Cellular Localization and Characterization of Pectic Enzymes of *Erwinia carotovora* subsp. *atroseptica*

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ABSTRACT

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Pectate lyase (PL) and polygalacturonase (PG) enzymes were isolated from the extracellular, periplasmic, and cytoplasmic fractions of *Erwinia carotovora* subsp. *atroseptica* strain SR-8 grown on a sodium polypectate minimal salts broth. The enzymes were purified and characterized for mode of action, ion requirements, and isoelectric point (p*I*). Six endoPLs with p*I*s of 10.2, 9.6, 9.5, 9.4, 9.2, and 8.9 were found in the extracellular fraction, along with endoPGs with approximate p*I*s of 10.7 and 3.9. The endoPLs produced unsaturated dimers as their final product, while the endoPGs produced saturated trimers. The periplasm contained the PLs with p*I*s of 9.5 and 9.4, and a small amount of the PG with a p*I* of 3.9. It also contained an exoPL (p*I* 7.1) that generated unsaturated dimers as its sole reaction product. The cytoplasm contained the PLs with p*I*s of 9.5 and 9.4, small amounts of the other PLs, and a large quantity of the PG with p*I* 3.9. Cation preferences differed among the enzymes isolated. The PL profile of *Erwinia carotovora* subsp. *atroseptica* is much more complex than previously realized, and unlike *Erwinia carotovora* subsp. *carotovora* and *Erwinia chrysanthemi*, this strain produces two endoPGs. The cellular location and physiological characteristics of the various enzymes may give clues to their role in pathogenicity.

Erwinia carotovora subsp. *atroseptica*, *Erwinia carotovora* subsp. *carotovora*, and *Erwinia chrysanthemi* cause soft rots and other diseases of plants throughout the world. These bacteria produce an array of extracellular enzymes that enable them to degrade plant cell wall and plant cell membrane components. In particular, pectic-enzyme production has been correlated with the maceration and cell death characteristic of soft-rot diseases (5,6,13).

The pectic enzymes of *E. chrysanthemi* and *E. c. carotovora* have been well studied. Both organisms produce multiple isozymes of pectate lyase (PL)(EC 4.2.2.2), and each also produces polygalacturonase (PG)(EC 4.2.2.9). *E. c. carotovora* produces an endoPG, while *E. chrysanthemi* produces an exoPG (EC 3.2.1.82). Purified endoPL and endoPG have been shown to macerate tissue and kill cells (5,6,13,26), but the roles of individual enzymes in

pathogenesis are not clear. Many PL genes (*pel*) from *E. chrysanthemi* and *E. c. carotovora* have been cloned, as has the PG gene (*peh*) from *E. c. carotovora* (6,11–13,20,25–27). The development of site-directed mutagenesis techniques has facilitated studies on the roles of the individual pectic enzymes in the pathogenesis of *E. chrysanthemi* and *E. c. carotovora* (4,10,22,26). However, in a recent study, an *E. chrysanthemi* isolate retained macerating ability even when all of the PL and PG enzymes thought to be produced by the wild type had been mutagenized (10). On closer physiological examination, more enzymes were discovered. This shows the need for physiological, as well as genetic studies, to determine all pathogenicity factors.

Less work has been done on the pectic enzymes produced by *E. c. atroseptica*. The pathogenicity of this organism is limited almost entirely to potatoes and differs from that of *E. chrysanthemi* and *E. c. carotovora*, which have broad host ranges (17). *E. c. atroseptica* has been shown to produce both extracellular endoPL and endoPG (18,21). Three extracellular endoPL enzymes have been isolated by sucrose-column isoelectric focusing (IEF) and demonstrated in overlays of polyacrylamide gels (PAGE) (18,21). One endoPL gene has been cloned from *E. c. atroseptica* and has a strong homologous relationship to an endoPL from *E. c. carotovora* (1).

