Synergism between *Erwinia* pectate lyase isoenzymes that depolymerize both pectate and pectin

Stefan Bartling,^{1,2} Christina Wegener² and Ole Olsen¹

Author for correspondence: Stefan Bartling (Carlsberg Laboratory). Tel: +45 33 27 53 33. Fax: +45 33 27 47 66. e-mail: bartling@biobase.dk

¹ Carlsberg Laboratory, Department of Physiology, Gamle Carlsberg Vej 10, DK-2500 Copenhagen, Denmark

² Federal Centre for Breeding Research on Cultivated Plants, Institute for Stress Physiology and Quality of Raw Materials, Institutsplatz, D-18190 Gross Lüsewitz, Germany

Phytopathogenic *Erwinia* bacteria cause tissue maceration by secretion of pectinolytic enzymes such as pectate lyase (PL). Sequencing of overlapping genomic fragments from *Erwinia carotovora* subsp. *atroseptica* established the organization of a 7.5 kbp region encoding PL isoenzymes. Two intergenic regions of 656 and 645 bp separate three enzyme coding regions of 1125 bp exhibiting approximately 80% positional identity. The promoters of each of the three genes contain a segment with high homology to the binding sequence of the *E. chrysanthemi* KdgR transcription repressor, implying similar mechanisms of gene regulation in the two bacterial species. Separate expression of the *pel* genes in the *Escherichia coli*-pT7-7 system and purification of their products yielded PLs at 7–33 mg (l culture)⁻¹ with greater than 95% purity. Availability of the recombinant enzymes allowed determination of the kinetic differences amongst the PL isoforms, PL1, PL2 and PL3. The results show that PL is not strictly confined to depolymerization of pectate since each isoenzyme more readily degrades 31% esterified pectin. Addition of isoenzyme combinations revealed no synergism with respect to degradation of pectate or 31% esterified pectin. However, addition of enzyme combinations containing PL3 enhanced the activity towards 68% esterified pectin, against which individual PL activities were low, by up to 64%. These data suggest that the combination of PL isoenzymes extends the range of pectic substrates which the bacterium can degrade.

Keywords: pectate lyases, synergism, isoenzymes, *Erwinia*

INTRODUCTION

Plant pectic substances have a major function in cell-wall cementing in the middle lamellae between adjacent primary cell walls and can be regarded as filler substances within the primary cell wall (reviewed by Varner & Lin, 1989). Accordingly, depolymerization of pectic components causes cell separation, release of cell-wall-bound proteins and leakage of electrolytes from protoplasts resulting in cell lysis. Phytopathogenic bacteria of the genus *Erwinia* penetrate and colonize plant tissues causing

soft-rot diseases by pectolytic, cellulolytic and proteolytic degradation of host cell walls (Pérombelon & Kelman, 1980). Given the complex and variant structure of pectin, its enzymic degradation is accomplished by the synergistic action of enzymes such as pectin methylesterase (PME), pectin lyase (PNL), polygalacturonase (PG) and pectate lyase (PL). Bacterial synthesis of enzyme isoforms may additionally help to ensure the degradation of pectin in a variety of environments or provide a more advantageous regulatory strategy to the bacteria.

PLs, which play a major role among the pectolytic enzymes (Collmer & Keen, 1986), have been distinguished from PNLs by their specificity for pectate, and not pectin, although both enzymes degrade their substrate by β -elimination resulting in an unsaturated C-4–C-5 bond at the nonreducing end of the α -1,4 linked products (Rombouts & Pilnik, 1980). Despite the

Abbreviations: *Eca*, *Erwinia carotovora* subsp. *atroseptica*; *Ecc*, *Erwinia carotovora* subsp. *carotovora*; *Ech*, *Erwinia chrysanthemi*; PG, polygalacturonase; PGA, polygalacturonic acid; PL, pectate lyase; PME, pectin methylesterase; PNL, pectin lyase.

The EMBL accession number for the nucleotide sequences of the three *pel* genes reported in this paper is X81847.

differences, sequence comparisons imply similarities between the three-dimensional enzymic structures (Yoder *et al.*, 1993). Several homologous and recombinant PL enzymes have been purified for use in tissue maceration analyses, but there are limited data available regarding their mode of action, and it has not yet been determined whether pectins of intermediate esterification could function as substrate for PL.

