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Differential Depolymerization Mechanisms of Pectate Lyases Secreted by *Erwinia chrysanthemi* EC16†

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The four pectate lyases (EC 4.2.2.2) secreted by *Erwinia chrysanthemi* EC16 have been individually produced as recombinant enzymes in *Escherichia coli*. Oligogalacturonates formed from polygalacturonic acid during reactions catalyzed by each enzyme have been determined by high-performance liquid chromatography analysis. PLa catalyzes the formation of a series of oligomers ranging from dimer to dodecamer through a random endolytic depolymerization mechanism. PLb and PLc are trimer- and tetramer-generating enzymes with an identical combination of endolytic and exolytic mechanisms. PLe catalyzes a nonrandom endolytic depolymerization with the formation of dimer as the predominant product. The pectate lyases secreted by *E. chrysanthemi* EC16 represent a battery of enzymes with three distinct approaches to the depolymerization of plant cell walls.

The phytopathogenic activities of many *Erwinia* species are related to their abilities to macerate nonlignified tissues during infection. The initiation of the maceration process involves the depolymerization of pectin and pectate components of the rhamnogalacturonan fraction of the cell walls. Strains of *Erwinia chrysanthemi* effect this depolymerization through the secretion of a combination of pectin esterases and pectate lyases (PLs), along with a regulatory contribution by an exolytic pectate hydrolase activity (3, 5, 6, 8). In addition to these macerating activities, *E. chrysanthemi* strains secrete cellulolytic and proteolytic enzymes that may contribute further to the pathogenic process (2, 4, 14).

The application of molecular genetics has provided detailed information on the organization of the genes coding for pectinolytic enzymes in different strains of *E. chrysanthemi* and *Erwinia carotovora* as well as the regulation of their expression (3, 5, 8). Four structural genes coding for the PLs secreted by *E. chrysanthemi* EC16 have been cloned and sequenced (13). These have been described as catalyzing the endolytic depolymerization of polygalaturonate (PGA), with PLa, PLb, and PLc catalyzing the formation of unsaturated dimer, trimer, and tetramer, respectively, and PLe catalyzing the formation of dimer, tetramer, and larger oligomers (1). By using ion-pair reverse-phase high-performance liquid chromatography (HPLC) and a chromatographic system employing an automatic sample injector, the kinetics of the formation of individual unsaturated oligogalacturonates, ranging in degree of polymerization (DP) from DP 2 to 13, has been determined (10). By using this approach, the recombinant trimer-generating PLb derived from *E. chrysanthemi* EC16 has been shown to have a depolymerization mechanism similar to those of PLs secreted by other bacteria (11, 12). In this study, we have applied HPLC kinetic analysis to compare the depolymerization mechanisms of all of the extracellular PLs of *E. chrysanthemi* EC16 expressed individually as recombinant enzymes in *Escherichia coli*.

HPLC kinetic analysis of individual PLs. The genes coding for the different PLs were derived from *E. chrysanthemi* EC16 and expressed from plasmids in *E. coli* HB101 as previously described (7, 13). PLa, PLb, PLc, and PLe were expressed from plasmids pPEL812, pPEL344, pPEL410, and pPEL748, respectively. Batch cultures (1 liter in 2.8-liter Fernbach flasks) were grown with shaking (140 rpm; New Brunswick G10 gyrotary shaker) at room temperature in Luria broth containing ampicillin (50 μg/ml). Isopropyl-β-D-thiogalactopyranoside was added to a concentration of 1 mM when the culture was initiated (for the overexpression of PLa and PLc) or when the culture turbidity (optical density at 600 nm) was 0.5 to 0.7 (for the overexpression of PLb and PLe). Cells were harvested at early stationary phase (optical density at 600 nm = 2.1 to 2.4) by centrifugation at 5,500 rpm (GSA rotor; DuPont-Sorvall), and the pellets were washed by resuspension with 0.2 M Tris-HCl, pH 8.3, followed by recentrifugation. Periplasmic enzymes were released from cells following spheroplast formation (7, 15).