The extracellular pectic-lyase enzymes produced by *E. c. atroseptica* have not been well characterized, and the intracellular pectic enzymes have not been characterized at all. These enzymes are necessary for the catabolism of the pectic fragments and induction of the extracellular enzymes; thus, they are important for soft-rot pathogenesis. Also, while several pectic enzymes from *Erwinia* spp. have been localized to the periplasm (25,26), none have been purified from it. The intracellular pectolytic enzymes that have been purified from *Erwinia* and other bacterial species were isolated from the total intracellular fraction (3,23). We have isolated and characterized the cytoplasmic, periplasmic, and extracellular pectic enzymes produced by *E. c. atroseptica* strain SR-8. A preliminary report of this research has been published (8).

MATERIALS AND METHODS

Cultural conditions. *E. c. atroseptica* strain SR-8 was obtained from A. Kelman, University of Wisconsin (16). Media for enzyme extraction contained 0.5% sodium polypectate (NaPP)(Ral Tech Scientific Services, Madison, WI) and minimal salts (MS) (23). Bacteria were grown at 30 C overnight in a shaking water bath at 90 rpm. A 1-ml sample was then transferred to each of five flasks that contained 200 ml of NaPPMS, and was incubated until early stationary phase. The bacteria were maintained on NaPPMS agar at 4 C.

Enzyme extraction and purification. Bacterial cells were collected by centrifugation at 20,000 *g* for 20 min at 4 C. Pectic enzymes isolated from the supernatant were designated as extracellular enzymes. The bacterial pellet was resuspended in a volume of 0.05 M Tris-HCl buffer (pH 8.0) equal to that of the pellet and was recentrifuged twice at 15 C to get rid of any remaining NaPP.

The periplasmic fraction was isolated by a modification of the lysozyme-EDTA treatment (12). The cell pellet was resuspended in 0.2 M Tris-HCl (pH 8.0) (10 ml/2 g of cells) and diluted with 2 vol of the same buffer. Sucrose (1.5 vol of 1.5 M, pH 8.0) was added, followed by the gradual addition of 1.5 mM EDTA (3.3 ml/10 ml). Egg-white lysozyme (1 mg/ml, Sigma Chemical Company, St. Louis, MO) was added to a final concentration of 60 μg/ml. The solution was diluted twofold with water and stirred for 30 min while on ice. $MgCl_2$ from a 1 M stock solution was added to a final concentration of 2 mM to stabilize the spheroplasts, which were then centrifuged at 4 C as described. The supernatant was used to isolate the periplasmic pectic enzymes.

The pelleted spheroplasts were resuspended in 5 mM Tris-HCl (pH 8.0), and lysed by ultrasonification with a Branson sonifier cell disrupter (Branson Ultrasonic Corp., Danbury, CT) with a medium tip at 30 W for 10 sec. The cell sonicate was centrifuged as described, and the supernatant was retained at 4 C for isolation of cytoplasmic enzymes.

To determine the degree of cross-contamination, each fraction was assayed for cyclic phosphodiesterase (15), which is found only in the periplasm, and β-galactosidase (7), which is confined to the cytoplasm.

Each fraction was brought to 95% saturation with $(NH_4)_2SO_4$ and centrifuged for 30 min, as described. The precipitate was suspended in distilled water and dialyzed overnight at 4 C from many liters of distilled water. After dialysis, the crude fractions were stored at −80 C until they were purified. Aliquots of the dialyzed fractions (0.5 mg extracellular, 1 mg periplasmic, or 15 mg cytoplasmic) were applied separately to a 1.6- × 16-cm diethylaminoethyl (DEAE) cellulose (DE 53, Whatman Inc, Clifton, NJ) column as described by Stack et al (23). Three-milliliter fractions were collected and assayed for pectic-enzyme activity.

