Purification of the acidic pectate lyase and nucleotide sequence of the corresponding gene (pelA) of Erwinia chrysanthemi strain 3937

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The pelA gene from Erwinia chrysanthemi strain 3937, which encodes the acidic pectate lyase, PLa, has been sequenced and characterized. The structural gene consists of a 1179 bp open reading frame encoding a polypeptide of 41 555 Da, which includes an N-terminal signal peptide. The deduced amino acid sequence shows a protein very similar to some PLs already sequenced. Cloning of the pelA gene behind the lacZ promoter of the vector pTZ19R allowed overexpression of PLa into a derivative of strain 3937 deleted of the other pel genes. The mature protein was obtained in milligram amounts from the supernatant of this strain and at homogeneous purity after two purification steps. Its biochemical properties were similar to those of other PLs. Polyclonal antibodies raised against the purified PLa cross-reacted with the basic pectate lyase PLd, but not with PLe. The role of PLa in pathogenicity is discussed.

Introduction

The enterobacterium Erwinia chrysanthemi strain 3937, which causes soft rot disease on its natural host, Saintpaulia ionantha, produces and secretes a battery of depolymerizing enzymes including five isoenzymes of pectate lyase (PL) which can be differentiated by isoelectric focusing (Bertheau et al., 1984). PLs cleave internal glycosidic linkages in pectic polymers by β-elimination. Five pel genes organized in two clusters encode these five PL activities (Kotoujansky et al., 1985; Reverchon et al., 1986). The genes pelB and pelC are adjacent and encode the two 'neutral' isoenzymes, PLb and PLc. One 'acidic' and two 'alkaline' isoenzymes are produced respectively from the pelA, pelD and pelE genes, and form the second cluster. Each gene is transcribed from its own promoter (Diolez & Coleno, 1985; Reverchon et al., 1986), and transcription is induced in the presence of polygalacturonate (PGA).

Despite strong similarity between the PLa and PLe amino acid sequences (Tamaki et al., 1988), PLa shows differences from other secreted PLs of E. chrysanthemi. PLa has an acidic isoelectric point, has poor maceration activity on potato tuber (Garibaldi & Bateman, 1971; Barras et al., 1987; Tamaki et al., 1988), and its gene, pelA, is hardly induced by PGA (Reverchon et al., 1986). Nevertheless, PLa is essential for full pathogenicity of E. chrysanthemi 3937 on Saintpaulia plants (Bocca et al., 1988). Furthermore, no cross-reactivity was observed between PLa and others PLs with polyclonal (Pupillo et al., 1976) or monoclonal antibodies (Vergnet-Ballas et al., 1986; Y. Bertheau and others, unpublished).

No antibody against PLa has so far been obtained. To prepare an antiserum which recognizes PLa, the purification of the PLa isoenzyme was undertaken and is presented in this paper. To obtain PLa in its mature state, we chose to purify it from the culture medium of an E. chrysanthemi strain overproducing PLa.

Methods

Bacterial strains, plasmids, media and growth conditions: The bacterial strains and plasmids used in this study are shown in Table 1. Escherichia coli strains were grown in Luria broth medium (L medium) (Miller, 1972) at 37 °C. All Erwinia chrysanthemi strains used in this study derived from the wild-type strain 3937. E. chrysanthemi strains were

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M77808.
Table 1. Bacterial strains, plasmids and bacteriophage

<table>
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<th>Relevant characteristics</th>
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<td>E1006</td>
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<td>PMV4123 ΔpelB pelC pelD pelE perm</td>
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<td>PMV4124 PMV4123 derivative containing pPMV0161</td>
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<td>PMV4066 Insertion of O fragment in pelA, pelB</td>
<td>Boccara et al. (1988)</td>
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<td>PMV4125 PelA+ PelE- (Spec')</td>
<td>M. Boccara, unpublished</td>
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<td>PMV4116 Δpel</td>
<td>F. Renou</td>
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<td>Phage φEC2</td>
<td>Resibois et al. (1984)</td>
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| Plasmid pU63-3 pUC derivative containing plB
| G. Murphy |
| pTZ19R pUC derivative with T7 RNA polymerase promoter | Mead et al. (1985) |
| pPMV0161 pTZ19R derivative containing pelA on a Dra1 fragment of E. chrysanthemi 3937 in the Smal site (Ap') | This paper |

