

## Pectic Enzyme Complex from *Erwinia carotovora*: A Model for Degradation and Assimilation of Host Pectic Fractions

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### ABSTRACT

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The intracellular pectic enzyme complex of *Erwinia carotovora* (Isolate 14) is comprised of at least four pectate depolymerases, PD I, PD II, PD III, and PD IV. PD I, a single peak when isoelectric focused, has an isoelectric point (pI) of 9.4 and has the ability to depolymerize a sodium polypectate substrate in both an endo-lyase manner at pH 8.5 in the presence of Ca<sup>++</sup>, and in an endo-hydrolase manner at pH 6.0 in the presence or absence of divalent cations. To date the two activities of PD I enzyme(s) have been inseparable. PD I is the only pectic enzyme(s) that is found extracellularly. PD II (pI 8.0) and PD III (pI 6.3) exhibit exo-lyase activity over a broad pH

range (5.5–10.0), with optimal activity at pH 8.5. PD II, which also exhibits limited endo-like activity, requires Mn<sup>++</sup> for optimal activity, while PD III is enhanced by, but not dependent upon, divalent cations for activity. PDIV (pI 6.5), an oligogalacturonide lyase, converts unsaturated dimer and other oligouronides to, primarily, unsaturated monomer at pH 7.5–8.5 and to, predominately, D-galacturonic acid at pH 6.0. PD IV also exhibits very limited exo-lyase and exo-hydrolase activity on sodium polypectate. A model describing the possible mode of action of the pectic enzyme complex in *E. carotovora* is presented.

Phytopathogenic species of the genus *Erwinia* produce a variety of pectic enzymes, including endo- and exo- forms of lyases as well as hydrolases (11,13,17,19). An isolate of *E. chrysanthemi* was observed to produce four extracellular pectate lyases (6), separable only by isoelectric focusing. From another *E. chrysanthemi* isolate, two extracellular lyases were purified through isoelectric focusing (5,6). From both strains, the enzymes with isoelectric points near pH 9.2 exhibited an endo mode of depolymerization, macerated tissue rapidly, and caused cellular death. The lyases with pI's near pH 8.0 were exo-type enzymes, macerated tissue slowly, and also caused cellular death. Nasuno and Starr (17) reported that *E. carotovora* (isolate 153) synthesized a pectinesterase, a polygalacturonase, and a pectate lyase. Although separation of the lyase and hydrolase activities was difficult, complete separation was achieved. Further characterization of the purified lyase of isolate 153 led to the conclusion that there was one extracellular lyase and one intracellular lyase, both having identical properties (11). No exo-type enzymes were reported in this isolate. Subsequently, a second pectic enzyme, an oligogalacturonide lyase (EC 4.2.2.6) was isolated which was able to convert unsaturated digalacturonic acid into two molecules of 4-deoxy-L-threo-5-hexoseulose uronic acid (12). This enzyme reacted with a pectic acid substrate at 1/400 the rate of reaction with the unsaturated dimer.

*Erwinia carotovora* (isolate 14) had been shown to produce one extracellular pectic enzyme, an endo-pectate lyase (EC 4.2.2.2) (13,23). When isolated and purified, this enzyme induces changes in potato tubers which resemble soft rot symptoms. Subsequent research characterized a cAMP regulatory mechanism of this enzyme (8,14). Interpretation of this cyclic nucleotide work was, in part, based upon the assumption that there was only one intracellular pectic enzyme, corresponding to the one extracellular pectic enzyme (13,23). The purpose of this investigation was to isolate and partially characterize the intracellular enzyme(s) of *E. carotovora*,

isolate 14, that depolymerizes sodium polypectate. From the data obtained, a model system is hypothesized to illustrate the possible role and mechanisms of the *E. carotovora*-pectic enzyme complex in tissue maceration and utilization of the oligouronides formed.

### MATERIALS AND METHODS

**Cultural conditions.** EC14 *thr* (14), a threonine auxotrophic mutant of *Erwinia carotovora* (Jones) Holland, was used in all experiments and was maintained on nutrient agar slants supplemented with 0.5% yeast extract, 1.0% casein hydrolysate, and 0.05% glucose.

Media for enzyme extractions contained 50 ml of minimal salts (MS) (0.02% MgSO<sub>4</sub>, 0.15% KH<sub>2</sub>PO<sub>4</sub>, 0.715% Na<sub>2</sub>HPO<sub>4</sub>, and 0.3% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>) supplemented with 0.02% threonine and 1.0% sodium polypectate (NaPP). Bacteria were aerated at 30 C for 8–12 hr on a shaking water bath (150 rpm). A 1-ml sample was then transferred to 500 ml of the same medium and incubated on a rotary shaker (150 rpm) at room temperature. Aeration continued until mid- to late log phase was reached (OD<sub>600nm</sub> = 0.6–0.8 as measured in a Bausch and Lomb Spectronic 20 colorimeter).