Erwinia synthesize isoenzymic forms of PL encoded by multiple *pel* genes. In *Erwinia chrysanthemi* (Ech), five separate genes and their products have been characterized (Koutoujansky *et al.*, 1985; Reverchon *et al.*, 1986), while the genome of *E. carotovora* subsp. *carotovora* (Ecc) has been shown to encode four PLs (reviewed by Koutoujansky, 1987). Previous studies disagreed on the number of PLs in *E. carotovora* subsp. *atroseptica* (Eca), which primarily causes soft-rot and blackleg diseases of potatoes (Pérombelon & Kelman, 1980). Enzyme purification or isoelectric focusing of concentrated culture fluids indicated the presence of three (Ried & Collmer, 1986) or six (George *et al.*, 1991) PL isoenzymes, while McMillan *et al.* (1992) purified two isoenzymes which were secreted into the culture medium. No gene sequences have been reported. However, two and three genes encoding PLs were cloned from *E. carotovora* EC (Lei *et al.*, 1987, 1988) and *E. carotovora* Er (Yoshida *et al.*, 1992). The EMBL Data Library (release 40) included a total of 49 entries for gene sequences encoding PLs. Among these are plant genes for enzymes which have been hypothesized to participate in pollen tube growth in tomato (Wing *et al.*, 1989) and tobacco (Rogers *et al.*, 1992).

To further analyse the molecular basis for the degradation of pectic substances by *Eca*, we have undertaken characterization of the *pel* genes and the corresponding gene products. We report here the genomic sequence spanning three *pel* genes encoding PL isoenzymes. Each gene was separately expressed in *Escherichia coli*, and active enzyme purified and characterized. Interestingly, the results revealed that the highly homologous PLs depolymerize both pectate and pectin. Combinations of the enzymes were accordingly examined for synergism towards various pectins.

METHODS

Bacteria, vectors and culture conditions. *Eca* strain C18, propagated at 25 °C in Luria Bertani (LB) medium, was from the culture collection of the Institute for Stress Physiology and Quality of Raw Materials, Germany. *E. coli* strains used were DH5 α (Gibco-BRL) and BL21(DE3)pLysS (Novagen). Vectors were pUC18 (Vieira & Messing, 1982) and pT7-7 (Studier *et al.*, 1990). *E. coli* were grown at 37 °C in LB medium or, to facilitate purification of the recombinant PL enzymes, in M9 medium (Sambrook *et al.*, 1989) supplemented with 100 μ g ampicillin ml⁻¹ for transformed DH5 α cells or 100 μ g ampicillin ml⁻¹ and 25 μ g chloramphenicol ml⁻¹ for BL21(DE3)pLysS cells.

DNA isolations and sequencing. Standard procedures were used for purification of bacterial chromosomal DNA, digestion with restriction endonucleases, ligation and transformation, and amplification of DNA using PCR, as described by Sambrook *et al.* (1989). Genomic DNA purified from *Eca* C18 was partially

digested with *Sau*3AI. Following electrophoresis in a 0.8% agarose gel, DNA fragments of 2–4 kbp were recovered using Prep-A-Gene (Bio-Rad), ligated into pUC18 linearized with *Bam*HI and propagated in *E. coli* DH5 α . After growth on LB-agar plates containing 0.4% citrus pectin (P-9135, Sigma), zones with enzymically degraded pectin remained clear upon staining with 0.05% ruthenium red. Plasmid DNA from clones synthesizing active PL was purified using the Wizard System (Promega) or the Maxi Plasmid Kit (Qiagen) according to the suppliers' instructions. Sequencing of both DNA strands utilized a model 373A DNA Sequencer (Applied Biosystems). Computer analysis of DNA and protein sequences was carried out using the MicroGenie software (Beckman).