The 3-ml fractions from each DEAE peak that showed enzyme activity were pooled, and then all the DEAE peaks from each cellular location were combined and subjected to isoelectric focusing in an LKB 8101 Ampholine electrofocusing apparatus (LKB Producter AB, Romma, Sweden). The column contained 2.5% ampholine carriers of pHs 9–11 and 3.5–10 in a ratio of 8:1 (24). Electrofocusing was done at 5 C at 15 W with a 2,500 V maximum until the current had stabilized, usually in 40–48 hr. Fractions of 1.25 ml were collected in the regions of PL activity; 5-ml fractions were collected in the rest of the column. All were treated as previously described (23). Protein concentrations were determined by the Bio-Rad (Bio-Rad Laboratories, Richmond, CA) protein assay with bovine γ-globulin as a standard. The crude and purified enzymes were stored at −80 C.

Enzyme assays. Lyase activity was determined by the periodate-thiobarbituric acid assay (TBA) (18) with a substrate of 0.6% NaPP in 0.05 M Tris-HCl buffer (pH 8.5) that contained 0.5 mM EDTA and 1 mM $CaCl_2$ as described by Stack et al (23). Hydrolase activity was determined by the Nelson's reducing group analysis (14) with a substrate of 0.6% NaPP in 0.05 M phosphate buffer (pH 6.0), also described by Stack et al (23). One hundred microliters of enzyme-containing fraction was incubated with 100 μl of substrate at 30 C for 1 hr or for longer periods when necessary. Specific activity is expressed as units of activity per milligram of protein (23). To analyze enzyme reaction products, the products were separated on descending paper chromatograms with pyridine/ethyl acetate/water/acetic acid (5:5:3:1, v/v) as a solvent as described by Stack et al (23).

The pH optimum for enzyme activity was determined by the TBA assay or the reducing-group analysis with 0.6% NaPP in three buffer systems: 0.05 M acetate buffer (pH 4.5–5.5), 0.05 M phosphate buffer (pH 6.0–7.5), and 0.05 M Tris-HCl (pH 8.0–10.0). To determine the ions necessary for optimal lyase activity, 0.6% NaPP was dissolved in 0.05 M Tris-HCl (pH 8.5) and EDTA was added to a final concentration of 0.5 mM to chelate endogenous ions (23). Calcium, Co^{++}, Mg^{++}, or Mn^{++} ions (in the chloride form) were then added to a final concentration of 1.0 mM. These substrates were used with the TBA assay.

β-Galactosidase activity was assayed by measuring the hydrolysis of *o*-nitrophenyl β-D-galactopyranoside (ONPG) as described by Dobrogosz (7), and cyclic phosphodiesterase activity was determined by measuring the hydrolysis of bis-*p*-nitrophenyl phosphate (15).

RESULTS

Enzyme purification. The extracellular, periplasmic, and cytoplasmic fractions were well separated, as proven by the location of the marker enzymes, cyclic phosphodiesterase and β-galactosidase. Virtually all of the β-galactosidase (99.4%) was in the cytoplasmic fraction, with only 0.6% of the activity in the periplasm, and none in the supernatant. Ninety percent of the cyclic phosphodiesterase was found in the periplasmic fraction, while 7.3% was in the cytoplasmic fraction, and 2.5% was in the supernatant. These results are similar to previously published

reports (2,20,27), and indicate that the enzymes found in the extracellular fraction were truly extracellular enzymes and not the result of autolysis.

DEAE cellulose chromatography of each of the fractions resulted in two peaks of PL activity (Fig. 1), as indicated by the TBA assay. Peak 1 contained the highest activity and was eluted in the void volume, while peak 2 was eluted with 0.05–0.1 M NaCl. Peak 2 was barely discernible in the extracellular and cytoplasmic fractions, but was much larger in the periplasmic fraction. The Nelson's assay revealed a peak of activity from all three cellular locations; this peak coincided with PL peak 2. In addition, the periplasmic fraction contained a Nelson's assay peak of activity within the PL peak 1. The Nelson's assay usually indicates PG activity at lower pH values, but it can also reveal any PL activity products generated under the acidic conditions. PLs that are active at these unusual conditions have been reported (23).