**Enzyme extraction and purification.** Cells from the liquid shake cultures described above were collected by centrifugation (20,000 g at 4 C for 20 min) using a GSA rotor in a Sorvall RC2-B refrigerated centrifuge. The supernatant fluid was saved for isolation of extracellular enzyme. The bacteria were resuspended in an equal volume of 0.05 M Tris-HCl buffer (pH 8.0) and recentrifuged to wash away any residual extracellular enzymes. The pelleted cells were resuspended in 1–2 ml of the same buffer and lysed by ultrasonification with a Branson Sonifier Cell Disrupter microtip at 30 W for 3 min. The cell sonicate was centrifuged (20,000 g at 4 C for 20 min) and the supernatant fluid was saved for further purification of the intracellular pectic enzymes. Purification was performed according to the method of Mount et al (13).

The crude enzyme preparation, either intracellular or extracellular, was brought to 50% saturation with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, centrifuged at 20,000 g at 4 C for 30 min, and the precipitate was

discarded. The supernatant fluid was then brought to 95%  $(\text{NH}_4)_2\text{SO}_4$  saturation and centrifuged as before. The resulting precipitate was resuspended in 1–2 ml of distilled water ( $\text{dH}_2\text{O}$ ) and dialyzed overnight at 4 C against several liters of  $\text{dH}_2\text{O}$ . The dialysate was applied to a 2.5 × 25-cm diethylaminoethyl (DEAE) cellulose column which had been equilibrated with 0.05 M Tris-HCl (pH 8.0) and eluted at 4 C with 30 ml of the same buffer followed by a NaCl stepwise gradient. Five-milliliter fractions were collected and assayed for pectic enzyme activity.

Fractions from each peak showing lyase activity were pooled and subjected to isoelectric focusing in an LKB 8101 Ampholine electrofocusing apparatus (LKB-Producter AB, Bromma, Sweden) and an LKB 3371 D DC power source. The column contained ampholine carriers with a range of pH 3–10 and electrofocusing was carried out at 4 C for 72–96 hr, until the voltage had stabilized. Five - milliliter fractions were collected and the pH was measured immediately. These fractions were dialyzed overnight at 4 C against several liters of  $\text{dH}_2\text{O}$  and analyzed for lyase activity.

Protein concentrations were determined by the method of Lowry et al (10) with bovine serum albumin as a standard.

**Enzyme assays.** Lyase enzyme activity was measured by the periodate-thiobarbituric acid assay (TBA) (20), the Nelson's reducing group analysis (18), and/or the viscometric assay (1,2). For initial screening of enzyme activity throughout purification, the substrate was 1.2% NaPP in 0.05 M Tris-HCl buffer (pH 8.5) containing  $2 \times 10^{-4}$  M  $\text{Ca}^{++}$  ( $\text{CaCl}_2$ ). For both the reducing group and TBA assays, 100  $\mu\text{l}$  of enzyme solution was incubated with 100  $\mu\text{l}$  of substrate at 30 C for 1 hr or varying times. In order to detect hydrolase activity at low pH, the reducing group analysis

mentioned above was employed, but utilizing a modified substrate consisting of 1.2% NaPP (in 0.05 M phosphate buffer, pH 6.0) pretreated with 0.5 mM EDTA to chelate any endogenous divalent ions. For the TBA assay, an optical density increase of 0.3 at  $A_{549}$  indicated the formation of 0.1  $\mu\text{mole}$  of unsaturated oligouronide from NaPP as determined in our laboratory using known amounts of unsaturated reducing sugars. For screening of column fractions, a unit of enzyme activity was expressed as the amount of enzyme yielding 0.1  $\mu\text{mole}$  of unsaturated product in 1 hr. Otherwise, activity was expressed as microkatal per kilogram of protein, where one katal is equal to one mole of unsaturated product formed per second. For hydrolase activity, one katal is equal to one mole of reducing sugars formed per second using D-galacturonic acid as the standard. For the viscometry assay, 5.0 ml of substrate (1.2% NaPP in 0.05 M Tris-HCl, pH 8.5, containing 0.1 mM  $\text{Ca}^{++}$  or  $\text{Mn}^{++}$ ) plus 1.0 ml of enzyme solution were reacted in an Ostwald-Fenske 300 viscometer and a unit of depolymerization activity was expressed as the reciprocal of the time in minutes for a 50% loss in viscosity times  $10^3$  (1,2). Specific activity is units of activity per milligram of protein. Autoclaved enzyme and/or  $\text{dH}_2\text{O}$  were used as controls.

**Characterization of enzymes.** The pH optimum of enzyme activity was determined by the TBA assay or the reducing group analysis utilizing 1.2% NaPP in four buffer systems: 0.05 M acetate buffer (pH 4.5–5.5), 0.05 M phosphate buffer (pH 6.0–7.0), 0.05 M Tris-HCl buffer (pH 7.5–9.0), and 0.05 M NaOH-glycine buffer (pH 9.5–10.0). Enzyme-substrate reaction mixtures were incubated at 30 C for 1 hr or for various time intervals.

To determine ion dependency for enzyme activity, 1.2% NaPP was dissolved in 0.05 M Tris-HCl buffer at pH 8.5, or 0.05 M phosphate buffer at pH 6.0, followed by the addition of EDTA to a final concentration of 0.5 mM to chelate endogenous ions.  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$ , or  $\text{Mn}^{++}$  was then added to a final concentration of 1.0 mM. These specific substrates were used with the TBA, reducing group, and viscometry assays to determine ionic requirements and in all further characterization studies.

