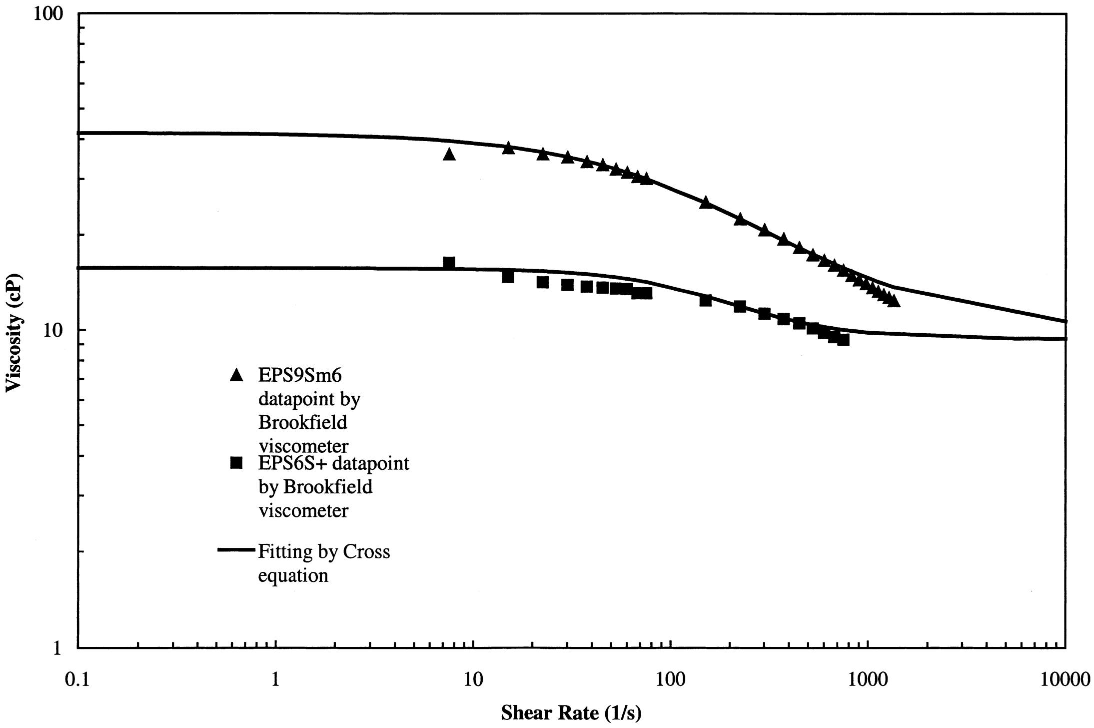


Fig. 6. The viscosity of EPS6S + and EPS9Sm6 (5 mg/ml in 0.86 M NaCl) measured with the Brookfield viscometer at 25 °C and fitted by the Cross-equation as stated in the text.

the EPS6 family. It is therefore proposed that these EPSs collapse and are present in a flexible conformation with increase in the ionic strength of the solvent. The influence of the pyruvate unit would be to induce a stiffer conformation in the EPS6 family.

Two hydrodynamic coefficients can be derived from the measurements of molecular weight by LS and from the viscometric determinations that give support for these proposed conformations. The conformation of a polymer is reflected in the RMS and M relationship:

$$\mathrm{RMS} \propto M^{\alpha} \tag{2}$$

where usually, the exponent α is 1 for rod-like molecules, 0.33 for spheres, 0.5 for random coils in a theta solvent, and 0.5–0.8 for random coils in a good solvent (Beri, Walker, Reese, & Rollings, 1993; Cassasa & Berry, 1989; Flory, 1953; Yau, Kirkland, & Bly, 1979). The molecular weights of the EPS6, EPS9 family and EPS Teranera fractions bear a linear relationship to their RMS (EPS9Sm6 shown in Fig. 4) and plots of log RMS–log M exhibit slopes of 0.4–0.6 (Table 2), implying that all the EPSs are flexible coils in aqueous solution. However, this does not give much further detail about the flexibility of the polymer.

