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Note

Structure and hydrodynamic properties of the extracellular polysaccharide from a mutant strain (RA3W) of *Erwinia chrysanthemi* RA3

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Abstract—The structure of the extracellular polysaccharide (EPS) produced by *Erwinia chrysanthemi* strain RA3W, a mutant strain of *E. chrysanthemi* RA3, has been determined using low pressure size-exclusion and anion-exchange chromatographies, high pH anion-exchange chromatography, glycosyl linkage analysis, and 1D ^{1}H NMR spectroscopy. The polysaccharide is structurally similar, if not identical, to the family of EPS produced by such as *E. chrysanthemi* strains Ech9, Ech9Sm6, and SR260. The molecular weight of EPS RA3W by ultracentrifugation (sedimentation equilibrium) and light scattering is compared with those of other *E. chrysanthami* EPSs, as are the viscometric properties.

Keywords: *Erwinia* spp.; Extracellular polysaccharide; Structure; Hydrodynamic properties

Erwinia chrysanthemi spp. are gram-negative bacterial phytopathogens that cause soft rot in a number of plants.[1–6] A considerable amount of work has been performed on their taxonomy, serology, host range,[2–5,7–12] and structures of the produced EPS.[13–19]

The isolation of a spontaneous mutant (named RA3W) from *E. chrysanthemi* RA3 (isolated from onion, *Allium* spp.) is shown to be related by ribotyping[19] to its parent strain, but differs significantly in the color of the culture and the formation of EPS. When grown on nutrient agar or modified YS-glucose agar the mutant is white and produces EPS, whereas the parent RA3 is black and produces no EPS. The finding of a spontaneous, EPS-producing mutant is physiologically significant and relevant to the pathogenicity of the two cultures where the presence of EPS can be a modifying factor.

Fractional precipitation of the EPS RA3W from a saline solution with ethanol into two fractions achieved some separation in the distribution of molecular size, but gave no indication of the presence of two EPS as had been seen in the EPSs produced by some other *Erwinia* spp.[20,21] Examination of the monosaccharide composition, glycosyl linkage analysis (Table 1), and 1D ^{1}H NMR spectra (Table 2 and Fig. 1) of both fractions show that the presence of a hexasaccharide repeating-unit in the EPS RA3W is essentially identical to those measured for EPS9, EPS9Sm6, and EPS SR260, demonstrating the similarity of the EPS RA3W to those of EPS produced by *E. chrysanthemi* strains SR260, Ech1, and Ech9.[13,14,17,18,22] The structural resemblance in the

Abbreviations: A_2, the second thermodynamic (osmotic pressure) virial coefficient; dn/dc, specific refractive index increment; EPS, extracellular polysaccharide; EPS9 etc., EPS produced from *E. chrysanthemi* Ech9, etc.; GLC, gas–liquid chromatography; GLC–MS, gas–liquid chromatography–mass spectrometry; HPAEC-PAD, high pH anion-exchange chromatography with pulsed amperometric detection; I, ionic strength; LS, light scattering; M, molecular weight; M_{app}, the apparent molecular weight; M_w, weight average molecular weight; R_w, weight average root mean square radius; rms, root mean square radius; SEC, size-exclusion chromatography; $[\eta]$, intrinsic viscosity; η_0, viscosity at zero shear rate; $[\eta]_0$, intrinsic viscosity at zero shear rate.

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Table 1. Glycosyl linkage analyses of EPS RA3W

Me sugar[a]	RA3WF1	RA3WF2	SR260[b]
2,3,4-Me_3Rha	0.8	1.0	0.8
2,4-Me_2Rha	1.7	1.7	1.9
2,4,6-Me_3Glc	1.0	1.0	1.0
2,3,6-Me_3Glc[c]			1.0
2,6-Me_2Man	1.1	1.1	0.8
2,3-Me_2GlcA[d]	0.5	0.5	

[a] 2,4-Me_2Rha = 1,3,5-tri-*O*-acetyl-1-deuterio-2,4-di-*O*-methyl Rha-ol.
[b] Refs. 13,14,18.
[c] Glucose derived from carboxyl-reduced GlcA.
[d] Observed as 1,4,5,6-tetra-*O*-acetyl-1,6,6-trideuterio-2,3-di-*O*-methyl Glc-ol derived from the reduction of the methyl ester of 2,3-Me_2GlcA with super-deuteride and etc.