**Identification of reaction products.** Reaction products were separated on descending paper chromatograms with pyridine-ethyl acetate-water-acetic acid (5:5:3:1, v/v) as solvent (11,21). After incubation of reaction mixtures at 30 C for varying times, 50- $\mu\text{l}$  samples were applied to Whatman No. 4 filter paper and allowed to migrate for 10–12 hr at room temperature. The chromatograms were developed with saturated silver nitrate in acetone and 0.5 N NaOH in ethanol (22).

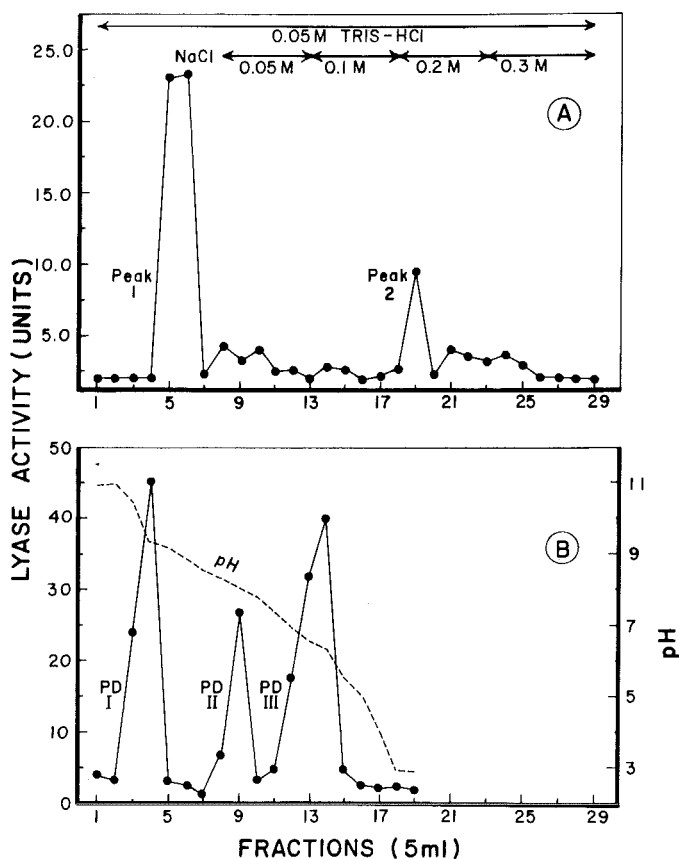
The chain length of the enzymatic breakdown products of NaPP was determined by comparison of the migration rate of the products on paper chromatograms to the migration rate of standard, D-galacturonic acid ( $R_{\text{gal}}$  value). These  $R_{\text{gal}}$  values for the breakdown products were compared to those reported in the literature using the identical solvent system (12,15,16). Chain length of selected reaction products was determined by comparing the ratio of uronic acid units, as determined by the carbazole analysis (3), to free reducing groups (18).

**Chemicals.** All chemicals used in this investigation were purchased from Sigma Chemical Company, P.O. Box 14508, St. Louis, Mo 63178.

## RESULTS

**Enzyme purification.** Chromatography of the intracellular pectic enzymes on DEAE cellulose resulted in two TBA-positive fractions (Fig. 1A). Peak 1 contained the highest activity and was eluted in the void volume, and peak 2 was eluted with 0.2 M NaCl. Isoelectric focusing (ampholine range, pH 3–10) of peak 1 yielded three TBA-positive fractions with isoelectric points of 9.4, 8.0, and 6.3 (Fig. 1B). In this communication, these three pectate depolymerases are referred to as PD I (pI 9.4), PD II (pI 8.0), and PD III (pI 6.3). As previously shown (13,23), DEAE cellulose chromatography and isoelectric focusing of extracellular pectic enzyme preparations resulted in a single TBA-positive enzyme with a pI of 9.4, and is referred to here as PD Ia. PD II and PD III were never detected in any extracellular enzyme extractions.

Purification of peak 2 from DEAE cellulose by isoelectric



**Fig. 1.** Purification of the intracellular pectic enzymes from *Erwinia carotovora* by DEAE cellulose chromatography and isoelectric focusing. Enzyme activity was analyzed by the periodate-thiobarbituric acid assay (20). Units are defined as the amount of enzyme causing a change in  $A_{548\text{nm}}$  of 0.1 in 4 hr. A, DEAE cellulose chromatography of the 50–90%  $(\text{NH}_4)_2\text{SO}_4$  enzyme preparation. Enzymes were eluted by a stepwise gradient of 0–0.3 M NaCl in 0.05 M Tris-HCl buffer (pH 8.0). B, Isoelectric focusing of DEAE cellulose peak 1 in an ampholine range of pH 3–10.

focusing produced one TBA-positive fraction with an isoelectric point of 6.5 (author's data, unpublished). This enzyme, designated as PD IV, was detected only in intracellular extractions.

