ELSEVIER

Carbohydrate Research 266 (1995) 153–159

CARBOHYDRATE RESEARCH

Extracellular polysaccharide of *Erwinia chrysanthemi*

James S.S. Gray [a], Theodore A.W. Koerner [a,b], Rex Montgomery [a,*]

[a] *Department of Biochemistry, College of Medicine, University of Iowa, Iowa City, IA 52242, USA*

[b] *Department of Pathology, College of Medicine, University of Iowa, Iowa City, Iowa 52242 USA*

Received 26 April 1994; accepted 12 July 1994

Keywords: Polysaccharide, extracellular; *Erwinia chrysanthemi*, extracellular polysaccharide of

The extracellular polysaccharide (EPS) produced by *E. chrysanthemi* pv. *zeae* strain SR260, a strain pathogenic to corn, has a branched hexasaccharide repeat unit consisting of a glucomannorhamnan backbone [→3)-β-D-Glc*p*-(1→4)-α-D-Man*p*-(1→3)-α-L-Rha*p*-(1→] and a trisaccharide side-chain [α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→4)?-GlcA*p*-(1→] attached to O-3 of the mannose in the backbone [1]. All the anomeric configurations, except that of the rhamnosyl-glucosyluronic linkage in the side chain, were unambiguously assigned by a combination of 1 and 2D NMR spectroscopy. The tentative assignment of the rhamnosyl-glucosyluronic glycosyl linkage as α-L is now proven.

1. Experimental

Methods.—Details of the bacterial strain, production, and purification of the EPS, proton NMR, and general methods were as described previously [1].

Smith degradation and purification of the low-molecular-weight products.—Carboxyl-reduced EPS (24 mg) was dissolved in water (20 mL), filtered, and subjected to Smith degradation as described previously [1]. The products were separated by gel chromatography on a ToyoPearl HW40S (TosoHaas, Montgomeryville, PA) column (1.6×51 cm) eluted with water at a flow rate of 18 mL h^{-1} and fractions (1 mL) were collected. Aliquots (25 μL) of each fraction were analyzed for carbohydrate by the phenol–H_2SO_4 method [2]. The leading edge fractions of the major low-molecular-weight peak (F1) and those of

* Corresponding author.

SSDI 0008-6215(94)00250-9

the trailing edge (F3) were pooled and lyophilized. A fraction between the major peak and its shoulder was separately lyophilized (F2).

The purity of the pooled low-molecular-weight peaks were checked by high pH anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD) on a Dionex BioLC (Sunnyvale, CA) on both a PA1 and a MA1 column. The PA1 column (4.6 mm × 25 cm) was eluted at a flow rate of 1 mL min^{-1} with the following gradient: 18 mM NaOH isocratically for 25 min followed by an increase to 100 mM NaOH between 25 and 30 min; finally, a linear NaOAc gradient of 0–500 mM in 100 mM NaOH was run between 30 and 65 min. Detection was by PAD after post-column mixing of the effluent with 300 mM NaOH (0.6 mL min^{-1}). The purity of the low-molecular-weight fragments was also determined by chromatography on a MA1 column (4.6 mm × 25 cm) eluted isocratically with 400 mM NaOH at a flow rate of 0.4 mL min^{-1} with no post-column mixing with 300 mM NaOH.

Methylation analysis.—F1 and F3 (50 μg of each) were methylated by the NaOH–Me_2SO procedure described by Anamula et al. [3]. A portion of each methylated compound was removed for GLC and GLC–MS analyses; the remainder was hydrolyzed and the products converted to the per-*O*-methylated alditol acetates [1] and analyzed by splitless injection on a DB5 capillary column (30 m × 0.25 mm; J&W, Folsom, CA) with He as carrier gas (20 cm s^{-1}) in a Hewlett–Packard 5890 Series II gas chromatograph (Hewlett–Packard, Avondale, PA) equipped with either an FID or a Hewlett–Packard 5971A mass selective detector. The following temperature program was used to analyze methylated F1 and F3: 60°C for 3 min increased to 300°C at 20°C min^{-1}, and held at 300°C for 20 min; the temperature program for analysis of the per-*O*-methylated alditol acetates was as follows: 60°C for 3 min increased to 180°C at 20°C min^{-1}, and held at 180°C for 2 min. This was followed by an increase to 220°C at 5°C min^{-1} and held for 8 min; thereafter it was increased to 240°C at 5° min^{-1} and held at 240°C for 9 min. The injector temperature was held at 245°C and the detector at either 320°C (high-temperature program) or 260°C (low-temperature program).