* J. L. Aymeric, LCB, Marseille, France; F. Renou, INA, Paris, France; G. Murphy, IPS, Norwich, UK.

grown at 30 °C in L medium or in PL-inducing medium (per litre: KH₂PO₄, 3 g; Na₂HPO₄, 0.5 g; MgSO₄·7H₂O, 0.2 g; (NH₄)₂SO₄, 3 g; Casamino acids (Difco), 5 g; polygalacturonic acid (Sigma), 5 g; adjusted to pH 7) (Bertheau et al., 1984). PL-producing bacteria were screened on PGT agar, comprising M9 agar medium (Miller, 1972) supplemented with 5 g glyceral, 1 g yeast extract and 5 g sodium PGA 1-2. Antibiotics were used at the following concentrations: ampicillin 50 µg ml⁻¹, chloramphenicol 20 µg ml⁻¹ and spectinomycin 25 µg ml⁻¹.

Preparation of phage lysates. Transduction with phage φEC2 was performed according to Resibois et al. (1982).

DNA analysis. Large-scale preparations of plasmid DNAs were made from clear lysates followed by two centrifugations in a caesium chloride/ethidium bromide density gradient (Maniatis et al., 1982). Mini-scale preparations of plasmid DNAs were made from Escherichia coli cells by the rapid boiling method of Holmes & Quigley (1981). After restriction, the DNA was electrophoresed in 0.8% agarose gels in Tris/borate buffer (Maniatis et al., 1982). Restriction enzymes and T4 DNA ligase (BRL) were used according to the supplier's recommendations.

DNA sequencing. DNA sequencing was performed by the dideoxy chain-termination method of Sanger et al. (1977) using the Sequenase kit (United States Biochemical Corporation). The pelA-containing fragment was digested by the restriction enzymes Sau3A, TaqI, Alul and HpalI. The resulting fragments were cloned in E. coli cells by the rapid boiling method of Holmes & Quigley (1981). After restriction, the DNA was electrophoresed in 0.8% agarose gels in Tris/borate buffer (Maniatis et al., 1982). Restriction enzymes and T4 DNA ligase (BRL) were used according to the supplier's recommendations.

Cup-plate assays. Activities of PLa, proteases, cellulases and pectin methyltransferase were tested by the cup-plate method as described by Bertheau et al. (1984).

PL assays. PL assays were performed on supernatants of cells grown to late exponential phase in PL-inducing medium. PL activity was determined by the release from PGA (Sigma) of unsaturated products that absorb at 235 nm (Moran et al., 1968) in 0.1 M-Tris buffer pH 8.6, 5 x 10⁻⁴ M-CaCl₂. One unit of PL activity was defined as 1 µmol unsaturated products liberated min⁻¹ (mg bacterial dry weight)⁻¹.

Preparation of concentrated supernatants. Strain PMV4124 was grown in PL-inducing medium at 30 °C to OD₆₀₀ = 2. The culture was centrifuged at 6000 g and then filtered successively through 8 µm, 0.8 µm, 0.45 µm and 0.22 µm filters (Millipore) and concentrated against polyethylene glycol (Carbowax 20M, Touzard and Matignon) in dialysis tubing. After concentration, the bags were extensively dialysed against 25 mM-imidazole/HCl buffer pH 7.4, for purification, or 100 mM-Tris/HCl buffer pH 8.6, for analytical purposes. A final concentration of 100-fold was achieved. Protein concentration was measured according to Bradford (1976) with bovine serum albumin (Boehringer Mannheim) as a standard. This crude supernatant was stored at 4 °C.

Purification of PLa. Step 1: chromatofocusing. Two millilitres (2 mg protein) of the 100-fold-concentrated crude extract in 25 mM-imidazole/HCl buffer pH 7.4 was loaded onto an HPLC chromatofocusing column (Mono P HR5/20, Pharmacia) equilibrated in the same buffer for 20 min. The adsorbed protein fractions were eluted after 18 min washing, using 55 ml polybuffer 7-4 (Pharmacia) diluted 10-fold and adjusted to pH 3.5. The column was then regenerated with 5 ml 1.5 M-NaCl (flow rate 1 ml min⁻¹). Fractions of 1 ml were collected and PL activity tested by the cup-plate assay. PL-active fractions were pooled, concentrated against polyethylene glycol and dialysed against 100 mM-Tris/HCl buffer pH 8.6.