**Characterizations of PD I through IV.** Both purified PD I and PD Ia had isoelectric points of 9.4. As previously demonstrated with PD Ia (23), PD I exhibited optimal activity at pH 8.0–8.5 in the presence of 1.0 mM Ca<sup>++</sup>. Although there was slight lyase activity in the presence of 1.0 mM Mn<sup>++</sup> (Table 1), Ca<sup>++</sup> was definitely the preferred ion. No activity was detected in the presence of 1.0 mM Mg<sup>++</sup> or in the absence of ions. A random mode of substrate depolymerization was indicated by the rapid decrease in viscosity of a NaPP solution (Table 2), and by the yielding of several, different-sized oligouronides as reaction products (Table 3). These products were found to be unsaturated (Table 4). Estimates of PD I reaction product sizes ranged from unsaturated trimer to unsaturated hexamer, as indicated by similar R<sub>gal</sub> values of previous reports (12, 15, 16). In reaction mixtures at pH 6.0 in the presence of 1.0 mM Ca<sup>++</sup>, Mn<sup>++</sup>, or Mg<sup>++</sup>, or when no ions were supplemented, isoelectric-focused purified PD I and PD Ia rapidly depolymerized sodium polypectate in a random fashion, forming many different-sized reaction products (Table 3). As determined by a positive reducing group analysis and negative TBA reaction (Table 4), these

products were saturated. Heat inactivation studies of PD I demonstrated significant differences between the hydrolase and lyase activities (Fig. 2). When PD I was pretreated at 80 C for various time intervals prior to reaction with sodium polypectate, hydrolase activity was completely lost after 6 min, while lyase activity was only reduced by 50%. Even after 15 min of heat pretreatment, 25% of the lyase activity was still evident. Other than by heat inactivation, we have not been able to separate lyase and hydrolase activities.

PD II (pI 8.0) exhibited activity from pH 5.5–10.0 with optimal activity at pH 8.5 in the presence of 1.0 mM Mn<sup>++</sup>. Enzyme activity was moderate or very low when reaction mixtures were supplemented with 1.0 mM Mg<sup>++</sup> or Ca<sup>++</sup>, respectively (Table 1). When endogenous ions were chelated with EDTA, no activity was detected. Under optimal conditions, PD II was much less effective than PD I in reducing the viscosity of NaPP (16-fold difference in specific activities) (Table 2). Chromatographic analysis of PD II reaction mixtures that had been buffered at pH 8.5 and supplemented with 1.0 mM Mn<sup>++</sup> revealed two products (Table 3). While trace amounts of an oligouronide with an R<sub>gal</sub> of 0.42 was produced, the predominant product (R<sub>gal</sub> = 0.72) was found to be an unsaturated digalacturonic acid (dimer) (see PD III below). Although PD II appears to exhibit an exo-mode of

TABLE 1. Effect of divalent cations on the pectate lyase activity of three intracellular pectate depolymerases from *Erwinia carotovora*

Purified enzyme	EDTA	Lyase specific activity <sup>a</sup>		
		Ca <sup>++</sup>	Mn <sup>++</sup>	Mg <sup>++</sup>
PD I	0	1,972	119	0
PD II	0	17	181	44
PD III	19	27	28	28

<sup>a</sup> Reaction mixtures contained 100 μl of isoelectric focused enzyme plus 100 μl of 1.2% sodium polypectate in 0.05 M Tris-HCl buffer, pH 8.5 which had been pretreated with 0.5 mM EDTA. Specific ions (Ca<sup>++</sup>, Mn<sup>++</sup>, or Mg<sup>++</sup>) were then supplemented to a final concentration of 1.0 mM. Mixtures were incubated at 30 C for 1 hr, and analyzed by the TBA assay (20). Specific activity is expressed as microkatal per kilogram of protein. One katal is equal to one mole of unsaturated product formed per second.

TABLE 2. The abilities of three intracellular pectate lyases from *Erwinia carotovora* to decrease in the viscosity of sodium polypectate<sup>a</sup>

Purified enzyme	Ionic conditions	Specific activity <sup>b</sup>
PD I	Ca <sup>++</sup>	1,020
	Mn <sup>++</sup>	<1.0
PD II	Ca <sup>++</sup>	<1.0
	Mn <sup>++</sup>	64
PD III	Ca <sup>++</sup>	<1.0
	Mn <sup>++</sup>	<1.0

<sup>a</sup> One milliliter of isoelectric focused enzyme was added to 5 ml of 1.2% sodium polypectate (NaPP) in 0.05 M Tris-HCl, pH 8.5 buffer plus 1.0 mM Ca<sup>++</sup> or Mn<sup>++</sup> in an Ostwald-Fenske 300 viscometer. Depolymerization of NaPP was measured over time.

<sup>b</sup> Specific activity is the reciprocal of time (minutes) to cause a 50% reduction in viscometry times 10<sup>3</sup>, per milligram of protein (1,2).

TABLE 3. Chromatographic mobilities of products formed from the degradation of sodium polypectate by pectate depolymerase from *Erwinia carotovora*<sup>a</sup>