Methylated F1 and F3 were also analyzed by direct probe chemical ionization mass spectrometry in a Nermag R 10-10 mass spectrometer (Paris, France) with ammonia as the ionizing gas. The ion-source temperature was 200°C and the ionizing voltage was 95 eV.

2. Results and discussion

As described previously [1], a high-molecular-weight and a low-molecular-weight fragment was isolated by chromatography of the first Smith degradation products on ToyoPearl HW40S. A shoulder was found to be present on the trailing edge of the low-molecular-weight peak. Appropriate fractions F1 and F3 were pooled and found to be better than 90% pure by HPAEC–PAD.

Despite the apparent difference in elution times on the PA1 and MA1 columns for F1 and F3, the only products detected in the hydrolyzates of both compounds were equimolar amounts of rhamnose and erythritol.

Methylation analysis of F1 revealed the presence of 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylrhamnitol and 3-*O*-acetyl-1,2,4-tri-*O*-methylerythritol. Quantitation of both partially meth-

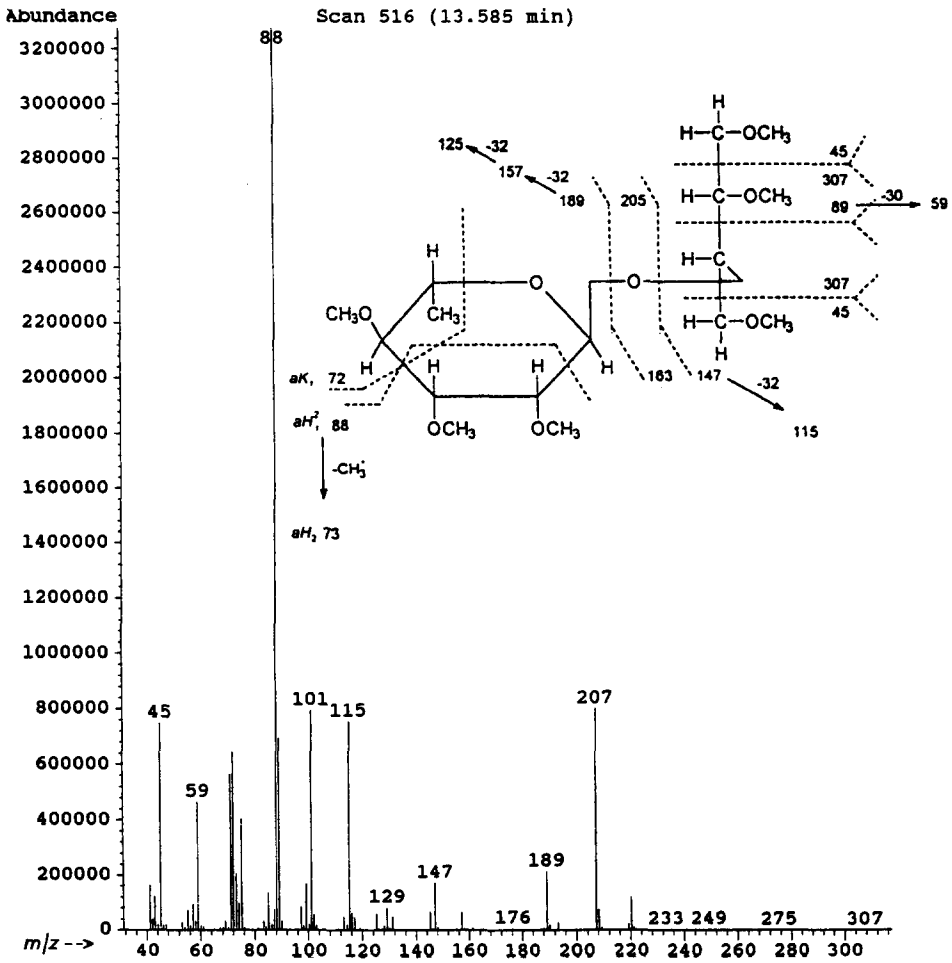


Fig. 1. EIMS spectrum of F1. Inset: the predicted fragmentation pattern of per-*O*-methylated 2-*O*-α-L-rhamnosylerythritol. Possible primary fragmentation patterns are indicated as cleavages between adjacent C-bonds.