Expression and purification of recombinant PLs. The coding regions of *pel*1, *pel*2 and *pel*3 were amplified by PCR using primers incorporating sequences for cloning into pT7-7 (Studier *et al.*, 1990). DNA sequencing confirmed the identity of each insert. *E. coli* BL21(DE3)pLysS cells harbouring the respective expression vectors were propagated in a 10 l Bioreactor (MBR). At OD₆₀₀ 0.4–0.8, the culture was made 0.4 mM in isopropyl β -D-thiogalactopyranoside to induce synthesis of PL. The culture fluid containing secreted PL was separated from the cells by filtration using Minicross (Microgon) membranes. Remaining PL was liberated from the periplasmic space of the concentrated cells by multiple freeze–thaw cycles, and subsequently combined with the culture fluid before ultrafiltration and concentration utilizing a Pellicon (Millipore) cassette. After anion-exchange chromatography using diethylaminoethyl cellulose DE53 (Whatman), unbound proteins were concentrated in a Minitan (Millipore) filtration unit and chromatographed on a S-Sepharose (Pharmacia) cation-exchange column. Elution was performed by applying a combined pH/salt gradient (20 mM sodium acetate, pH 5.0 to 20 mM Tris/HCl, pH 8.0, and 0 to 500 mM NaCl). The fractions exhibiting PL activity were combined and subjected to gel-filtration chromatography employing Superdex 200 (Pharmacia).

Protein concentration determinations and electrophoresis. Protein concentrations were determined by dye-binding, using bovine serum albumin as a standard (Bradford, 1976). Enzyme purity was assessed by SDS-PAGE (Laemmli, 1970) using 12.5% (w/v) ExcelGel (Pharmacia) polyacrylamide gels. Protein bands were visualized by staining with Coomassie Brilliant Blue R-250. Isoelectric focusing of the purified PLs employed Isogel pH 3–10 (FMC) agarose gels according to the manufacturer's instructions.

N-terminal amino acid sequencing. The purified PL isoenzymes were sequenced by automated Edman degradation in a model 470A Sequenator (Applied Biosystems); 100 pmol of the individual enzymes were used.

Enzyme assays, substrate specificity analysis and synergism studies. All measurements of PL activity were performed at least in triplicate. Product formation was followed continuously in a computer-assisted model 8452A (Hewlett-Packard) diode array spectrophotometer equipped with a thermostat. Unless otherwise indicated, the experiments were carried out at 25 °C during time courses of 2–5 min by monitoring the increase in A_{236} caused by the formation of unsaturated products released during the enzymic degradation of polygalacturonic acid (PGA) (P-1879, Sigma). Activity on pectin was determined by using a similar procedure but substituting 31%, 68% or 93% esterified citrus pectin (P-9311, P-9436, P-9561, Sigma) for PGA. An increase of 5.2 A_{236} units min⁻¹ corresponds to the formation of 1 μ mol unsaturated products min⁻¹ (Nasuno & Starr, 1966) or one enzyme unit (EU). To avoid alkaline breakdown of the pectic compounds, the substrate was freshly prepared immediately prior to the measurements by dissolving it in 100 mM

Tris/HCl, pH 8.5 (pH 8.0 for PL2), 0.1 mM CaCl₂. Enzyme samples of 15 µl containing 0.04 EU were added to solutions containing 985 µl substrate. To reduce measurement of unspecific disintegration of pectin, recordings were initiated after 30 s of preincubation. For determination of K_m and V_{max} values, the PL isoenzymes were incubated with 0.02–0.10% (w/v) PGA or 31% esterified pectin. The choice of the temperature for measurements of kinetic data (45 °C) was a compromise between the optimum for PL1 and PL3 (40 °C) and that for PL2 (50 °C). Subsequent calculations employed the direct linear plot according to Cornish-Bowden & Eisenthal (1978); 0.1% (w/v) substrate was used for analyses of substrate specificity and synergism.

Scanning electron microscopy. The individual PLs were infiltrated by vacuum into potato (*Solanum tuberosum*) tuber discs of 30 mm diameter and 5 mm thickness. Each enzyme was applied at 2 EU ml⁻¹. After 30 min incubation at 25 °C, the macerated tissue was washed five times with deionized water. The separated cells were air-dried and examined by scanning electron microscopy employing a Rem-Cam Scan 44 microscope.