Isoelectric focusing of the extracellular DEAE fractions produced four major PL peaks with isoelectric points (p*I*) of 10.2,

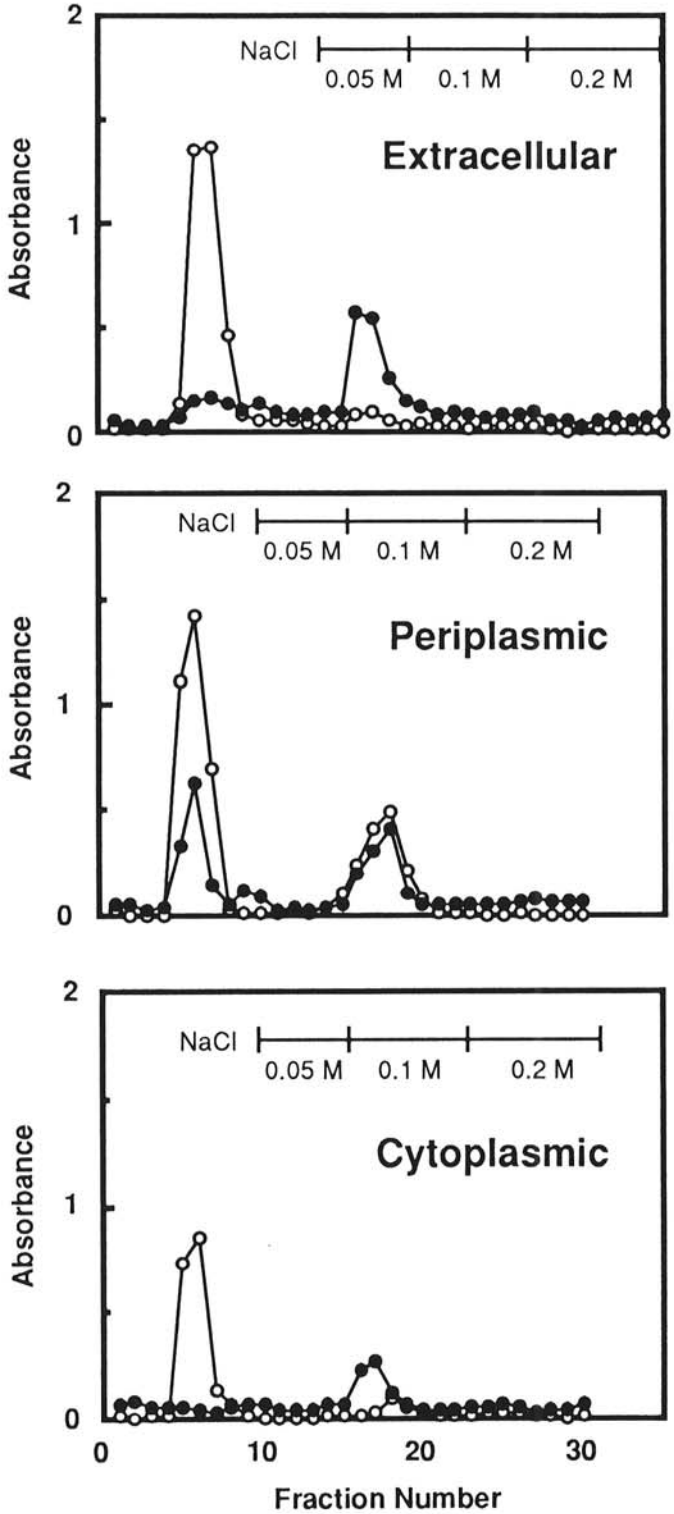

Fig. 1. DEAE cellulose column chromatography of extracellular, periplasmic, and cytoplasmic pectic enzymes. The $(NH_4)_2SO_4$ preparation was applied to a DEAE column, and enzyme was eluted by a stepwise gradient of 0–0.2 M NaCl in 0.05 M Tris-HCl buffer, pH 8.0. Fractions were incubated with 0.6% sodium polypectate for 1 hr and assayed by the TBA assay (18) for pectate lyase activity at pH 8.5 (○) and by the Nelson's reducing sugar assay (14) for polygalacturonase activity at pH 6.0 (●).