Reaction conditions <sup>b</sup>	R <sub>gal</sub> values <sup>c</sup>	Unsaturated product formed <sup>d</sup>	Probable product
PD I/PD Ia, pH 6.0 ± Ca <sup>++</sup>	0.68 (t) <sup>e</sup> , 0.35, 0.19, 0.1, 0.04	–	s <sup>e</sup> dimer - hexamer
PD I/PD Ia, pH 8.5 + Ca <sup>++</sup>	0.42, 0.22, 0.09, 0.04	+	u trimer-hexamer
PD II, pH 6.0 + Mn <sup>++</sup>	0.42(t), 0.22(t), 0.09(t), 0.04(t)	+	u trimer - hexamer
PD II, pH 8.5 + Mn <sup>++</sup>	0.72, 0.42(t)	+	u dimer and trimer
PD III, pH 6.0 ± Ca <sup>++</sup> or Mn <sup>++</sup>	0.72	+	u dimer
PD III, pH 8.5 ± Ca <sup>++</sup> or Mn <sup>++</sup>	0.72	+	u dimer
PD IV, pH 8.5 + Ca <sup>++</sup>	0.72, 0.42(t)	+	u dimer and trimer
PD IV + PD III product, pH 8.5 + Ca <sup>++</sup>	1.42	+	u monomer
PD I–PD IV, pH 8.5 + Ca <sup>++</sup> or Mn <sup>++</sup>	1.42, 0.72	+	u monomer and dimer
PD I–PD IV, pH 6.0 ± Ca <sup>++</sup> or Mn <sup>++</sup>	1.42(t), 1.0, 0.72, 0.42, 0.20, 0.04	±	u monomer-trimer D-galacturonic acid s and/or u tetramer and hexamer

<sup>a</sup> Descending paper chromatography on Whatman No. 4 paper was carried out for 10–12 hr in pyridine, ethyl acetate, water, and acetic acid (5:5:3:1, v/v). Chromatograms were developed with saturated silver nitrate in acetone and 0.5 N NaOH in ethanol and washed in 6 M NH<sub>4</sub>OH (21,22).

<sup>b</sup> Reaction mixtures contained 100 μl of substrate (1.2% sodium polypectate in 0.05 M Tris-HCl buffer, pH 8.5 or 0.05 M phosphate buffer, pH 6.0, 0.5 M EDTA, and Ca<sup>++</sup> or Mn<sup>++</sup> to a final concentration of 1.0 mM) and 100 μl of enzyme. Incubation was for 1–5 hr (except PD IV; 16 hr) at 30 C. PD I–IV are intracellular pectate depolymerases and PD Ia is an extracellular depolymerase.

<sup>c</sup> R<sub>gal</sub> values indicate the ratio of product migration to the distance migrated by a D-galacturonic acid standard.

<sup>d</sup> Unsaturated products in reaction mixtures were determined by the periodate-thiobarbituric acid assay (20).

<sup>e</sup> Letters indicate (t) = trace amounts, u = unsaturated, s = saturated.

depolymerization of NaPP under alkaline conditions, a limited endo-depolymerization of substrate is evident when reacted in the acidic range. When reaction mixtures were run at pH 6.0 with  $Mn^{++}$ , trace amounts of unsaturated products with  $R_{gal}$  values similar to those of PD I were observed (Table 3). Unlike PD I, PD II always showed a lyase mode of action.

The pH activity profile for PD III was similar to that of PD II. Enzyme activity was enhanced slightly by supplemented ions ( $Ca^{++}$ ,

TABLE 4. Hydrolase and lyase activities of PD I<sup>a</sup> from *Erwinia carotovora*

Substrate conditions <sup>b</sup>	Specific activity <sup>c</sup>	
	Unsaturated product <sup>d</sup> ( $\times 10^3$ )	Reducing product <sup>e</sup> ( $\times 10^3$ )
NaPP (pH 8.5) + $Ca^{++}$	12.7	12.9
NaPP (pH 6.0) + $Ca^{++}$	0.00	12.5

<sup>a</sup>PD I is an intracellular endo-pectate depolymerase.

<sup>b</sup>One hundred microliters of isoelectric focused PD I plus 100  $\mu$ l of 1.2% sodium polypectate (NaPP) in 0.05 M Tris-HCl buffer, pH 8.5 or 0.05 M phosphate buffer, pH 6.0 plus 1.0 mM  $Ca^{++}$  were incubated at 30 C for 1 hr. Products were analyzed by the TBA (20) or Nelson's free reducing group assays (18).

<sup>c</sup>Specific activity is expressed as microkatals per kg protein.

<sup>d</sup>One katal is equal to one mole of unsaturated oligouronide formed from NaPP per second.

<sup>e</sup>One katal is equal to one mole of free reducing sugar released from NaPP per second.

TABLE 5. Characterization of an unsaturated dimer and an unsaturated monomer formed by pectate depolymerases III and IV (PD III and IV) from *Erwinia carotovora*

Compound	Migration of product formed <sup>c</sup>	COOH/CHO <sup>d</sup>	Unsaturated product formed <sup>e</sup>	Uronic acid residues per chain length
Unsaturated dimer <sup>a</sup>	$R_{gal}$ 0.72	1.9:1.0	+	2
Unsaturated monomer <sup>b</sup>	$R_{gal}$ 1.42	0.97:1.0	+	1

<sup>a</sup>Product formed by the reaction of PD III with sodium polypectate at pH 8.5 plus 1.0 mM  $Mn^{++}$ .

<sup>b</sup>Product formed by the reaction of PD IV with  $R_{gal}$  0.72 product formed by PD III at pH 8.5 plus 1.0 mM  $Mn^{++}$ .

<sup>c</sup> $R_{gal}$  is the ratio of the distance migrated on a paper chromatogram (see text) of an unknown product to the distance migrated by a D-galacturonic standard.

<sup>d</sup>Estimation of uronic acid residues and free reducing groups as determined by carbazole (3) and reducing group analysis (18), respectively.