Step 2: hydrophobic interaction chromatography. The fractions mentioned above were added to an equal volume of 3 M-(NH₄)₂SO₄ and loaded onto a hydrophobic interaction HPLC column (Phenyl Superose HR5/5, Pharmacia), equilibrated with 1.7 M-(NH₄)₂SO₄ in 50 mM-Tris/HCl pH 8.6. Elution was done with a decreasing linear gradient of (NH₄)₂SO₄ in the same buffer (flow rate 0.5 ml min⁻¹). The fractions exhibiting PL activity were pooled, further concentrated and dialysed using Centricron 10 microconcentrators (Amicon), and stored at 4 °C.
Isoelectric focusing (IEF). PL isoenzymes were analysed in the supernatant of stationary-phase bacteria grown in PL-inducing medium. Fifteen microfilters of each fraction were layered on a thin polyacrylamide gel and electrofocusing was performed in a pH gradient from 3 to 10 (Pharmacia). PL activities were assessed directly on the gel by an overlay technique according to Bertheau et al. (1984).

Polyacrylamide gel electrophoresis. Electrophoresis under denaturing conditions (SDS-PAGE) was performed according to Laemmli (1970), with a 2% (w/v) stacking gel and a 12% (w/v) running gel, using the Mini-PROTEAN II dual slab cell system (Bio-Rad). Gels were fixed and proteins stained according to the silver nitrate method of Merrill et al. (1981). To enhance subsequent renaturation of PL, bovine serum albumin (Boehringer Mannheim) at 20 μg ml⁻¹ was incorporated in the resolving gel when the gels were to be stained for enzyme activity (Lacks & Sprinholm, 1980). The gels were then incubated in several changes of 10 mM-Tris/HCl pH 8 and a 6% (w/v) solution of naphthol As-Mx phosphate conjugate (Biosys). Blots were developed at 37°C in a 0.5% M-Tris/HC1 buffer pH 7.5 for 1 h before being assayed by the overlay technique of Bertheau et al. (1984).

Origin and production of antibodies. Polyclonal antibodies were produced in two rabbits by a modification of the method described by Vaitukaitis (1981). The first immunization consisted of 10 intradermal multipoint injections of 100 μl (2.5 μg purified enzyme). Then, on a further four occasions, at 20 d intervals, one subcutaneous injection (1 ml, 25 μg of purified enzyme) was performed. Serum samples were taken just before each injection.

Immunoblotting. Proteins were electrotransferred from SDS-PAGE or IEF gels to nitrocellulose membranes (BA 85, 0.45 μm, Schleicher and Schuell) according to Towbin et al. (1979). Proteins bound to the nitrocellulose membrane were stained with 0.5% (w/v) Ponceau red S solution in water. Immunological detection of proteins on nitrocellulose was performed at room temperature. PLa was detected by incubation with the appropriate antiserum, followed by goat anti-rabbit IgG alkaline phosphatase conjugate (BioSys). Blots were developed at 37°C in a mixture (1:1, v/v) of 0.4% (w/v) fast red TR salt (Sigma) in 0.2 M-Tris/HCl buffer pH 8 and a 6% (w/v) solution of naphthol As-Mx (Sigma) in distilled water.