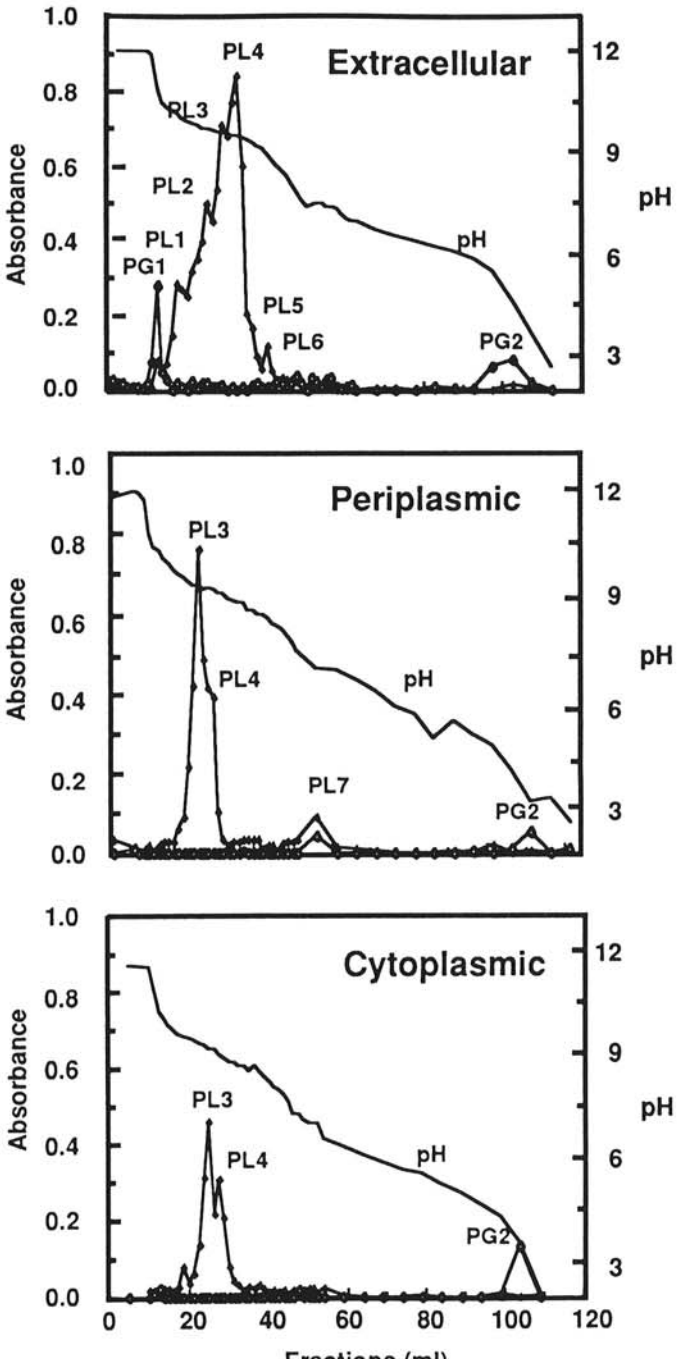

Fig. 2. Isoelectric focusing of extracellular, periplasmic, and cytoplasmic pectic enzymes. Fractions of 1.25 ml were collected in high pH regions to resolve the PL enzymes, while 5-ml fractions were collected in the rest of the column. For this reason, the fractions are shown by the total milliliters collected at that point, instead of by fraction number. Pectate lyase (◇) and polygalacturonase (◆) enzymes were incubated with 0.6% sodium polypectate for 4 hr and assayed as described for Figure 1.

9.6, 9.5, and 9.4, and two smaller peaks with p*I*s 9.2 and 8.9 (Fig. 2). These enzymes are referred to as PL1–6 (p*I*s 10.2–8.9). Two peaks of PG activity also were present at p*I* 10.7 and 3.9; these enzymes are referred to as PG1 (10.7) and PG2 (3.9). PG1 sometimes contained trace amounts of unstable PL activity.