ylated alditol acetates, particularly that of the tri-*O*-methylerythritol, was poor due to their volatility. GLC–MS analysis of the per-*O*-methylated F1 (before hydrolysis and conversion to the alditol acetates) gave a peak eluting at 13.57 min with only a trace (<5%) of per-*O*-methylated F3, eluting at 14.97 min, being observed. The EIMS (Fig. 1) was consistent with the structure of the per-*O*-methylated derivative of rhamnosylerythritol, the predicted structure from the Smith degradation of carboxyl-reduced EPS from *E. chrysanthemi* strain SR 260. The origin of the primary ions are depicted in the inset to Fig. 1. Other ions present in the mass spectrum arose from the rhamnose residue by the pathways outlined by Kochetkov and Chizhov [4], Lönngren and Svensson [5], and Lindberg et al. [6], involving a combination of primary and secondary fragmentations, often in concert with the migration of methoxy groups from one carbon atom to another. Following the nomenclature proposed by Kochetkov and Chizhov [4], the following fragments derived from the rhamnosyl residue are observed in the mass spectrum of per-*O*-methylated F1: aA_1, aA_2, and aA_3 (*m/*

Table 1
^{1}H NMR data [a] and residue assignments for the rhamnosylerythritol and its 2-hydroxyethyl-1,3-dioxolane derivative derived by Smith degradation of the carboxyl-reduced EPS from *E. chrysanthemi* pv. *zeae* strain SR260

Residue Symbol	Assignment [b]	Chemical shifts (ppm) and apparent coupling constants (Hz)					
		H-1 ($J_{1,2}$)	H-2 ($J_{2,3}$)	H-3 ($J_{3,4}$)	H-4 ($J_{4,5}$)	H-5 ($J_{5,6}$)	H-6 ($J_{6,6'}$)
Fraction F1 (2)							
E	Erythritol	3.630 (3.4)	3.890 (6.6)	3.725 (5.8)	3.630		
		3.742 (5.1)			(3.5)	3.742	
		$^2J_{1,1'}$ -11.9			$^2J_{4,4'}$ −12.3		
R	α-L-Rha*p*	4.927 (1.7)	3.989 (3.4)	3.802 (9.7)	3.451 (9.7)	3.870 (6.3)	1.279 (<0.1)
Fraction F3 (3)							
E	Erythritol	4.047 (7.1)	4.365 (6.1)	(3.4)	3.751		
		4.019 (6.1)		3.931 (4.3)	3.715		
		$^2J_{1,1'}$ −8.4			$^2J_{4,4'}$ −12.5		
R	α-L-Rha*p*	5.009 (1.7)	3.989 (3.4)	3.801 (9.8)	3.449 (9.7)	3.855 (6.3)	1.280 (<0.1)
D	2-Hydroxyethyl-1,3-dioxolane		4.990 (3.3)	3.680			

[a] Obtained at 600 MHz at 298 K.
[b] Assigned according to the procedures of Koerner et al. [9] using 600-MHz COSY-45 spectra and integrated 1D spectra for each compound.

z 189, 157, and 125, respectively), aJ_1 (*m/z* 207), aB_2 (*m/z* 131), aF_1^2, and aG_1^4 (*m/z* 101), H_1 (*m/z* 88), and K_1 (*m/z* 72).

CIMS analysis of the per-*O*-methylated F1 with ammonia as the ionizing gas gave an M+1 ion of *m/z* 353 and an M+NH_4 ion of *m/z* 370. These values are in excellent agreement with the calculated molecular weight, 352, of the per-*O*-methylated F1.

The 1D NMR spectrum of F1 revealed the presence of a single anomeric signal (δ 4.927; $J_{1,2}$ 1.7 Hz) and three methyl protons (δ 1.279, $J_{5,6}$ 6.3 Hz) (Table 1), similar to values found previously for α-L-Rha*p*1 → 3Rha-ol. Analysis of the COSY-45 and NOESY-45 2D spectra allowed all the signals in the 1D NMR spectrum to be assigned (Table 1). Inspection of the NOESY-45 spectrum of F1 revealed cross-peaks between R-1 and E-3, confirming the Rha-(1 → 4)-GlcA linkage in the side chain of the original polysaccharide as C-3 of the erythritol is derived from C-4 of the reduced GlcA of the EPS side chain. Cross-peaks between R-3 and R-4 but not between R-1 and R-5 were also observed in the NOESY-45 spectrum. These interactions are expected for a Rha residue involved in an α-L linkage. Moreover, the downfield shift of the resonance due to R-5 also confirms the α-L linkage between Rha and GlcA.