PLa and PLb were purified from periplasmic fractions by chromatofocusing, as previously described for the purification of analogous activities from *E. chrysanthemi* P860281 (10). PLb was obtained in the preelution buffer fraction, while PLa was obtained in the early fractions of the 1.0 M NaCl wash. The periplasmic fraction containing PLc was dialyzed (Spectrapor 1, 6,000- to 8,000-molecular-weight cutoff) against 5 mM Tris-HCl, pH 6.0, at 5°C overnight, bound to a CM-Sephadex column (1 by 20 cm) equilibrated with the same buffer, and released upon elution with the same buffer containing 0.2 M NaCl. PLe was purified following adsorption to Biogel A 1.5m (Bio-Rad) as described by Keen and Tamaki (7). Purified preparations were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% acrylamide slab gels according to the procedures described by Laemmli (9); proteins were stained with Coomassie brilliant blue R-250. PLb, PLc, and PLe proteins were detected as single polypeptide bands having apparent molecular masses of 37.5, 38.9, and 44.5 kDa, respectively. PLa showed a prominent band of 45.4 kDa and several other less prominent bands. On the basis of their mobilities as determined by SDS-PAGE, these proteins correspond to those previously characterized as pure en-

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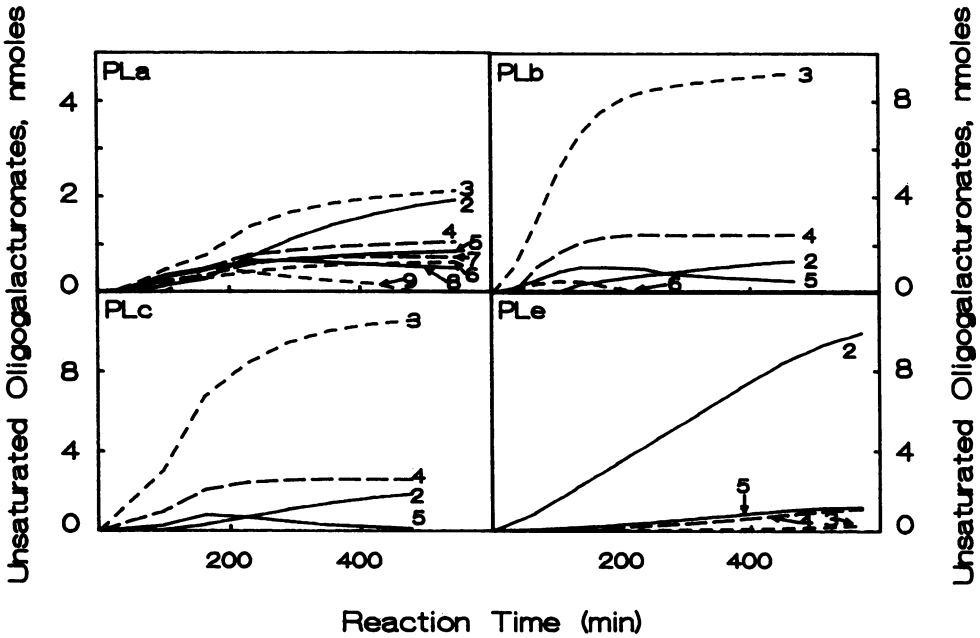

FIG. 1. HPLC kinetic analysis of individual product formation for *E. chrysanthemi* EC16 recombinant PLs. Reaction mixtures contained 0.1% PGA in buffer A and 4.57 U of PLa per ml, 5.38 U of PLb per ml, 2.61 U of PLc per ml, or 3.42 U of PLe per ml. Injections of 0.05 ml were made from reaction mixtures, incubated at room temperature, with a WISP automatic sample injector. Separations of individual products occurred on a C-18 column which was eluted with ion-pairing solvent at 1.0 ml/min with 30-min run times. Specific chromatographic conditions and detection methods were the same as those described previously (10). Numbers beside each curve identify the DP value for each product.

zymes (3, 13). On the basis of specific enzyme activity, PLa was judged to be approximately 50% pure.