Table 2. NMR data for EPS RA3W and EPS SR260

Residue of inferred linkage in the native EPS	δ_H (ppm)[a] of anomeric proton		
	RA3WF1	RA3WF2	EPS SR260[b]
-4)-α-D-GlcpA-(1-	5.190	5.189	5.185
-3)-α-L-Rhap-(1-	5.170	5.168	5.162
-3,4)-α-D-Manp-(1-	5.049	5.048	5.047
α-L-Rhap-(1-	5.005	5.004	5.003
-3)-α-L-Rhap-(1-	4.786	4.783	4.772
-3)-β-D-Glcp-(1-	4.546	4.546	4.544

The spectra were obtained in D_2O containing 1% NaCl, 65 °C.
[a] Chemical shifts relative to acetone (δ_H 2.225 ppm).
[b] Refs. 13,14,18.

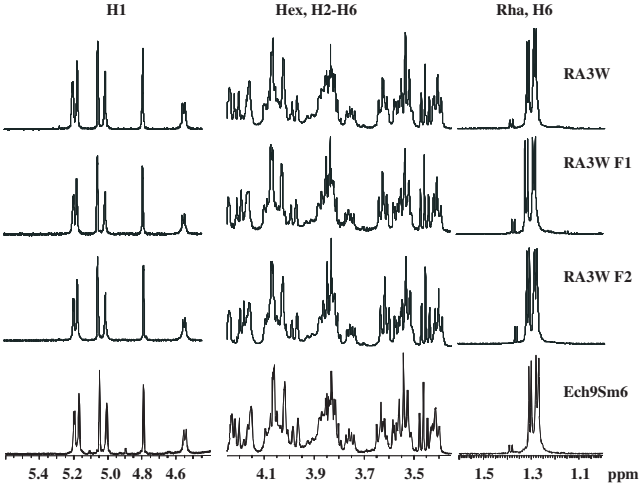
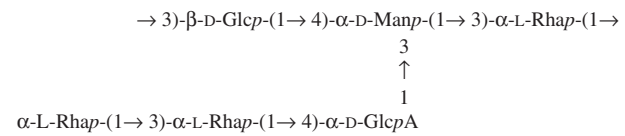


Figure 1. 1D ^{1}H NMR spectra of EPS RA3W, its fractions and EPS9Sm6. The spectra were acquired at 338 K and 600.14 MHz in D_2O containing 1% NaCl.

primary repeating-unit of these EPSs is also reflected by the HPAEC-PAD analyses of the oligosaccharides produced by the same partial acid hydrolysis condition and by the 1D ^{1}H NMR spectra analysis of the ring-proton resonances, particularly those that are well separated from their neighbors (Fig. 1). A comparison of the glycosyl residue compositions and ^{1}H NMR data for the EPSs from *E. chrysanthemi* strain RA3W and from strain SR260 and Ech9Sm6 suggests that the two polysaccharides have identical glycosyl sequences:



Thus RA3W can be included in the same chemical family of strains SR260, Ech1, Ech9, and Ech9Sm6.

EPS RA3W demonstrates characteristics of pseudoplastic (shear-thinning) and non-thixotropic flow, where the viscosity η decreases reversibly with the increase of shear rate.[23] The EPS RA3W by the Brookfield viscometer shows a tendency to reach the Newtonian plateau at the lowest shear rate that could be measured. The resulting viscosity extrapolated to zero shear rate (η_0) was obtained by fitting of the Cross-equation,[24] where the fitting gave curves close to the measured data points.

Using Wyatt Technology's ASTRA V4.73.04 software program, the relation between the rms and molecular weight (M) across the entire elution peak were obtained and the intrinsic viscosity was calculated by Flory–Fox theory,[25,26] with a ϕ value of 2.87×10^{23}. From the data summarized in Table 3, the viscosity calculated from the light scattering studies are close to the viscosity at zero shear rate obtained by Brookfield viscometer using the Cross-equation.

The intrinsic viscosity [η] of EPS RA3W as determined by capillary viscometer is almost independent of pH between 4 and 9, but decreases over the pH range of 1–4 or 9–13. The intrinsic viscosity is affected little at the ionic strength of 0.5 M and above and this decrease could be principally due to the equilibrated formation of free carboxylic acid and carboxyl anion in acidic and alkaline condition, respectively.