The flexibility of a polyelectolyte in solution, by an empirical approach, was developed by Smidsrod and Haug (1971) who studied the effect of ionic strength on $[\eta]$. By plotting $[\eta]$ against $I^{-0.5}$, the slope S of the line and the value of $[\eta]$ at an ionic strength 0.1 M, denoted as $[\eta]_{0.1}$, are related:

$$S = B[\eta]_{0.1}^{\rho} \tag{3}$$

where the average ρ is approximately 1.3. The lower values of B are associated with stiffer chains. Although it is reported that the method is not very reliable and the physical meaning of the B value is not very clear (Tobitani & Ross-Murphy, 1997), it is still widely applied to approximate the flexibility of polysaccharide chains in solution (Cescutti, Paoletti, Navarini, & Flaibani, 1993; Flaibani, Leonhartsberger, Navarini, Cescutti, & Paoletti, 1994; Higashimura, Mulder-Bosman, Reich, Iwasaki, & Robijn, 2000; Jampen, Britt, & Tung, 2000; Lai, Tung, & Lin, 2000; Lapsin & Pricl, 1995).

The B values of all EPSs, listed in Table 2, all fall between 0.01 and 0.14, which suggests intermediate flexibilities. Polysaccharides with similar B values to the EPS6 family (B values 0.01–0.02) are pectin (0.02), *Zoogloea ramigera* polysaccharide (0.02) (Lapsin & Pricl, 1995), and villain (0.03) (Higashimura et al., 2000); for EPS9Sm6 (B value 0.05) are alginate (0.04) (Smidsrod, 1970), chitosan (0.04), λ-carragenan (0.05) (Lapsin & Pricl, 1995), carboxymethylcellulose (0.065) (Schneider & Doty, 1954), hyaluronate (0.07) (Cleland, 1968); for EPS9, EPS80, and EPS Teranera fractions (B values 0.09–0.14) is xanthan (Ac:Pyr = 1:1, B value 0.12) (Lapsin & Pricl, 1995).

The RMS ~ M relation also points that the EPS9Sm6 of the higher molecular weight has a more rigid conformation than the other three EPSs in the EPS9 family, which is

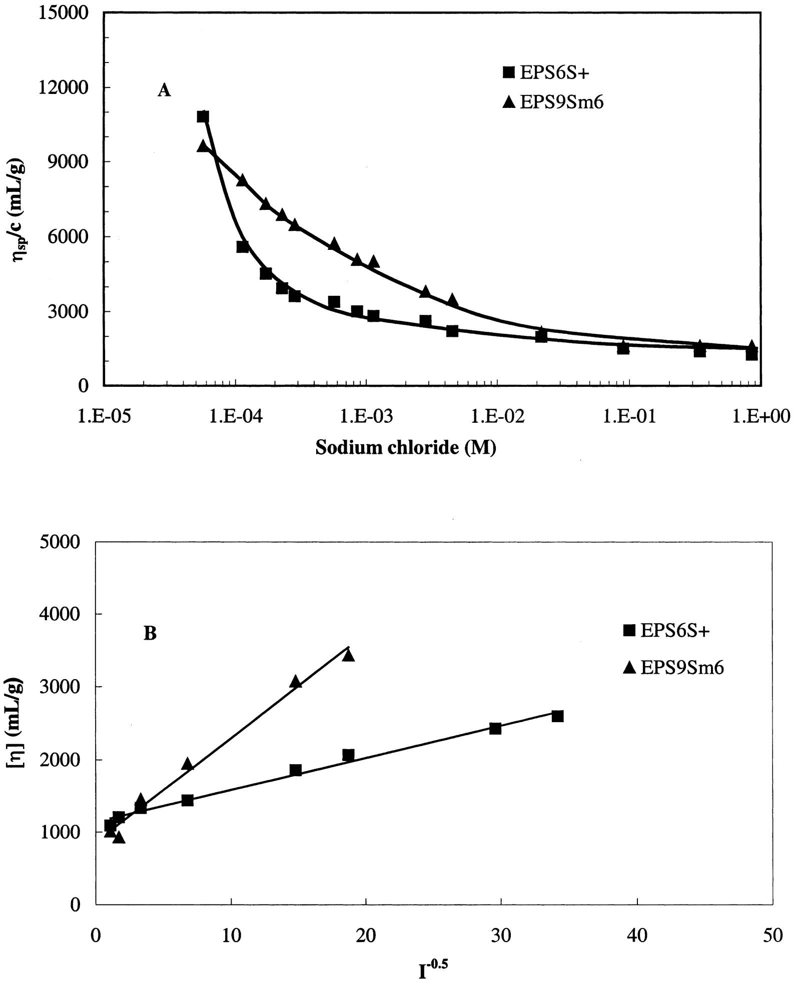


Fig. 7. The effect of salt concentration and ionic strength ($I^{-0.5}$) on the reduced viscosity (η_{sp}/c) and intrinsic viscosity [η] of EPS6S + and EPS9Sm6 using capillary viscometer 102 (average shear rate 400 s^{-1} and maximum shear rate 600 s^{-1}) at 25 °C; the EPS concentration is 0.1 mg/ml. (A) (η_{sp}/c) versus salt concentration; (B) [η] versus $I^{-0.5}$.