RESULTS AND DISCUSSION

Structural organization of the *pel* genes

Previous work has shown that *Eca* produces at least three PL isoenzymes (Ried & Collmer, 1986). However, the structural organization of the corresponding genes was

Fig. 1. Genetic organization of a 7.5 kbp region of *Eca*. Open reading frames encoding three isoenzymes are demarcated by open boxes. The locations of restriction sites used for cloning of individual gene fragments are indicated.

not analysed. Expression cloning using *E. coli* as host for plasmids directing synthesis of active *Eca* PLs followed by alignment of four overlapping cloned DNA fragments allowed the establishment of the primary structure for a contiguous sequence of 7.5 kbp encoding three PL isoenzymes (Fig. 1), each consisting of 374 amino acids. Alignment of the enzyme-coding regions revealed a homology of around 80%, with the differences randomly scattered throughout the sequences. Unlike *Ech*, in which one of the two *pel* gene clusters encodes an acidic PL (PL A) and two alkaline forms (PL D and PL E), the triple gene cluster unveiled in *Eca* encodes three alkaline forms (see below). The tandem organization has also been described for *Erwinia* genes encoding proteases (Boyd & Keen, 1993) and Out proteins (Reeves *et al.*, 1993). However, the functional significance for this tandem arrangement remains elusive. Given that isoelectric focusing may resolve isoenzymes derived from minor post-translational modifications, e.g. N-terminal modification, deamidation or phosphorylation, the results also agree well with previous analyses employing isoelectric focusing of *Eca*-synthesized PLs, where the presence of at least three and possibly six extracellular isoforms was identified (George *et al.*, 1991). Similarly, three isoenzymes have been identified in the closely related bacterium *Ecc* (Ried & Collmer, 1986).

The DNA sequence of the individual *pel* genes 5' to the ATG translation initiation codons is shown in Fig. 2. A typical ribosome-binding sequence (RBS) precedes each start codon, while segments exhibiting homology with binding sequences for prokaryotic –35 and –10 transcription factors (Studnicka, 1987) are located further upstream. The low homology of the –10 region with the consensus sequence could suggest binding by a special sigma factor (Harley & Reynolds, 1987). Together, these results indicate that each gene constitutes a single independent transcription unit. This is further supported by the presence of sequences with high homology to the identified consensus sequence for the binding of the *Ech* KdgR transcription repressor (Nasser *et al.*, 1994), which negatively regulates several genes encoding enzymes involved in pectin degradation (Hugouvieux-Cotte-Pattat & Robert-Baudouy, 1992). The appearance of common regulatory sequences suggests that factors controlling transcriptional activity of *pel* genes are shared by the *Eca* and *Ech* bacteria. Thus, as in *Ech*, the *Eca pel* genes are likely to be inducible by growth on pectin-containing media (Condemine *et al.*, 1986).

<i>pel1</i>	GGCTTTATAGACAAACCTTATTTTCATTTTGTGAACAGCCCTTTTAAATTACACA	GGGTGTG TAG T GATAAATACCC	AAAAAATTCATGTCAAGGAGAGTAC ATAATG
<i>pel2</i>	GGTTTTTCAC CAAAATTGAAATTCATTTTGTGAAGAAA TTTACACTTGTACATC	GGGCATAG GGATCGATAAATGCC	ATGAAATTCATTTCCAGGAGACAGTGATAATG
<i>pel3</i>	GGGATTTTCA ATAAACCTTGATTTTATTTTGAACACA CCAAATAATATA	ATCTGGGTTATGTGGAT	CATAAATGCCAAACAAAATTCATTTCCAGGAGAGTACCCTAATG
	KdgR (62–77%)	–35 (100%)	–10 (50%)
			RBS (100%)

Fig. 2. Control regions for *pel* expression in *Eca*. The underlined sequences indicate putative KdgR (repressor of genes encoding pectolytic enzymes) binding sites (Condemine & Robert-Baudouy, 1991), (–35, –10) promoter motifs and ribosome-binding sites (RBS; Studnicka, 1987). The percentages given below the underlined sequences denote the degree of homology to the consensus sequences, in the case of the KdgR binding sites referring to the consensus established by Nasser *et al.* (1994).

Fig. 3. PL amino acid sequence analysis. The deduced sequences of *Eca* PL1, PL2 and PL3 are shown together with that of *Ech* PL C (Tamaki *et al.*, 1988). Except for the signal sequence, conserved amino acids (defined as those important for structure and function in *Ech* PL C; Yoder *et al.*, 1993) are boxed.