Methylation analysis of F3 revealed the presence of 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylrhamnitol and 1,2,3-tri-*O*-acetyl-4-*O*-methylerythritol, which suggest that positions 1 and 2 of the erythritol residue had been substituted in addition to the substitution at position 3 due to linkage to the rhamnose residue. The molecule was stable to the alkaline conditions of the methylation procedure and susceptible to acid hydrolysis as evidenced by the production of rhamnose and erythritol and its per-*O*-methylated alditol after hydrolysis of F3 and per-*O*-methylated F3 by 2 M CF_3CO_2H. The third component in F3 was not detected by the various analyses performed.

The 600-MHz 1D ^1H NMR spectrum of F3 was similar to that of F1 (Table 1) except for the downfield shift of E-1 and E-2 and the appearance of two new signals at δ 4.990 ($J_{1,2}$ 3.3 Hz) and δ 3.680 ($J_{1,2}$ 3.3 Hz). Analysis of the COSY-45 and NOESY-45 spectra (Table 1) revealed that these two signals form part of an isolated system consisting of one methine group and one methylene group, consistent with a –CH–CH_2OH residue in the molecule as already argued. The downfield shift of E-1 and E-2 (from δ 3.630 to δ 4.047 for E-1 and δ 3.890 to δ 4.365 for E-2) and a minor shift for E-3 and E-4 indicate that the O-1 and O-2 of the erythritol have been substituted. Inspection of the NOESY-45 spectrum of F3 shows cross-peaks between E-1 and E-2, E-1, and D-1, and E-2 and D-1, data consistent with the formation of a 1,3-dioxolane ring between O-1, O-2 of the erythritol and glycolaldehyde. The EIMS (Fig. 2) of the per-*O*-methylated F3 revealed a low-intensity ion of *m/z* 379 possibly corresponding to M − 1. This finding supports the possibility that one is dealing with with an acetal in the form of a 1,3-dioxolane ring, as Marshall and Williams [7] and Chizhov et al. [8] observed that the alditol 1,3-dioxolanes readily lose a proton, probably from the acetal-C, using EIMS. Should this be the case with F3, the molecular weight of the per-*O*-methylated derivative is 380, as calculated. CIMS analysis of F3 gave a very strong M + 1 ion (*m/z* 381) and an M + NH_4 ion (*m/z* 398). These values support the molecular weight determined from the EIMS of per-*O*-methylated F3.

The difference in mass between the per-*O*-methylated F1 and F3, after correcting for the methyl groups, is 44. Analysis of the ^1H NMR spectrum of F3 reveals the presence of an isolated spin system containing one methine and one methylene group. This information, together with the information from the mass spectral studies indicates that a hydroxyethyl group, –CH–CH_2OH, which has a calculated molecular mass of 44, is present in F3. These data, together with the susceptibility of the adduct to acid hydrolysis, indicates that F3 possesses a 2-hydroxyethyl-1,3-dioxolane formed between O-1 and O-2 of the rhamnosylerythritol.

The EIMS of per-*O*-methylated F3 (Fig. 2) and its predicted EI fragmentation pattern is consistent with the structure inset to Fig. 2. Many of the ions, particularly those derived from the rhamnose residue, are common to those derived from per-*O*-methylated F1 (Fig. 1). The unique ions to per-*O*-methylated-F3, viz *m/z* 379 (M − 1), 335 (M − 45), 248 (aH_1^1 and aF_1^1), 235 (aJ_1), 205 (aA_1), and 175 (F3-2,3,4-tri-*O*-methyl rhamnopyranose) define the mass not only of the intact molecule but also of its components.

There is no evidence that the 1,3-dioxolane ring occurs naturally in the polysaccharide obtained from *E. chrysanthemi* strain SR 260 and it is concluded that F3 is derived by transacetalation of the rhamnosylerythritol–glycolaldehyde adduct during work-up of the Smith degradation products [10].