<sup>e</sup>Double bond formation was detected by the TBA assay (20).

TABLE 6. Activity of DEAE cellulose purified PD IV under acidic and alkaline conditions as indicated by the formation of unsaturated oligouronides or the release of free reducing sugars from sodium polypectate (NaPP)

Substrate <sup>a</sup>	Specific activity <sup>b</sup>	
	Unsaturated product <sup>c</sup>	Reducing product <sup>d</sup>
NaPP (pH 8.5) + $Ca^{++}$	875	950
NaPP (pH 8.5)	67	50
NaPP (pH 6.0) + $Ca^{++}$	544	1,353
NaPP (pH 6.0)	67	1,100

<sup>a</sup>Substrates contained 100  $\mu$ l of 1.2% NaPP in either 0.05 M Tris-HCl buffer, pH 8.5, or 0.05 M phosphate buffer, pH 6.0 and with or without 1.0 mM  $Ca^{++}$ . Reaction mixtures were incubated at 30 C for 16 hr with 100  $\mu$ l of enzyme.

<sup>b</sup>Specific activity is expressed as microkatals per kilogram of protein.

<sup>c</sup>One katal is equal to one mole of unsaturated oligouronide formed from NaPP per second as measured by the periodate-thiobarbituric acid assay (20).

<sup>d</sup>One katal is equal to one mole of free reducing sugar released from NaPP per second as measured by the free reducing group analysis (18).

$Mn^{++}$ ,  $Mg^{++}$ ) but did not require these ions for activity (Table 1). Irrespective of substrate ionic conditions, PD III decreased the viscosity of NaPP in a manner indicating an exo-mode (terminal) of substrate depolymerization (Table 2). Analysis of chromatograms revealed the formation of one product ( $R_{gal} = 0.72$ ) from PD III reaction mixtures at pH 6.0 or 8.5 with or without supplemented ions (Table 3). This product was found to be unsaturated and had a ratio of free reducing groups to uronic acid units of 1:2, indicating that the product formed was an unsaturated dimer (Table 5). Acid hydrolysis of the dimer resulted in the migration of two spots on paper chromatograms; one corresponding to a D-galacturonic acid standard and one corresponding to a 5-formyl-2-furancarboxylic acid (4).

PD IV (pI 6.5), unlike the other pectate depolymerases, was unstable after DEAE cellulose chromatography unless immediately lyophilized and stored at -20 C. Isoelectric focusing of PD IV resulted in the recovery of only 30% of the DEAE cellulose purified preparation. Within 2 hr after isoelectric focusing, no activity was detected, even if storage conditions were at -20 C. Therefore, experiments dealing with PD IV were performed with the more stable DEAE cellulose purified enzyme fraction.

In the presence of NaPP and 1.0 mM  $Ca^{++}$ , PD IV exhibited optimal lyase activity from pH 7.5-8.5. Trace amounts of two oligouronides ( $R_{gal} = 0.42$  and 0.72) were produced from reaction mixtures at pH 8.5, indicating an exo-type of pectate depolymerization (Table 3). PD IV lyase activity at pH 8.5 is  $Ca^{++}$ -dependent (Table 6). Reaction mixtures at pH 6.0, with or without  $Ca^{++}$ , indicated predominantly hydrolytic activity. However, in order to obtain sufficient amounts of products for analysis, reaction mixtures of PD IV with NaPP at either pH had to be incubated for at least 16 hr.

Moran et al (12) reported an oligogalacturonide lyase which exhibited great affinity for oligouronides, especially unsaturated dimer, over pectin as a substrate. When PD IV was reacted with the unsaturated dimer produced by PD III, a single product with an  $R_{gal}$  value of 1.42 was formed (Table 5). This product was highly reactive in the TBA assay and yielded a 1:1 ratio of uronic acid units to free reducing groups, indicating an unsaturated monomer (Table 5). Based on this data, PD IV is tentatively classified as an oligogalacturonide lyase (EC 4.2.2.6).

When PD IV was reacted against NaPP in conjunction with isoelectric focused PD I, PD II, and PD III, the smallest products

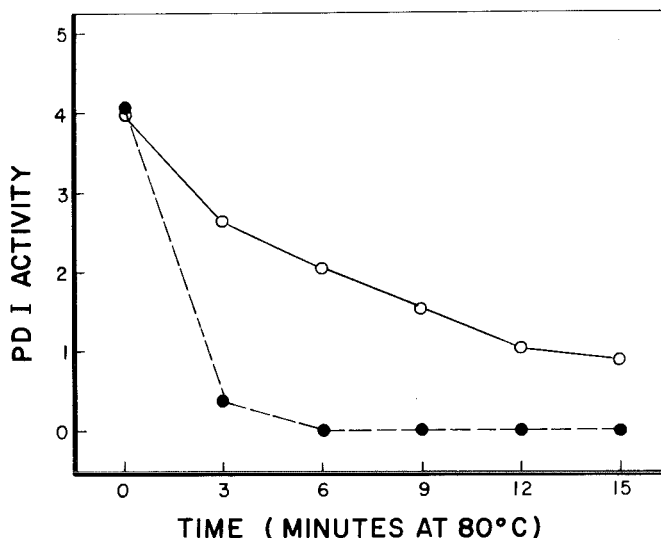


Fig. 2. Heat inactivation of isoelectric-focused purified PD I from *Erwinia carotovora* by preincubating the enzyme at 80 C for various time intervals. Hydrolase activity (●---●) was measured at pH 6.0 by the free reducing group analysis (18). Lyase activity (○—○) was assayed at pH 8.5 with  $Ca^{++}$  by the periodate-thiobarbituric acid assay (20). Specific activity is expressed as  $\mu$ moles of product per hour per milligram protein. Each specific activity unit is equal to 278 microkatals per kilogram of protein.