The periplasmic fractions contained large peaks of PL3 and PL4 and an additional PL with p*I* 7.1 (PL7). A small amount of PG2 was also present. The cytoplasmic fraction contained peaks of PL3, PL4, PG2, and small amounts of the other pectic enzymes. Most of the intracellular PL activity is localized in the periplasm. Isoelectric focusing of a portion of the periplasmic fraction gave larger amounts of PL activity than isoelectric focusing of the entire cytoplasmic fraction.

The IEF profiles presented here, which show all of the enzymes isolated, are single representatives of several replicates. Enzymes which seem poorly resolved on these IEF profiles, such as PL5, were characterized from other IEF runs that gave better resolution of those particular enzymes.

Characterization of enzymes. All of the extracellular PLs (PL1–6) produced several oligouronides of different sizes (Table 1) as reaction products, indicating an endo mode of NaPP depolymerization. The sizes of these products varied from unsaturated dimers to unsaturated pentamers, as indicated by R_{gal} values similar to those previously determined in this laboratory (23). The PL with a p*I* of 7.1 (PL7), which was present only in the periplasm, produced a single reaction product, indicating an exo mode of depolymerization. The R_{gal} of this product was 0.70, which corresponded to an unsaturated dimer (23).

The cation dependency and preferences of the isolated enzymes varied. Most of the extracellular PLs had dual preferences for Ca^{++} and Co^{++} ions, although PL2 and PL3 preferred Ca^{++} to any other ion (Table 2). All enzymes gave some activity with Mg^{++} as a cofactor, but most had little or no activity with Mn^{++}, except for PL1 and PL7. The extracellular PLs all appeared to require ions for activity; there was some relatively low activity in the absence of ions.

The PL7 enzyme had activity over a pH range of 5.5–10 and, although more active in the presence of Ca^{++}, Mg^{++}, Co^{++}, or Mn^{++} ions, did not require cofactors for activity.

The PGs had similar characteristics. Each produced a series of saturated oligomers at pH 6.0, indicating an endo mode of hydrolytic depolymerization (Table 1). The smallest oligomer produced was a saturated trimer.

TABLE 1. Chromatographic mobilities of products formed from the degradation of sodium polypectate by pectic enzymes[a]

Purified enzyme[b]	R_{gal} values[c]	Unsaturated product[d]	Probable product formed
PG1	0.33, 0.18, 0.09	–	s[e] trimer-pentamer
PG2	0.33, 0.18, 0.09	–	s trimer-pentamer
PL1	0.70, 0.43, 0.18, 0.08	+	u dimer-pentamer
PL2	0.70, 0.43, 0.18, 0.08	+	u dimer-pentamer
PL3	0.70, 0.43, 0.18, 0.08	+	u dimer-pentamer
PL4	0.70, 0.43, 0.18, 0.08	+	u dimer-pentamer
PL5	0.70, 0.43, 0.18, 0.08	+	u dimer-pentamer
PL6	0.70, 0.43, 0.18, 0.08	+	u dimer-pentamer
PL7	0.70	+	u dimer

[a] Descending paper chromatograph on Whatman no. 4 paper was carried out for 14–16 hr in pyridine/ethyl acetate/water/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 then washed in 6 M NH_4OH (23). Reaction mixtures contained 100 μl of substrate (0.6% sodium polypectate in 0.05 M Tris-HCl buffer, pH 8.5, containing 0.5 mM EDTA, and Ca^{++} to a final concentration of 1.0 mM for PL and 0.05 M phosphate buffer, pH 6.0, containing 0.5 mM EDTA) for PG, and 100 μl of enzyme. Incubation was at 30 C for varying amounts of time.

[b] PG = polygalacturonase enzyme; PL = pectate lyase enzyme.

[c] R_{gal} values indicate the ratio of product migration to the distance migrated by D-galacturonic acid standard.

[d] Unsaturated products in reaction mixtures were determined by the TBA assay (18).

[e] u = unsaturated; s = saturated.