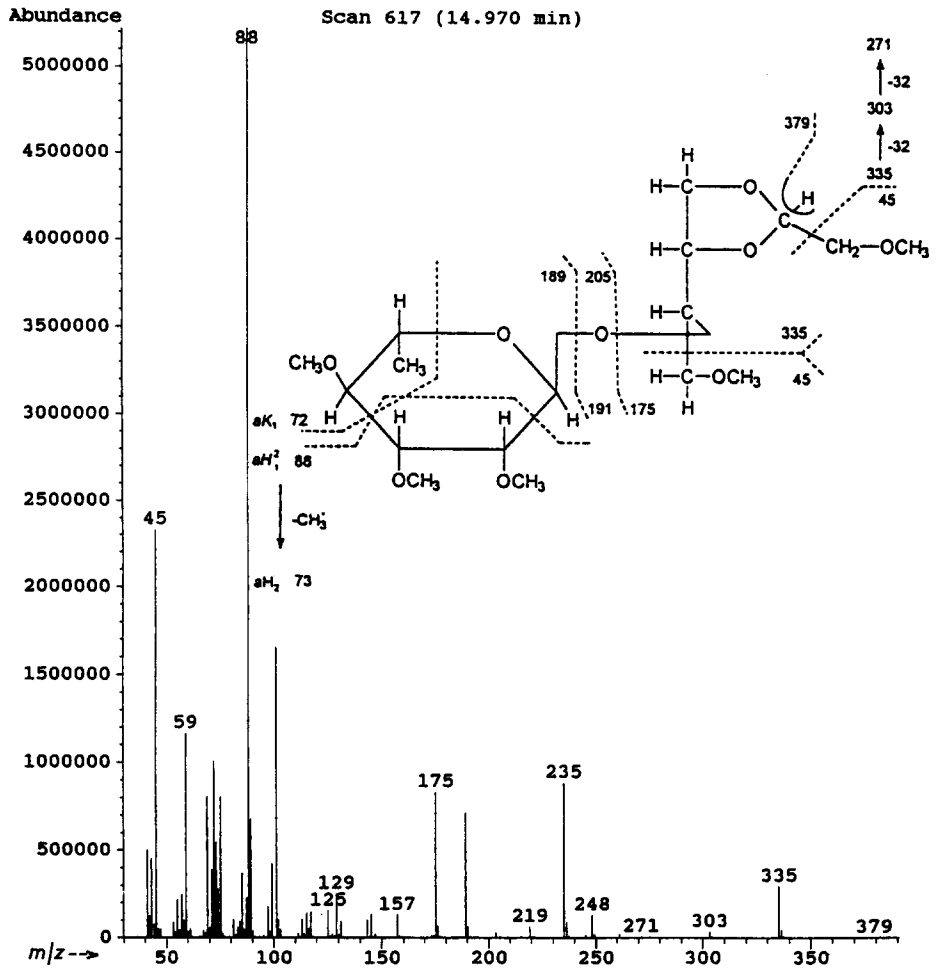
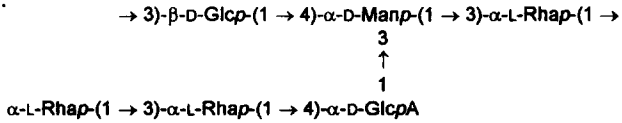


Fig. 2. EIMS spectrum of F3. Inset: the predicted fragmentation pattern of per-*O*-methylated 2-*O*-α-L-rhamnosyl-2-hydroxyethyl-1,3-dioxolane.

Nontheless, the 3-*O*-α-L-rhamnosylerythritol, which is common to both F1 and F3, supports the original proposal [1] that the configuration of the Rha-GlcA in the side chain is indeed α-L, and that the structure of the EPS produced by *E. chrysanthemi* pv. *zeae* strain SR 260 is:



Acknowledgments

The authors wish to thank the Biotechnology Byproducts Consortium (USDA grant No. 91-34188-5943) and the Carbohydrate Structure Facility for the use of its equipment. We also thank John Snyder for the ^{1}H NMR spectra and the GM–MS Facility for the Cl spectra.

References

[1] J.S.S. Gray, J.M. Brand, T.A.W. Koerner, and R. Montgomery, *Carbohydr. Res.*, 245 (1993) 271–287.
[2] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350–356.
[3] K.R. Anumula and P.B. Taylor, *Anal. Biochem.*, 203 (1992) 101–108.
[4] N.K. Kochetkov and O.S. Chizhov, *Adv. Carbohydr. Chem.*, 21 (1966) 39–93.
[5] J. Lönngren and S. Svensson, *Adv Carbohydr. Chem. Biochem.*, 29 (1974) 41–106.
[6] B. Lindberg, F. Lindh, J. Lundsten, and S. Svennson, *Carbohydr. Res.*, 254 (1994) 15–23.
[7] J.T.B. Marshall and D.H. Williams, *Tetrahedron*, 23 (1967) 321–333.
[8] O.S. Chizhov, L.S. Golovkina, and N.S. Wulfson, *Carbohydr. Res.*, 6 (1968) 143–149.
[9] T.A.W. Koerner, J.H. Prestegard, and R.K. Yu, *Methods Enzymol.*, 138 (1987) 38–59.
[10] P.A.J. Gorin and J.F.T. Spencer, *Can. J. Chem.*, 43 (1965) 2978–2984.