Protein homology and structure

Alignment of the deduced protein sequences unveiled homologies of 88.3%, 85.9% and 81.9% for PL1:PL2, PL1:PL3 and PL2:PL3, respectively (Fig. 3). When compared with the protein sequence of *Ech* PL C, which has been crystallized and found to fold into a novel domain motif designated the parallel β -helix (Yoder *et al.*, 1993), all three PL isoenzymes exhibit conserved residues which have been identified as important for enzyme integrity. Thus, there are four Cys residues in positions 93, 176, 350 and 373, indicating the presence of two disulphide bonds. Linear stacks of amino acids, including a novel asparagine ladder, form the core of the *Ech* PL C. The Asn residues of the ladder are also conserved throughout the *Eca* PL isoenzymes (Fig. 3). Moreover, the residues Asp-131, Glu-166 and Asp-170, suggested by Yoder *et al.* (1993) to be involved in Ca^{2+} binding essential for PL activity, correspond to Asp-152, Glu-187 and Asp-191, respectively, in the *Eca* PLs. Major differences between *Ech* PL C and the investigated *Eca* enzymes are confined to regions assigned to loop structures protruding from the central core of the enzymes. Accordingly, PLs synthesized and secreted by *Eca* are expected to fold into a conformation similar to the *Ech* PL C counterpart.

Purification of recombinant PL isoenzymes

The conservation in *Eca* of three genes encoding PL raised questions regarding the functional significance of three isoenzymes. Differences in isoenzymic properties can be expected to elucidate factors contributing to the

depolymerization of various complex plant pectin substances.

In order to obtain pure enzyme, the protein-coding region of each *pel* gene was amplified by PCR and placed under transcriptional control of the strong T7 bacteriophage ϕ 10 promoter in the pT7-7 vector (Studier *et al.*, 1990). While PL1 and PL3 were properly expressed in *E. coli*, synthesis of PL2 was only obtained with a plasmid construct containing the coding region inverted with respect to the ϕ 10 promoter. Suitable RBS and (−10, −35) promoter motifs were identified in the lower strand of the pT7-7 polylinker region (data not shown), and can apparently effect transcription and translation of *pel*2.

PL1, PL2 and PL3 were purified to homogeneity from concentrated 10 l cultures by ion-exchange chromatography followed by gel-filtration chromatography. The procedure yielded 159 mg PL1, 78 mg PL2 and 330 mg PL3, corresponding to recoveries of 18%, 11% and 15%, respectively.

Analysis of the first 15 N-terminal residues of the purified enzymes resulted in sequences corresponding to residues 23–37 deduced from the DNA sequences. The absence of secondary sequences in the preparations confirmed their purity (data not shown). Given the close relationship between the Gram-negative enterobacteria *E. coli* and *Eca*, these results indicate that PL1, PL2 and PL3 are synthesized as pre-proteins in their homologous host. The 22-residue sequences preceding the mature N-termini (Fig. 3) resemble typical signal sequences with a charged N-terminus, a central hydrophobic core and a processing

Fig. 4. Protein purification. SDS-PAGE analysis following expression of *Eca* PL1, PL2 and PL3 in *E. coli*. Lanes: A, combined culture fluid and filtered cell contents; B, unbound fraction after DE53 anion-exchange chromatography; C, protein fraction eluted from S-Sepharose cation-exchange column chromatography; D, protein fraction after Superdex 200 gel filtration. Molecular mass standards are indicated.

site consistent with the $(-3, -1)$ rule for signal peptidase cleavage (von Heijne, 1985). The high conservation of the signal sequences, with 22, 21 or 20 identical residues among the three enzymes, is notable.

SDS-PAGE under reducing conditions of purified PL1, PL2 and PL3 yielded single protein bands that corresponded to a molecular mass of 42 kDa (Fig. 4). A mass of 38 kDa has been calculated from the deduced amino acid sequences. Similar divergences have also been reported for other PL enzymes, e.g. *Ech* PL E (Tamaki *et al.*, 1988) and PL A (Favey *et al.*, 1992). Assuming that *Eca* PLs fold into a compact stable structure similar to *Ech* PL C (Yoder *et al.*, 1993), this would suggest that undenatured local structural elements of the PLs result in lower enzyme mobilities during electrophoresis.

Isoelectric focusing revealed a pI of > 10 , similar to the basic *Ech* PLs (Ried & Collmer, 1986). *Ech* also secretes PL A with a pI of 4.6, exhibiting poor maceration activity on potato tuber tissue, but yet being essential for unlimited bacterial pathogenicity (Favey *et al.*, 1992). The functional implication for the absence of an acidic PL isoform in *Eca* remains to be elucidated.