The products formed during the lyase-catalyzed depolymerization of PGA were resolved and quantified by reverse-phase ion-pair HPLC, using detection at A_{235} as a measure of the unsaturated residues generated at the nonreducing termini following each eliminative cleavage of a glycosiduronic bond (10). Reaction mixtures contained 0.1% PGA in buffer A (0.05 M Tris-HCl [pH 8.5]–0.2 mM $CaCl_2$). One unit of enzyme activity is defined as that which catalyzes the formation of 1 nmol of unsaturated product per min at room temperature. Kinetic data (Fig. 1) for the formation of individual products are presented as computer-derived graphs (without data curve fitting) of the amounts of individual products detected in samples injected every 30 min throughout the course of reactions. These show the essential identities of the reactions catalyzed by PLb and PLc, as well as the unique aspects of the reactions catalyzed by PLa and PLe. PLa appears to be randomly endolytic, with some preference for the formation of trimer and dimer, and eventual loss of octamer and nonamer. PLb and PLc catalyze the formation of trimer as the predominant product throughout the reaction, tetramer and pentamer early in the reaction, and dimer at the apparent expense of the pentamer. PLe produces dimer as the predominant product throughout the reaction, with much smaller amounts of pentamer and tetramer, which are also produced at a nearly constant rate throughout the reaction. Notable in the case of the PLe-catalyzed reaction is the absence of detectable trimer during the first 200 min; the later appearance of small amounts of trimer may occur at the expense of larger oligomers which are also being converted to dimer, tetramer, or pentamer as the preferred products.

Limit product formation. To identify limit products formed by the different PLs, portions of the reactions (Fig. 1) were incubated at room temperature and analyzed by HPLC after 24 and 40 h of incubation. Profiles for the 40-h incubation are presented in Fig. 2. While this incubation time was not necessarily sufficient to exhaustively digest substrate and generate true limit product for a given enzyme, there was little difference in the amounts of the individual products determined in the 40- and 24-h reactions. The products determined for the 40-h reactions therefore represent practical limits for comparison of the properties of the different PLs analyzed here. Table 1 lists the quantities of the accumulated products for each enzyme and compares the activities of each enzyme remaining after 24 h when measured after the addition of fresh substrate. In all cases, significant activity remained, further establishing that the products accumulated were near, if not true, limit products of the reaction. PLa retained the least activity (36%), while PLe retained the most (95%), with PLb and PLc retaining 50 and 44%, respectively. As in the case of the kinetic profiles (Fig. 1), PLb and PLc catalyze the formation of a nearly identical mixture of products, with trimer as the predominant (68 to 72%) product. In the PLa-catalyzed reaction, there accumulates a continuous series of products ranging from dimer to octamer. PLe catalysis is distinctive in the accumulation of dimer as 76% of the final product, with very little trimer (<2%). PLb and PLc were also similar in their abilities to convert most (86 to 91%) of the initial PGA substrate to detectable oligomers.

Structure and function relationships and comparisons with other PLs. The reverse-phase ion-pair HPLC kinetic analysis establishes the similarities and distinct differences of the PLs secreted by *E. chrysanthemi* EC16. Similarities and differences with respect to limit products formed by these enzymes were noted by Barras et al. (1) on the basis of the