Results

Sequence of the pelA gene

To achieve overexpression of PLa, sequencing of the pelA gene of strain 3937 was first undertaken. The pelA gene was shown to be included in a 1-6 kb DraI fragment, as PLa activity was detected in Escherichia coli strains harbouring pPMV0161. This fragment was sequenced. The data, summarized in Fig. 1, revealed a single long open reading frame of 1179 bp, which corresponds to the PLa preprotein. It was not possible to assign putative promoter sequences with assurance, since computer searches failed to disclose any typical E. coli promoter sequences 5’ to the coding region. However, several putative weak promoter sequences were present. Nuclease S1 mapping experiments have so far been unsuccessful as pelA RNA was found to be very unstable (C. Bourson, unpublished). Upstream from the pelA gene, we identified two putative KdgR-binding sites. The KdgR protein regulates many genes involved in the pectin degradation pathway (Condemine & Robert-Baudouy, 1987), and a consensus sequence for KdgR binding showing the typical symmetry of operator sites has been determined (Reverchon et al., 1989; Condemine & Robert-Baudouy, 1991). Furthermore, an AT-rich region, as described for other sequenced pel genes (Reverchon et al., 1989; Tamaki et al., 1988), was also observed in the 5’ untranslated end of the pelA gene. This region could be of importance for the regulation of the pel genes. A GC-rich palindromic sequence, followed by a T repeat, occurred after the translational stop codon of pelA (TTA). This sequence is typical of rho-independent transcription-termination sites (Von Hippel et al., 1984). All the PL genes of E. chrysanthemi so far sequenced possess a TAA stop codon and a rho-independent transcription-termination site (Schoedel & Collmer, 1986; Tamaki et al., 1988; Reverchon et al., 1989).

Upstream of the initiation codon we observed a typical Shine–Dalgarno sequence. The deduced amino acid sequence of the pelA gene corresponded to a polypeptide of 393 amino acids, with a calculated molecular mass of 41 555 Da, and included a typical N-terminal signal sequence. The cleavage site of this signal peptide was predicted to be between the two alanine residues at nucleotide positions 336 and 337 (Fig. 1). This was confirmed by determining the sequence of the 17 first amino acids of the purified mature protein. The mature PLa protein contains 361 amino acids, and has a calculated molecular mass of 38 412 Da and a calculated pl of 5.2. The deduced amino acid sequence of E. chrysanthemi 3937 PLa was compared with the published sequences of PLs from E. chrysanthemi strains EC16, B374 and 3937. Our sequence has considerable similarity (about 60%) with the PLd and PLe of these three strains and 90% similarity with the PLa of strain EC16. The similarity is greatest in the central part of the protein.

Construction of a strain overproducing PLa

To obtain a strain producing solely PLa, a mutant of strain 3937 deficient in the production of the PLb, PLc, PLd and PLe isoenzymes was constructed by using marker exchange-eviction mutagenesis with the npt-sacB-sacR cartridge as described by Ried & Collmer (1988). The clone PMV4122, which had suffered a 6 kb deletion covering the pelB and pelC genes and a deletion of 6·7 kb which removed the pelD, pelE and pem genes, was selected for PLa production (F. Renou & M. Boccara, unpublished).

The endoglucanase EgZ, secreted by E. chrysanthemi 3937, has a pl similar to that of PLa (Boyer et al., 1987). To eliminate EgZ protein, the celZ : :Ω mutation of strain
pelA gene of Erwinia chrysanthemi

Fig. 1. Nucleotide sequence of the 1.6 kb DraI fragment containing the pelA gene of E. chrysanthemi strain 3937. The deduced amino acid sequence of PLa is shown and selected restriction sites are specified. The putative Shine-Dalgarno sequence (SD) and KdgR-binding sites are underlined. The potential rho-independent termination sites are indicated by facing arrows representing the inverted repeat of G+C-rich dyad symmetry preceding the poly(T) sequence. The downward arrow indicates the signal sequence cleavage site of PLa.

E1006 (J. L. Aymeric, unpublished) was introduced into strain PMV4122 by transduction with phage φEC2. A Spc⁺ CelZ⁻ transductant was obtained and called PMV4123.

Finally, the 1.6 kb DraI fragment containing the pelA gene cloned in the SmaI site of pTZ19R (Mead et al., 1985) was introduced into strain PMV4123. When the lacZ promoter was present upstream of the pelA gene, the PLa activity in PL-inducing medium was 15-fold higher than when the DraI fragment was in the other orientation. One clone, PMV4124, was retained. The protein profiles after SDS-PAGE of supernatants of strains 3937, PMV4122 and PMV4124 grown in PL-inducing medium (see Fig. 3a) showed that PLa, as identified by its activity, represented the major protein in the culture medium of strain PMV4124 whereas it was hardly visible in the other two strains.