Substrate specificities of *Eca* PL isoenzymes

In plant cell walls, mainly in the middle lamellae, chemically diverse pectic substances are abundantly present (reviewed by Varner & Lin, 1989). Detailed analyses of potato tuber cell walls revealed pectic substances to account for approximately 50% of the total cell wall material. Around 20% of these pectins were found to be 43% esterified (Weber, 1976). Their unesterified form, i.e. pectate, is considered the natural substrate for PL. Highest enzymic activity towards this compound was measured at 50 °C and pH 8.0 for PL2, while PL1 and PL3 had a temperature optimum of 40 °C at pH 8.5. The

Fig. 5. Effect of substrate on enzyme activity. PL activities were measured towards PGA and citrus pectin. An activity of 0.04 EU of isoenzyme, corresponding to an increase in A_{236} of 0.2 min⁻¹ when utilizing PGA as substrate, was applied to each reaction mixture. No activity could be determined towards 93% esterified citrus pectin. The values shown are the mean $\pm$ SE of three separate determinations.

amount of products formed by the individual enzymes at 45 °C was virtually identical to that obtained at the respective temperature optima. Moreover, thermo-inactivation experiments revealed $> 80\%$ residual activity for each enzyme after 5 min incubation at 45 °C (data not shown). The K_m values were found to be 0.27 mg ml⁻¹ for PL1 and PL3, and 0.30 mg ml⁻¹ for PL2, similar to the K_m values of 0.20 and 0.32 mg ml⁻¹ for *Ech* PL B and PL C (Schoedel & Collmer, 1986). PL2 and PL3 displayed almost identical V_{max} values of 641 and 634 $\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$, while the V_{max} of 846 $\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ for PL1 hints at a significantly higher activity towards PGA for this enzyme.

Differences were also observed between the isoenzymes in the time courses of pectate depolymerization. PL1 displayed the highest initial velocity, which levelled off rapidly. While PL3 degraded less substrate in the first 80 s, the total degradation after 165 s of incubation was higher. The time course for degradation of pectate by PL2 was between those of PL1 and PL3. Although the results from the kinetic analyses were obtained by *in vitro* assays employing pure enzymes and substrates under defined conditions, it seems likely that they reflect the situation *in vivo*.

Further examination of depolymerization using various esterified pectins supported this assumption. Unexpectedly, application of identical amounts of enzyme units to PGA and esterified pectins revealed a significantly higher efficiency in degradation of pectin with 31% esterification (Fig. 5). Despite its low V_{max} value towards the PGA substrate, PL3 turned out to be more active than PL1 and PL2 in depolymerizing pectin with 31% esterification. Superiority was by 45% and 24%, re-

Fig. 6. Scanning electron micrographs of potato tuber tissue after incubation with recombinant PLs. Application of the individual enzymes caused different forms of cell wall breakdown. (a) Separation of tuber cells after degradation of the middle lamellae by the action of PL3. (b) The action of PL1 (and PL2, not shown) causes total tissue maceration with liberation of the cell contents, including starch granules. Bars, 100 µm.

spectively. Also pectin with 68% esterification was attacked by the three enzymes, albeit less efficiently (Fig. 5), PL3 again being more active than the other two isoforms. No degradation of pectin with 93% esterification could be detected. The K_m value towards 31% esterified pectin was 0.20 mg ml^{-1} for PL1 and PL2 and 0.10 mg ml^{-1} for PL3; the corresponding $V_{\max}$ values were 1228, 1041 and $2115 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$, indicating higher affinities to pectin than to pectate. Further evidence for the novel finding that the PLs depolymerize pectin more actively than pectate was provided by examination of electron micrographs (Fig. 6 and Wegener *et al.*, 1994). Separate application of the three *Eca* PLs to potato tuber tissue not only degraded the middle lamellae, but also effected disintegration of pectic components localized in the primary cell wall. The isoforms exhibited differences in degradation activity of potato tuber pectic material depending on the plant variety used. PL3 was the least active isoform towards esterified pectins of the potato cell walls, resulting in a breakdown of the middle lamellae. In contrast, incubation of tuber tissue with PL1 or PL2 gave rise to disintegration of single cells and liberation of the cell contents. These

results support the notion that *Eca* PLs *in vivo* exhibit major affinities to pectins of intermediate esterification, specifically contributing to the chemical composition of potato tuber cell walls indicated above. Possibly, various isoforms degrade pectin subdomains of diverse cell wall types.