DISCUSSION

We have isolated and characterized the pectic enzymes from the cytoplasmic, periplasmic, and extracellular fractions of *E. c. atroseptica* SR-8. The profile of enzymes produced by this organism is much more complex than previously thought; we found six endoPLs, an exoPL, and two endoPGs. All of the endoPLs (PL1–6) were found in the extracellular fraction, while PL3 and PL4 were also found in the cytoplasm and periplasm. The periplasm uniquely contained an exoPL (PL7). Both PGs were extracellular, but PG2 was also found in both the cytoplasm and periplasm.

The complexity of the extracellular pectate lyase profile of *E. c. atroseptica* is similar to that of *E. chrysanthemi* and *E. c. carotovora*, which produce a number of extracellular endoPLs. However, *E. c. atroseptica* is unique in producing two endoPGs. PG1 is similar to an endoPG produced by *E. c. carotovora*, although this enzyme produced trimers as its final reaction product, instead of dimers like the *E. c. carotovora* EC14 enzyme (21,23,26). The *E. c. carotovora* and *E. c. atroseptica* PGs were found to have identical p*I*s when run on PAGE gels; this suggests that the enzymes might be identical (21). PG2 has not been previously reported.

Two previous studies of *E. c. atroseptica* with preparative IEF or PAGE overlays identified three extracellular endoPLs (19,21), while we have obtained six. We were able to resolve these enzymes in 1.25-ml fractions collected from an IEF column, instead of the usual 5-ml fractions. In a previously published profile of the extracellular PL enzymes of strain SR-8, four peaks of PL activity were identified; 2.5-ml fractions were collected in those experiments (1). It is possible that the collection of fractions even smaller than 1.25 ml might have enabled the resolution of additional enzymes, but the enzyme quantity would have been too small to characterize accurately. However, this raises the concern that there may have been additional enzymes that were not resolved. Also, enzymes that are very unstable in highly purified form have been reported (23), and therefore additional enzymes could have been lost in isolation procedures.

It is not clear why fewer enzymes were previously observed with PAGE overlays (21). One reason could be that we used a longer incubation time to assay the IEF fractions, which enabled us to detect enzymes with lower amounts of activity, or those with poor activity utilizing the overlay substrate. Trollinger et al (25) were unable to detect a cloned *E. c. carotovora* endoPL on PAGE overlays, even though the clone definitely had detectable enzyme activity. Another possibility for the appearance of so many enzymes is that some of them could be the products of specific posttranslational modifications; this may be the case for several *E. chrysanthemi* PLs (6,13,21). Different cultural conditions, especially the pectate source, may account for the appearance of novel enzymes, as was found recently for *E. chrysanthemi*

TABLE 2. Effect of divalent cations on the activity of pectate lyase enzymes

Purified enzyme[b]	Specific activity (units per milligram of protein)[a]				
	EDTA[c]	Ca^{++}	Co^{++}	Mg^{++}	Mn^{++}
PL1	3 ± 0.9	36 ± 1.5	34 ± 0.8	17 ± 1.9	5 ± 1.2
PL2	3 ± 0.5	25 ± 1.1	15 ± 1.6	15 ± 0.9	1 ± 0.9
PL3	12 ± 0.8	62 ± 4.4	48 ± 4.9	37 ± 2.2	2 ± 1.2
PL4	10 ± 2.1	39 ± 5.8	36 ± 4.4	18 ± 4.4	0 ± 0
PL5	2 ± 0.2	20 ± 1.4	21 ± 1.4	9 ± 0.5	0 ± 0
PL6	2 ± 0.2	8 ± 0.1	8 ± 0.4	6 ± 0.2	0 ± 0
PL7	2 ± 0.1	5 ± 0.1	4 ± 0.1	4 ± 0.1	3 ± 0.1

[a] Reaction mixtures contained 100 μl of 0.6% sodium polypectate in 0.05 M Tris-HCl buffer, pH 8.5, which had been pretreated with 0.5 mM EDTA. Specific ions (Ca^{++}, Co^{++}, Mg^{++}, or Mn^{++}) were then supplemented to a final concentration of 1.0 mM. Mixtures were incubated at 30 C and analyzed by the TBA assay (18). One unit is equal to 1 μmol of D-galacturonic acid formed per hour.