The addition of calcium chloride and the resulting aggregation of molecules initiated at 0.1% (w/w), increases the intrinsic viscosity [η] of EPS RA3W and its fractions.

All of these viscometric properties are similar to many of the EPSs studied to date[20–22] and also xanthan.[27]

At the commencement of our studies of EPSs using light-scattering techniques, two dextran standards of molecular weight, determined by size-exclusion chromatography, were purchased: one of 2.4×10^6 Da (Dextran DXT2400 K, American Polymer Standards Co., Mentor, OH, USA) and the other of 36K Da (Dextran 1662, Sigma, St. Louis, MO, USA). The nominal value for the standard 36K Da was close to the molecular weight determined by either batch-mode LS or SEC/LS. However the Dextran DXT2400K, as deter-

Table 3. The molecular parameters and intrinsic viscosity ([η]) of RA3W and its fractions F1 and F2 at 25 °C

Sample	dn/dc	$M_w \times 10^{-6}$ (Da)	R_w (nm)	Slope[a] of log(RMS) versus log(M)	Polydispersity (M_w/M_n)	$[\eta]$[b] (mL g^{-1})	$[\eta]_0$[c] (mL g^{-1})	B[d]
EPS RA3W	0.157	2.3 ± 0.07	111.5 ± 1.4	0.55 ± 0.05	1.7	1058	894	0.11
RA3W F1	0.157	2.6 ± 0.16	138.1 ± 1.9	0.56 ± 0.05	1.6	1075	980	0.09
RA3W F2	0.157	1.2 ± 0.05	113.5 ± 1.8	0.51 ± 0.04	1.6	885	865	0.10
EPS9[d]	0.154	1.2 ± 0.05	91.5 ± 1.4	0.42 ± 0.06	1.6	779	860	0.09
EPS9Sm6[d]	0.157	2.1 ± 0.08	128.0 ± 1.9	0.56 ± 0.03	1.9	1855	1673	0.05

[a] RMS = KM^{α}, where exponent α is represented as the slope by $\log(\mathrm{RMS}) = \log(K) + \alpha \log(M)$.
[b] EPS solution of 1 mg mL^{-1} in 0.15 M Na_2SO_4 determined by SEC/LS.
[c] EPS solution of 1.0–5.0 mg mL^{-1} in 0.5 M NaCl determined by Brookfield viscometry.
[d] Ref. 22.

mined by the SEC/LS method, continued to give values (1.31–1.49 × 10^6 Da in 0.5 M NaCl, 0.15 M Na_2SO_4 or 0.1 M NaCl) lower than the reported molecular weight. The nominal value of the standard was attained only by the batch method LS (2.46 × 10^6 Da in 0.1 M NaCl). This led to a study of these dextrans and several EPSs from *E. chrysanthemi* strains by sedimentation equilibrium with the result (Table 4) that the values obtained by SEC/LS and batch method LS at a high ionic strength of 0.5 M were largely confirmed. Clearly the effect of ionic strength of the medium must always be examined when reporting the molecular weights of polysaccharides.

The molecular weight of EPS RA3W is similar to EPS9 and its mutant EPS9Sm6 (Table 3), noting that alcohol precipitation separates the EPS into the fractions of two different molecular sizes.

The slope of log(RMS) versus log(M), where RMS = KM^{α}, is an index of the conformation of polymer in solution, usually being 1.0 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 (Ref. 22 and references cited therein). The molecular weights of the EPS RA3W and its fractions bear a linear relationship to these slopes of 0.5–0.6 (Table 3), implying that the EPS RA3W and its fractions are flexible random coils in aqueous solution, similar to the EPS9 family.