Based on an extensive comparison of PL enzymes from various organisms, Barras *et al.* (1994) identified the sequence (S/A/T)-hWVDH-h (h representing I, L or V) in several PL and PNL enzymes, and suggested that it qualifies as a signature for pectate/pectin lyase activity. Since this sequence motif is highly conserved in the pectin- and pectate-degrading *Eca* PL1, PL2 and PL3 at positions 159–169 (Fig. 3), other subtle amino acid substitutions in substrate binding or catalytic residues may explain the differences in their substrate specificities. This is supported by recent results describing significant alterations in substrate affinities, acquired by changes of only amino acid side chains in the substrate-binding groove of two β -glucanases giving hydrolysis of either (1 → 3)- or (1 → 3, 1 → 4)- β -glucan (Varghese *et al.*, 1994).

Involvement of *Erwinia* PL isoforms in degradation of pectin was unexpected as the bacteria secrete PME, a deesterifying enzyme initially removing methoxyl groups from pectins (Rexová-Benková & Markovic, 1976). Its action yields methanol and PGA, which in turn is accessible to PGs and PLs (Plastow, 1988). Presumably, the action of PME occurs primarily towards highly esterified pectins (degree of esterification > 60%) or in concert with PG at the onset of pathogenesis. Due to alterations in environmental conditions during the progress of plant tissue degradation, an increase from pH 5.0 to pH 8.5 would accompany inactivation of PG (optimal activity for *Ecc* PG is at pH 5.5; Saarilahti *et al.*, 1990). Further pectin depolymerization possibly depends on the action of PL isoforms exhibiting affinities towards pectins, although an increase of pH in a continuing plant degradation could not be detected (data not shown). Thus, secretion of PL isoforms may be a specific adaptation of *Eca* to its main host plant potato and its numerous varieties. It remains to be established if similar enzyme specificities are characteristic for other *Erwinia* species. However, it has been suggested that *Ech* PL A partially depolymerized 20% esterified pectin (Favey *et al.*, 1992), while the *E. carotovora* EC153 *pel153* gene product preferred polypectate to pectin as substrate (Trollinger *et al.*, 1989).

Synergistic action between *Eca* PLs

Synergism is evident not only between enzymes utilizing identical substrates (e.g. cellulases; Irwin *et al.*, 1993; Vincken *et al.*, 1994), but also among enzymes with different specificities. For degradation of cell walls, strong synergism was found between pectolytic and cellulolytic enzymes (Renard *et al.*, 1991a, b). Since naturally occurring pectins are structurally heterogeneous in their side chain composition, degree of esterification and linkage frequency, synergism among pectolytic enzymes

Table 1. Depolymerization of PGA and pectin by PL isoenzyme combinations

Equal enzymic activities giving a total of 0.04 EU were applied to the substrate, followed by measuring the activity ($A_{236} \text{ min}^{-1}$). The expected activities are derived from the individual enzymic activities (data not shown). Values for synergism are calculated by dividing the measured activity by the expected activity; values > 1 indicate positive synergism. Results are the means $\pm$ SE of at least three measurements.

Enzyme combination	Substrate:								
	PGA			31 % esterified pectin			68 % esterified pectin		
	Activity measured	Activity expected	Synergism	Activity measured	Activity expected	Synergism	Activity measured	Activity expected	Synergism
PL1/PL2	0.177 $\pm$ 0.020	0.198 $\pm$ 0.013	0.89	0.229 $\pm$ 0.013	0.292 $\pm$ 0.009	0.78	0.022 $\pm$ 0.0015	0.023 $\pm$ 0.0009	0.96
PL1/PL3	0.213 $\pm$ 0.040	0.215 $\pm$ 0.013	0.99	0.263 $\pm$ 0.010	0.365 $\pm$ 0.005	0.72	0.037 $\pm$ 0.0016	0.027 $\pm$ 0.0015	1.37
PL2/PL3	0.181 $\pm$ 0.011	0.218 $\pm$ 0.007	0.83	0.290 $\pm$ 0.020	0.401 $\pm$ 0.013	0.73	0.033 $\pm$ 0.0014	0.025 $\pm$ 0.0020	1.32
PL1/PL2/PL3	0.187 $\pm$ 0.012	0.204 $\pm$ 0.012	0.92	0.231 $\pm$ 0.009	0.347 $\pm$ 0.020	0.67	0.041 $\pm$ 0.0013	0.025 $\pm$ 0.0005	1.64