[b] PL = pectate lyase enzyme.

[c] No cations were added.

(10). We must also consider the possibility of artifacts from the purification procedure; this is sometimes a concern when high-resolution IEF techniques are used (9). However, several of the *E. c. atroseptica* enzymes could be actual isozymes that differ in their cation preferences. It remains to be seen whether all of the enzymes are the products of separate genes.

The profile of the intracellular enzymes from *E. c. atroseptica* was less complex than that of the extracellular enzymes. Only two of the endoPLs and one of the endoPGs (PG2) were intracellular, although the periplasm contained an exoPL that was found nowhere else. This pattern of enzyme production differs from that of *E. c. carotovora* and *E. chrysanthemi*. Unlike *E. c. atroseptica*, *E. chrysanthemi* produces an exoPG. Also, three intracellular endoPLs have been purified from *E. chrysanthemi* (3,5,6). Several strains of *E. c. carotovora* have been shown to produce an exoPL that generates dimers and trimers as reaction products (6,23,27); we did not find a comparable enzyme in *E. c. atroseptica*. Also, the *E. c. carotovora* endoPG is both intra- and extracellular, while PG1 was found only in the extracellular fraction of *E. c. atroseptica* (23). *E. c. carotovora* 71 was shown to produce two intracellular PLs that were found predominantly in the periplasm (26), while we found three PLs in the periplasm of *E. c. atroseptica*.

The periplasmic exoPL was very similar to an intracellular exoPL with p*I* 6.3 isolated from *E. c. carotovora* EC14 (23). This enzyme also produced only dimers as a reaction product, had a very broad pH activity range, and did not require cations for activity. Recently, the gene for a periplasmic PL enzyme was isolated from *E. c. carotovora* SCRI193 (12). This enzyme was found to belong to a different gene family than an extracellular PL gene from the same strain. This gene family has not been detected in any of the *E. chrysanthemi* strains isolated to date (11). It will be interesting to see whether this enzyme is very similar or identical to the exoPL we have purified, especially because *E. chrysanthemi* has not been shown to produce an exoPL.

The cellular location and characterization of the pectate depolymerating enzymes may provide some clues to their roles in pathogenesis. All of the extracellular pectic enzymes depolymerize NaPP in an endo manner. Both endoPL and endoPG have been found to macerate tissue (6,13,22,26). The extracellular production of both types of enzymes may help to ensure the degradation of pectin in a variety of environments and give a distinct advantage to the pathogen. Production of multiple enzyme forms may allow for a more complex regulatory strategy or provide for higher levels of PL activity. Some of the enzymes may be more stable than others under adverse conditions in the host, and pectic enzymes have been shown to differ in their ability to damage host tissue (6,13,19).

In *E. c. atroseptica*, PL1, PL2, PL5, and PL6 are only found in the extracellular fraction. This suggests that they probably have a role in the degradation of plant tissue and perhaps in enzyme regulation, but not in the catabolism of the breakdown products of pectin. PL3 and PL4, found in all cellular locations, may have a function in catabolism. The exoPL is located solely in the periplasm where it may facilitate the production of unsaturated dimers from the oligouronides that have entered the cell. This may be important in the induction of PL synthesis, because the breakdown products of unsaturated dimers have been found to induce PL synthesis in *E. c. carotovora* and *E. chrysanthemi* (5,6).

The role of pectic enzymes in plant pathogenesis is much more complicated than previously thought (4,22). These enzymes have been studied most extensively in *E. chrysanthemi*, while much less is known about their role in the pathogenicity of *E. c. atroseptica*. The latter organism has a much more restricted host range than *E. chrysanthemi* or *E. c. carotovora* (17). It will be interesting to see whether the differences in enzyme production between *E. c. atroseptica* and the other bacteria can be correlated to differences in host range pathogenicity. Further study of the physiology and genetics of these enzymes should help to elucidate their roles in causing plant disease.

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