formed at pH 6.0 were D-galacturonic acid and trace amounts of unsaturated monomer (Table 3). At pH 8.5 this mixture of pectic enzymes produced only the unsaturated monomer as the smallest product. The unsaturated monomer and D-galacturonic acid were not formed by any combination of the intracellular purified enzymes unless PD IV was present. Although PD IV has not been extensively purified, this enzyme "complex" seems to act in a hydrolase or lyase manner yielding saturated and unsaturated monomers under different pH conditions.

It appeared that differential responses to divalent ion supplements could offer a means of separately detecting the presence of each enzyme in a mixture of all four. However, rate of substrate degradation, differential substrate affinity, and degree of reactivity of the various breakdown products made this very difficult with purified preparations and impossible with crude extracts.

### DISCUSSION

Previous investigations on *E. carotovora* have demonstrated inducibility and cAMP regulation of at least one endo-pectate lyase (8,14,24,25). In order to more fully define and understand pectic enzyme regulation and how these enzymes interrelate to the pathogenic process of plant cell wall hydrolysis, it was necessary to investigate all the pectic enzyme types produced by our *E. carotovora* isolate.

*E. carotovora* synthesized at least three intracellular pectate lyases, as well as one oligogalacturonide lyase. Only one of these enzymes, PD Ia was found outside of the bacterium. The data strongly suggest that PD I and PD Ia (endo-pectate lyase, pI 9.4, intracellular and extracellular, respectively), are identical. PD Ia was found in culture filtrates of nutrient media as well as in rotted potato tubers (author's data, unpublished). Of significance was the observation that a single isoelectric focused peak of PD I or PD Ia contained dual enzymatic activity. In reaction mixtures at pH 8.5

containing  $Ca^{++}$ , NaPP was cleaved in a lytic manner, resulting in formation of unsaturated oligouronides. At pH 6.0 in the presence or absence of ions, cleavage of NaPP was effected in a hydrolytic manner; only saturated products were produced. Both of these depolymerizations were performed in a random fashion. Purified PD I, when reacted with potato tuber disks at pH 6.0 or 8.5, caused tissues maceration and cellular death (author's data, unpublished). Unlike the hydrolase and lyase reported by Nasuno and Starr (17), we have not yet been successful in separating the two activities of PD I. However, temperature inactivation of PD I and PD Ia showed preferential loss of hydrolase activity over that of the lyase portion. Whether this indicates two separate enzymes with similar physical properties, or possibly the loss of a heat labile coenzyme or subunit necessary for hydrolase activity remains to be determined. It is not known why only PD Ia is found extracellularly. A positive recognition of PD I by a site on the membrane could be present which selectively transports PD I out of the cell. Some strains of *Erwinia chrysanthemi* produce several pectate lyases that are found extracellularly (6).

The two remaining intracellular pectate lyases, PD II and PD III, both produce unsaturated dimers over a broad pH range. PD II is dependent on divalent cations (especially  $Mn^{++}$ ) and shows some endo-like activity, although it exhibits predominately exo-activity. PD III is strictly an exo-enzyme and is enhanced by, but not dependent upon, divalent cations. Although the molecule which interacts at the DNA level has not been clearly identified, the unsaturated digalacturonic acid has been shown to efficiently induce endo-pectate lyase synthesis (7,24). As a result of PD II and PD III, unsaturated dimer is readily available for this induction process.

PD IV has been termed an oligogalacturonide lyase; it exhibited characteristics associated with those described for a similar enzyme isolated by Moran et al (12). Although PD IV could depolymerize NaPP in an exo-manner at a very slow rate, it easily converted unsaturated digalacturonic acid (at pH 8.5) into (presumably) 4-