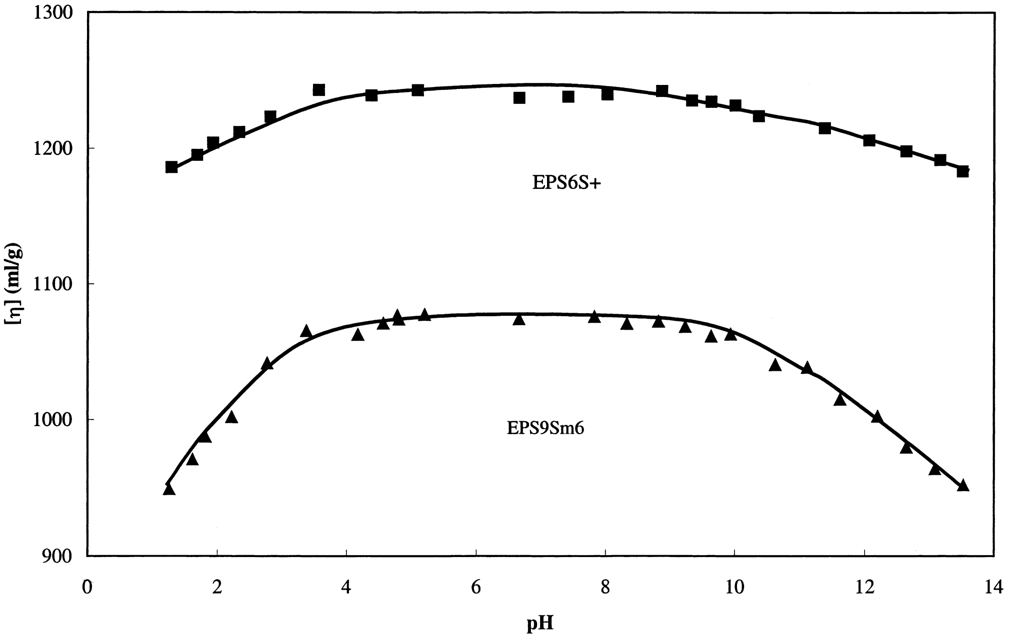


Fig. 8. The effect of pH on the intrinsic viscosity [η] of EPS9Sm6 and EPS6S +. The EPS concentrations are 1 mg/ml in 0.86 M NaCl using capillary viscometer 102 (average shear rate 400 s^{-1} and maximum shear rate 600 s^{-1}) at 25 °C.