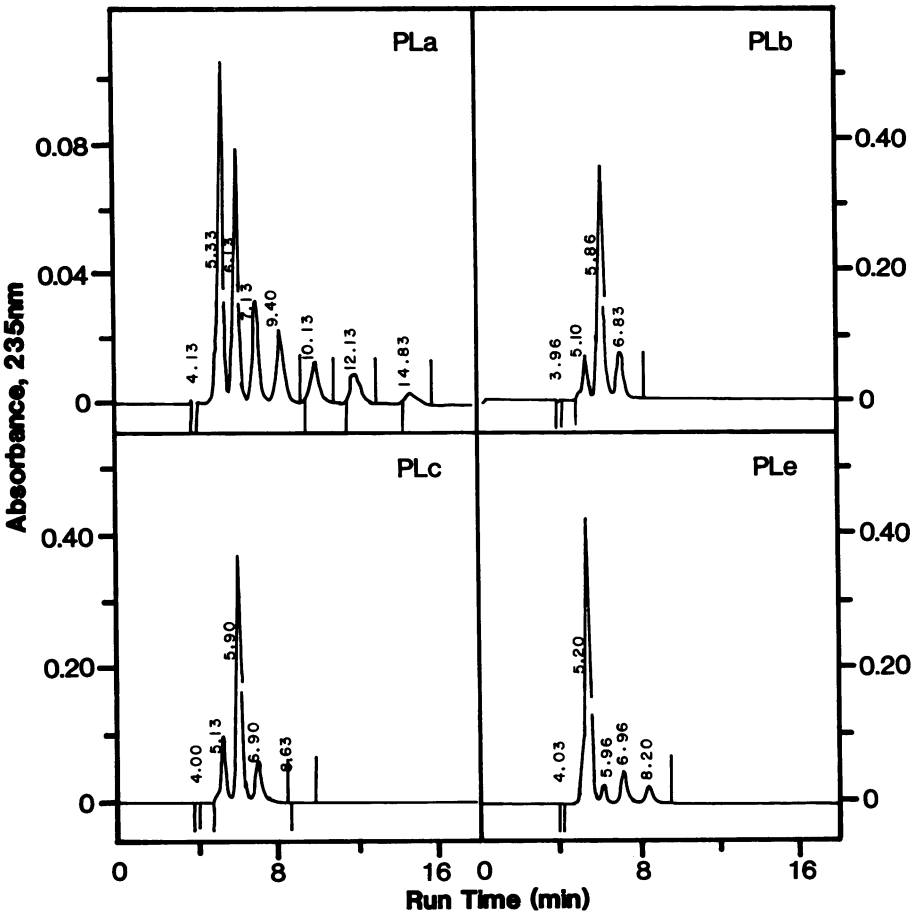

FIG. 2. HPLC profiles of limit reaction products generated for EC16 recombinant PLs. Reaction mixtures were the same as those described for Fig. 1. Samples of 0.05 ml were injected after incubation for 40 h.

separation and detection of the products following paper chromatography. The near limit products we have reported here are in agreement with their observations.

PLa catalyzes a randomly endolytic depolymerization of PGA and in this respect is similar but not identical to PL-CFC, an acidic PL secreted by another *E. chrysanthemi* isolate (10). While PL-CFC was purified to apparent homogeneity, it was not established as a single gene product (as was PLa expressed in *E. coli*) and thus resists a definitive assignment allowed for the process catalyzed by PLa. The limited conversion of substrate to product by PLa, compared with conversion by PLb, PLc, and PLe, may reflect the acidic properties of this enzyme as well as a possible preference for polymers over oligomers during the depolymerization process.

TABLE 1. Unsaturated oligogalacturonates accumulated during PL-catalyzed approach to limit products

PL	V_0[a]	V_{24}[a]	Conversion (%)[b]	Concn (mM) of unsaturated oligomer with Dp of[c]:						
				2	3	4	5	6	7	8
a	4.57	1.63	56	0.32	0.25	0.12	0.09	0.06	0.04	0.02
b	5.38	2.72	86	0.21	1.14	0.26				
c	2.61	1.15	91	0.32	1.19	0.24				
e	3.42	3.26	76	1.34	0.10	0.19	0.12			

[a] Initial velocities as nanomoles of unsaturated product per minute per milliliter of enzyme solution were determined on the recording spectrophotometer at the start of the reaction (V_0) at the same time that the HPLC analyses were initiated. V_{24} values were apparent initial velocities determined with 0.2 dilutions of the reaction mixtures (incubated for 24 h), which were reinitiated with the addition of fresh PGA to a concentration of 0.1%.