The flexibility of a polyelectrolyte in solution, by an empirical approach, was developed by Smidsrød and Haug[28] who studied the effect of ionic strength on [η]. By plotting [η] against ionic strength ($I^{-0.5}$), the slope S of the line and the value of [η] at the ionic strength 0.1 M, denoted as $[\eta]_{0.1}$, are related:

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

where the average ρ approximately 1.3. The B value reflects approximately the flexibility of polysaccharide chain in solution, the lower the B values the stiffer the chain of the polysaccharide (Ref. 22 and references cited therein). The low B values of the EPSs (Table 3) suggest intermediate flexibilities and confirm the flexible random coil conformation inferred from the LS measurement. Polysaccharides with similar B values are EPS9 family[22] and xanthan (Ac–Pyr = 1:1, B value 0.12).[29]

Table 4. The molecular weight determined by the sedimentation equilibrium and SEC/LS at 25 °C

EPS	Sedimentation equilibrium[a]		SEC/LS[b]
	A_2[c]×10^4 (mL mol g^{-2})	$M_w \times 10^{-6}$ (Da)	$M_w \times 10^{-6}$ (Da)
RA3W	5.90	1.89	2.08
RA3W F1	7.65	2.70	2.56
RA3W F2	10.10	1.49	1.24
Ech9	5.70	1.36	1.18
Ech9Sm6	12.30	2.11	2.08
Ech6	16.60	0.74	0.66
Ech6S+	10.10	0.60	0.62
Dextran 1662 (36K)	14.40	0.04	0.04
Dextran DXT2400 K	2.34	1.34	1.30 (2.46[d])
FF1	3.23	1.91	2.08[e]
FF2	3.68	1.28	1.25[e]
TPK1	14.00	0.75	0.59[f]
TPK2	3.02	0.17	0.28[f]
CU643	8.80	1.33	1.54
A350	8.65	1.33	0.99

[a] In 0.5 M NaCl.
[b] In 0.15 M Na_2SO_4.
[c] The second thermodynamic (osmotic pressure) virial coefficient.
[d] By batch method in 0.1 M NaCl.
[e] Ref. 21.
[f] Ref. 20.

1. Experimental

1.1. Preparation of extracellular polysaccharides

Extracellular polysaccharide was produced on a modified Scott's medium supplemented with 1.5% glucose and 1.5% Difco agar and isolated as described previously.[13,14,18] The crude EPS was precipitated twice from 5% (w/v) NaCl with 2 vol of EtOH, dialyzed against

three changes of distilled water, and lyophilized. The yield of the EPS from RA3W was about 2.4 g L^{-1} of medium.

1.2. Analytical and general methods

The methods used for chromatographic purification of the polysaccharide, glycosyl linkage analysis by methylation, GLC and GLC–MS analyses, 600 MHz 1D ^{1}H NMR spectroscopy, and the light scattering and viscometric analyses have been described previously.[13–19,22]

1.3. Purification of EPS

Fractional precipitation of EPS RA3W with ethanol resulted in two major fractions of EPS RA3WF1 (with 1.1 vol of EtOH) and EPS RA3WF2 (with 1.2 vol of EtOH), which were present in the approximate ratio of 2:1 by dry weight. Each fraction was further purified by low-pressure gel-permeation (ToyoPearl HW65F, TosoHaas, Montgomeryville, PA) and anion-exchange chromatographies (ToyoPearl DEAE-650 M). No neutral oligo- or poly-saccharides were present in the EPS preparations. HPAEC-PAD analysis of the monosaccharide composition (2 M TFA, 120 °C, 1 h) across the polysaccharide peaks from both chromatographies revealed that the EPS is homogeneous and both fractions have the same composition.

1.4. Ultracentrifugation

The EPSs were dissolved at the desired concentration (0.1–1 mg mL^{-1}) in 0.5 M NaCl and dialyzed against the same solvent for 3 days. The equilibrium sedimentation measurements were made at 25 °C using a Beckman optima XLI ultracentrifuge with the Rayleigh interference optical system, equipped with 6-channel cells. Equilibrium sedimentation was reached at rotor speed of 3000–5000 rpm (600–2000*g*) after approximately 72 h. The apparent molecular weight (M_{app}) data was obtained[30] with the parameters of weight average partial specific volume of 0.6 mL g^{-1} used for the EPSs[31–34] and the density (1.02) of the 0.5 M NaCl taken from data reported in the literature.[35] M_{app} was extrapolated to zero concentration to account for thermodynamic nonideality, according to the equation[30]

$$1/M_{app} = 1/M_w + 2A_2C$$

with a set of three concentrations, where M_w is the weight-average molecular weight and A_2 the second thermodynamic (osmotic pressure) virial coefficient (mL mol g^{-2}), obtained from the slope of M_{app} versus concentration.

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