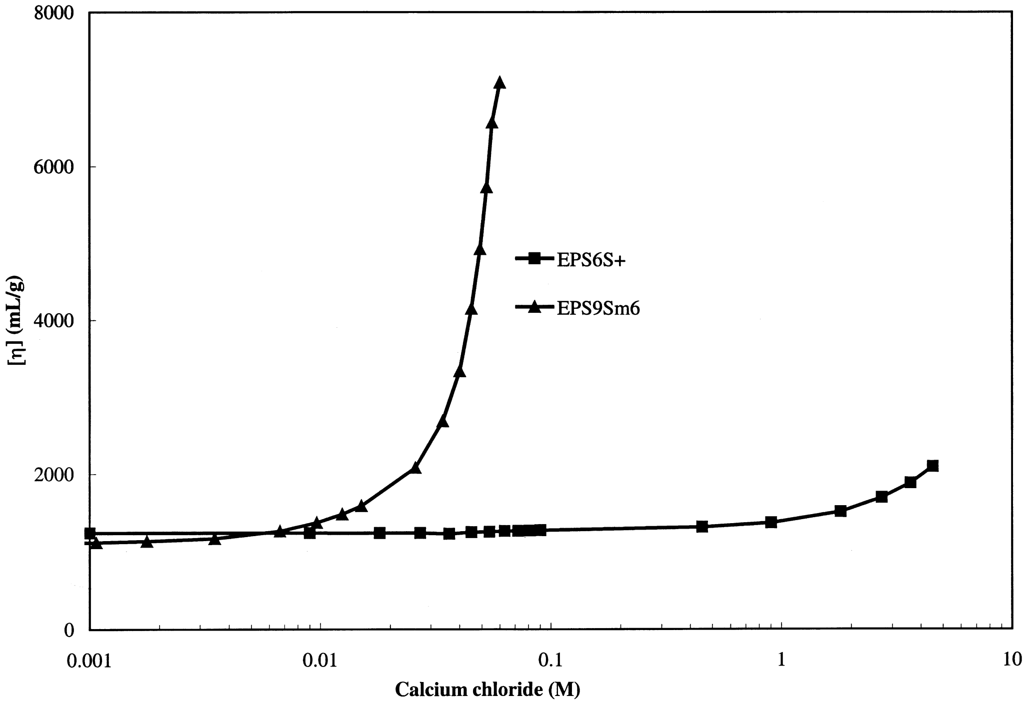


Fig. 9. The effect of Ca^{2+} on the intrinsic viscosity $[\eta]$ of EPS9Sm6 and EPS6S + . The EPS concentrations are 1 mg/ml in 0.86 M NaCl using capillary viscometer 102 (average shear rate 400 s^{-1} and maximum shear rate 600 s^{-1}) at 25 °C.

further supported by the B parameter (Smidsrod & Haug, 1971) noted above. Thus, the flexibilities are somewhat different, in keeping with their structures.

It is interesting to note that the streptomycin-resistant strain in Ech9 family has an EPS with higher M_w than EPS from the parent, which is also seen in the two EPSs of the Ech6 family although not to the same extent. Also with EPS6 and EPS6S + there is correlating indications of less flexibility than the EPS9 family in that the log RMS–log M slopes are larger, 0.6–0.7, and the B parameters are 0.01–0.02. This is again consistent with the proposed influence of the additional charge per repeating unit in the EPS6 and EPS6S + , but not in the similar EPS Teranera PK-2 where the side chain is shorter. In fact, the hydrodynamic property of the EPS Teranera PK-2 is more similar in this respect to the EPS9 family.

4.3. Viscometric studies

All the EPSs studied here show pseudoplastic, polyanionic, non-thixotropic flow characteristics, so that the capillary viscometric measurements of these EPS solutions cannot readily lead to conformational conclusions and are used only for purposes of qualitative comparisons.

The pseudoplastic flow characteristics (shear-thinning) of the EPSs, where the viscosity η decreases with the increase of shear rate (Tanner & Walter, 1998), is similar to that of xanthan (Fig. 5) and many other polysaccharides (Cottrell, 1980). Compared with xanthan, the most commercially used gum, aqueous solutions of EPS9Sm6 and EPS6S + have a similar viscosity while the other EPSs are less viscous. In 0.86 M NaCl, all these polysaccharides have smaller viscosities.

Using Wyatt Technology's ASTRA V4.73.04 software program, which uses Flory–Fox equation (Flory & Fox, 1951; Ptitsyn & Eizner, 1959), the intrinsic viscosity can be calculated from the RMS ~ M data determined by SEC/LS. R_w was obtained after extrapolation to zero polymer concentration, thus in the region of Newtonian flow at zero shear rate, so the calculated intrinsic viscosity is that at zero shear rate. From the data listed in Table 2, the calculated zero shear viscosities using the Cross-equation (Eq. (1)) are close to the viscosities calculated from the LS studies. Furthermore, for the EPS9 family there is a linear relationship, although over a relatively narrow range of M_w, for M_w/R_w, and $M_w/[\eta]_0$ properties (Fig. 10), supporting their similar conformations in solution.

The intrinsic viscosity $[\eta]$ of all EPSs in 0.86 M NaCl solution is almost independent of pH between 4 and 9 (Fig. 8), with decreases of $[\eta]$ over the pH range of 1–4 or 9–13, resulting from slight hydrolysis and confirmed by the fact that viscosity cannot recover from the pH of 1–13 (data not shown).

Although in many respects solutions of the EPSs of each family behave similarly and proportionately to their molecular size (Fig. 10), it is interesting that there is difference between the two EPS families in their reactions with Ca^{2+}. The $[\eta]$ of EPS9, EPS9Sm6, and EPS Teranera PK-1 in 0.86 M NaCl increases significantly with increase in Ca^{2+} concentration, due to the cross-linking of molecules, as demonstrated by increases in M_w by batch LS. This is in contrast to the behavior of EPS6, EPS6S + ,