[b] Percent conversion for the 40-h reaction of the initial PGA substrate was estimated from the combined mass of the individual oligogalacturonates quantified by HPLC.

[c] Unsaturated oligogalacturonates with DPs of 2 to 8 were quantified (see Fig. 2 profiles) as area units from 0.025-ml injections of 40-h reaction mixtures, converted to nanomoles by comparison to a trimer standard, and corrected to concentration in the reaction mixture.

Both PLb and PLc catalyze an initial endolytic depolymerization of PGA and then rapidly convert products of DP 5 and greater to near limit products of trimer with smaller amounts of tetramer and dimer. This trimer-generating process is also catalyzed by the slightly basic enzyme secreted by *E. chrysanthemi* P860281 (PL-CFB), as well as the acidic enzymes secreted by *Clostridium populeti* and *Lachnospira multipara* (11, 12), which also catalyze the nearly complete conversion of PGA to trimer, dimer, and tetramer. The essentially identical depolymerization process catalyzed by PLb and PLc is not unexpected, since the structural genes coding for these show sequence homology equal to 84% amino acid identity (13). The tandem location of these nearly homologous genes, each with its own promoter, has suggested a relatively recent gene duplication event, with little evolutionary change in primary sequence. Those changes

that have occurred seem to have no effect on the eliminative depolymerization of PGA and may be at least tentatively dismissed as candidates for site-directed mutagenesis studies that are anticipated to define the structural requirements of a particular depolymerization mechanism. It is possible that these changes may affect the activity toward more complex substrates, e.g., rhamnogalacturonan integrated into the cell wall, and comparisons of the activities of these enzymes on such complex polymers seem warranted.

PLe shows a unique depolymerization profile, with the formation of dimer as the predominant product throughout the course of the reaction. The formation of oligomers larger than DP 6 is transient and indicates an initial endolytic cleavage generating oligomers of DP 7 and greater which are in turn rapidly depolymerized to the smaller oligomers. The nearly constant rate of formation of dimer, tetramer, and pentamer throughout the 500-min incubation (Fig. 1) is difficult to explain in terms of an exolytic mechanism. An enzyme that might bind to a terminus and reach in to cleave out a dimer, tetramer, and pentamer would be expected to have as an option the ability to cleave out a trimer as well. The complete absence of trimer in the early stages of the reaction thus argues against an exolytic mechanism accounting for the continued formation of tetramer and pentamer. The preferred formation of pentamer over tetramer and hexamer suggests a nonrandom endolytic process in which the enzyme recognizes a domain of a certain size on the PGA and then selectively cleaves at a point sufficiently distal from the binding site to form the ratio of tetramer/pentamer/hexamer that is observed. The formation of dimer then either requires a separate exolytic process or a single endolytic process in which a complete trimer sequence is not accessible to bound enzyme within the confines of the secondary structural configuration of the PGA in solution. These different possibilities may be distinguished through further examination of the depolymerization process in different environments, as well as site-directed mutagenesis studies.

The DNA sequences of the *pelA* and *pelE* genes have established 62% amino acid identity for the PLa and PLe enzymes (13). This relatively high degree of homology, coupled with the tandem position of the *pelA* and *pelE* genes, suggests, as in the case of the *pelB* and *pelC* genes, a relatively recent gene duplication. If this is the case, the evolutionary changes that have occurred have provided very specific changes in the depolymerization mechanism, along with changes in the pI of the protein products and upstream regulatory sequences. The very basic property of the PLe (pI 10), along with its depolymerization mechanism, may account for its potent macerating activity, especially in relation to PLa (1, 13).

Computer programs comparing the primary structures, deduced from nucleotide sequences, with predicted secondary structures have not revealed domains that can be considered characteristic for an enzyme displaying a particular depolymerization mechanism. Current information of the complete sequences for the structural genes *pelA*, *pelB*, *pelC*, and *pelE* provides an opportunity to apply site-directed mutagenesis to identify specific domains involved in binding and catalysis.

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