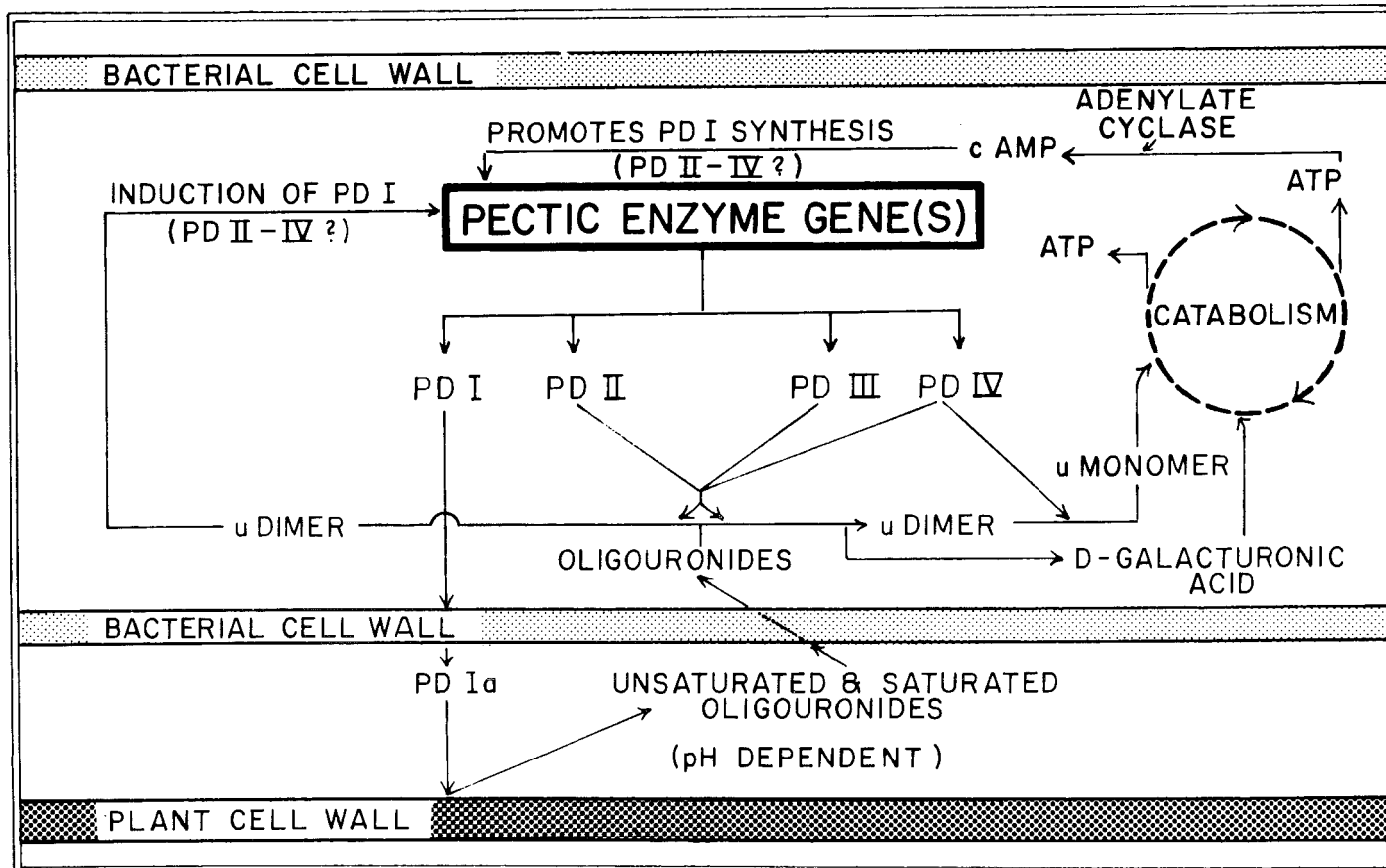


Fig. 3. A proposed model system for the pectic enzyme complex of *Erwinia carotovora* and its interaction with plant cell walls.

deoxy-L-threo-5-hexoseulose uronic acid, the unsaturated monomer. At pH 6.0, PD IV can convert the breakdown products of PD I through III into D-galacturonic acid, the saturated monomer. Both the saturated and unsaturated monomers can be assimilated into the energy-yielding food chain (9,12). Because of problems with the stability of the highly purified enzyme preparation, PD IV was not as fully characterized as PD I through III.

Summarizing the data from our own work and from other studies on *E. carotovora*, we are presenting one hypothesis upon which further work and evaluation of the functions of PD I through IV could be based. This model is described below and illustrated in Fig. 3.

Low basal levels of PD I could be transported from the bacteria to the plant cell wall. Because of the lytic and/or hydrolytic action of PD Ia enzyme(s), tissue could be macerated over a broad range of pH and ionic conditions. Saturated or unsaturated oligouronides (depending upon pH) could then be transported into the bacteria to be acted upon by PD II through IV. PD II and PD III have the ability to convert the oligouronides into unsaturated dimer, which can serve as both a food source to be further metabolized, and as an inducer of the synthesis of, at least, PD I (7,24). PD IV could then convert unsaturated dimer and other oligouronides to unsaturated monomer and D-galacturonic acid for subsequent cell metabolism (9,12). A portion of the ATP resulting from fermentation or oxidative phosphorylation could be converted by adenylate cyclase into cAMP, which, along with the readily available inducer (unsaturated dimer), is necessary for promoting increased synthesis of, at least, PD I (8,14).

Another possibility may be that PD II through IV, although independently capable of depolymerizing oligouronides, are intermediates in the synthesis of PD I. Even if intermediates, PD II through IV may still function in vivo in the metabolism of oligouronides and formation of the inducer of PD I, since our experiments demonstrate that PD I alone cannot produce the inducer, an unsaturated dimer. Whether or not there is an operon or operons containing genes for some or all of the pectic enzymes, and whether or not these enzymes are under the same or similar control mechanisms, remains to be established. However, it is evident from this study that the bacterium has the necessary enzyme machinery for depolymerization of the pectic acid fraction of plant cell walls over a broad pH range and under varying ionic conditions, for the utilization of the breakdown products of maceration as a source of energy, and for the generation of the necessary molecules to maintain the synthesis and activity of these enzymes.

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