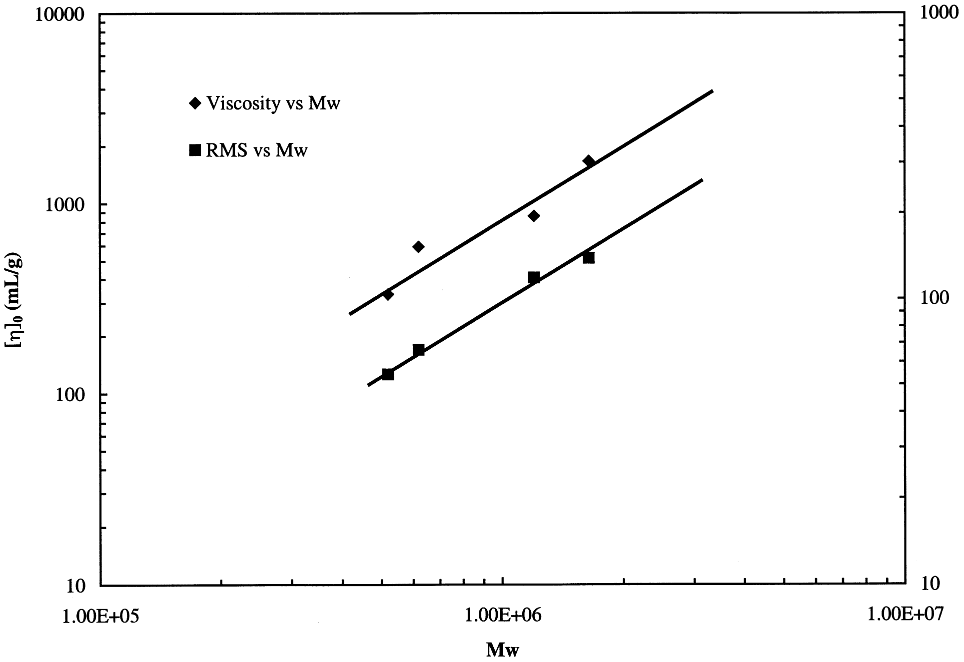


Fig. 10. The relationship of the molecular weight (M_w) with RMS and the intrinsic viscosity at zero shear rate $[\eta]_0$ for EPS9 family.

and EPS Teranera PK-2 in 0.86 M NaCl where the first additions of Ca^{2+} have little influence on the $[\eta]$ or M_w. It is apparent that in the EPS6 family the initial complexation of the Ca^{2+} occurs between the proximate carboxylate groups of the pyruvate and uronic acids in the same molecule, with extramolecular complexation and a resulting increase in viscosity occurring only after later additions of Ca^{2+}. Only extramolecular complexes with Ca^{2+} can occur with the EPS9 family and EPS Teranera PK-1.

5. Conclusions

Many of the *E. chrysanthemi* spp produce EPS, the role of which in host-specificity and virulence is not well known. There is some evidence that EPS production is necessary for infection of plants when the pathogen is present at low levels (Condemine, Castillo, Passeri, & Enard, 1999).

The EPSs can be produced on agar plates or in liquid culture, where the yields are sometimes poor. One means of increasing the yield of EPS is to isolate hyperproducing strains by treatment with antibiotics. *E. chrysanthemi* Ech9Sm6, a hyperproducer of EPS, is a streptomycin-resistant strain of the potato phytopathogen, Ech9. The structure of the EPS from Ech9Sm6 has now been shown to be similar to that of the parent. Similarly, the isolation of a streptomycin mutant from *E. chrysanthemi* Ech6 (named Ech6S +) is a hyperproducer of EPS and by ribotyping to be closely related to its parent strain, Ech6 (Gray et al., 2000). It is not clear how the streptomycin resistance has resulted in the hyperproduction of the EPS.

It was proposed that the strains with similar ribotypes might produce similar EPSs (Gray et al., 2000), which has shown to be the case for the two families reported in the present study, using exhaustive chemical procedures for one member EPS in each family and comparisons of monosaccharide composition, oligosaccharide production, and 1D ^{1}H-NMR spectroscopy to show the identity of repeating units for the other EPSs in the family. The validity of such structural comparisons has been well established for these types of EPS (Yang et al., 2001a; Yang et al., 1996). However, the repeating units of any polysaccharide only suggest the molecular characteristics. Modeling programs point to conformations that by analogy with other well-studied polysaccharides can suggest physicochemical properties and structure/function relationships, which have been sought for several EPSs in these studies.

Hydrodynamic examinations showed different viscosities and R_w within the EPSs of each family and there was a linear relationship of these values and their M_w. Within each family, the proposed flexible coil conformations were supported by physical measurements. The stiffness of the coils was greater for the pyruvated molecules in the EPS6 family. Like many other examples of similar polysaccharides, their pseudoplasticity was most evident in the EPS of higher M_w and R_w, but did not seem to be related to the stiffness parameters.

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