displaying either identical or different modes of action might facilitate pectin depolymerization (Rexová-Benková & Markovic, 1976). To test this possibility it was examined what effect exogenous PLs would have on the depolymerization of pectin.

All possible enzyme combinations were added to solutions containing either PGA or pectin, followed by determination of product formation. Table 1 shows that no increased activities were detected when using PGA or 31% esterified pectin. The measurements gave activity values of 70–90% of the sum of the single enzyme activities. Although the monoenzymic degradation of 68% esterified pectin was low compared to the less esterified compounds, combination of some PLs revealed significantly enhanced depolymerization of the substrate. Interestingly, synergism only occurred in enzyme combinations containing PL3, resulting in up to 64% increase in activity. PL1 and PL2 together showed no higher degradation of pectin compared to depolymerization by the individual enzymes (Table 1). The closer linkage and length of the substrate side chains may influence enzymic breakdown and emphasize differences in substrate binding or cleavage specificities between the PL isoforms. It is noteworthy that an exo-acting PL from *Ecb* has been suggested to cleave highly esterified pectin (Brooks *et al.*, 1990). A similar enzyme is probably present in *Eca*, where it may function in concert with PLs to promote efficient degradation of pectin.

More detailed information was obtained by examining the time-course for product formation following degradation of pectin with enzyme combinations (Fig. 7). PL1 and PL2 together caused high initial activities followed by significant reduction after around 150 s. No increase of product formation was obtained when the reaction mixture was supplemented with additional PL1 or PL2 enzyme after 165 s, suggesting exhaustion of available substrate. On the other hand, supplementation with PL3, either initially or after 165 s, produced a significant

Fig. 7. Depolymerization of 68% esterified pectin. The reactions were initiated by applying combinations of two PLs, each isoenzyme at a concentration of 0.12 EU: ●, PL1 and PL2; ▲, PL1 and PL3; ■, PL2 and PL3. Arrows indicate addition of the third isoenzyme at a concentration of 0.12 EU. Further amounts of enzyme added did not result in enhanced degradation of pectin. Individual points represent the mean of three measurements differing by less than 5%.

enhancement of pectinolysis. These data show that PL1 and PL2 generate products which can be further degraded by PL3, implying differences in substrate binding and action patterns among the isoenzymes, with PL3 being less affected by steric features of the substrate, such as esterification and sidechain length. Because of the relatively low activity towards 68% esterified pectin, it

remains to be established if these observations are of significance for the degradation of pectin *in vivo*.

Multiplicity of PLs

The reason why *erwinias* have acquired multiple genes encoding PLs is an intriguing question. It could be argued that pectate, which has been supposed to be the natural substrate for PL, is chemically homogeneous and therefore would not necessitate multiple enzymes for its degradation. Multiple genes encoding PL could, however, result in elevated synthesis of the required enzymes for efficient depolymerization of pectate. Alternatively, it seems likely that the various PL isoenzymes hydrolyse α -(1 $\rightarrow$ 4) bonds situated in different physical environments within the heterogeneous cell wall pectin. This is supported by our results showing that not only do the *Eca* PL isoenzymes depolymerize pectin of intermediate esterification more efficiently than pectate (Fig. 5), but they also reveal synergism for degradation of 68 % methylated pectin (Table 1). The results obtained by measuring product formation following addition of multiple purified PL isoenzymes to pectate or pectin (Table 1, Fig. 7) imply that PL1 and PL2 produce substrates for the action of PL3, the multiple genes thus providing the organism with selective advantages.

Several compounds function as inducers for expression of *Ecb pel* genes (summarized by Sauvage & Expert, 1994). Among these are pectate oligomers and polymers. Since our results indicate differences in the action of PL isoenzymes, it will be of interest to study the effect on *pel* gene expression of adding specific pectic products to *Eca* cultures.

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