Purification of PLa

Titration curves in polyacrylamide gels showed that PLa had a strong charge variation around its isoelectric point (data not shown). Thus we chose chromatofocusing for the first step of purification.

After chromatofocusing, PL activity appeared in the 42-44 min fractions (Fig. 2a). These fractions were pooled, concentrated and dialysed for the next purification step. No protease, cellulase or pectin methyl-esterase activities were found in these fractions. Silver-nitrate-stained IEF and SDS-PAGE gels showed purity of PLa after this step (Fig. 3a, b). However, the PLa-containing fractions were contaminated with ampholines present in the polybuffer used for elution.

To remove the ampholines, hydrophobic interaction chromatography was used. The ampholines are not fixed onto the hydrophobic gel, whereas the adsorbed PLa activity eluted at the end of the decreasing (NH₄)₂SO₄ gradient (Fig. 2b). A single protein peak with PLa activity was obtained.
Purity of the protein was checked with silver-nitrate-stained SDS-PAGE and IEF gels (Fig. 3a, b). Using this two-step purification procedure, PLα was obtained at homogenous purity but with only 11% recovery of PLα activity (Table 2).

**Biochemical properties of the PLα**

Using a purified preparation of the mature PLα protein isolated from the supernatant fraction of strain PMV4124, the N-terminal residue was shown to be alanine. This classical cleavage site presumably accounts for the fact that PLα is efficiently exported to the periplasm of *Escherichia coli* cells (Reverchon et al., 1985). The purified enzyme migrated in SDS-PAGE with an apparent molecular mass of 44 kDa. DNA sequence data gave a molecular mass of 39 kDa for the mature protein. This discrepancy was also observed for PLε and PLα of *E. chrysanthemi* EC16 (Tamaki et al., 1988). The behaviour of PLε was previously suspected to be due to its relatively high pI; however, this cannot be applied to PLα. A pI of 4.6 was consistently found in equilibrated IEF gels (Fig. 3b), this being different from the value of 5.2 obtained from the DNA sequence and chromatofocusing data. IEF gels and chromatofocusing were performed at different temperatures, at 4 and 20 °C, respectively, which might explain the observed difference in pI values.

An increase in pectin esterification (more than 20% of galacturonic acid residues methoxylated) led to less...
efficient degradation of the substrate by PLa (data not shown). This result was expected, since PLs cleave internal glycosidic linkages by β-elimination after demethylation of the pectic polymer by pectin methyl-esterase. PLa caused a rapid decrease in the viscosity of PGA as measured in an Ostwald viscosimeter with a concomitant production of unsaturated products (a viscosity decrease of 50% corresponds to an $A_{235}$ less than 0.1), suggesting random cleavage of the substrate (data not shown). PLa was most active at pH 8.6 and 50 °C in 0.1 M-Tris/HCl, 5 × 10^{-4} M-CaCl$_2$. The activity was significantly stimulated by Ca$^{2+}$ (5 × 10^{-4} M-Ca$^{2+}$ increased the activity 15-fold compared to the control without Ca$^{2+}$), a property which is shared by bacterial and fungal PLs. No other monovalent and divalent cations tested, including Na$^+$, K$^+$, Mg$^{2+}$ and Mn$^{2+}$ (as chloride salts), stimulated PLa activity. Addition of 0.1 mM-EDTA or 0.5 mM-Hg$^{2+}$ completely inhibited PLa activity. The initial velocity for the reaction allowing the formation of unsaturated oligogalacturonides from PGA, at different concentrations of PGA, in 0.1 M-Tris/HCl buffer pH 8.6, 5 × 10^{-4} M-CaCl$_2$ and at 30 °C, was determined by spectrophotometric assay. From a Lineweaver–Burk plot of the data, the apparent $K_m$ and $V_{max}$ were estimated to be 0.43 mg ml$^{-1}$ and 483 U ml$^{-1}$, respectively. These results are similar to those obtained with PLb and PLc of $E$. chrysanthemi strain CUCPB1237 (Schoedel & Collmer, 1986).

**Characterization of antibodies raised against PLa**

Polyclonal antibodies were raised against purified PLa. Immunoblotting after IEF of concentrated supernatant (1 μg protein) of strain 3937 revealed two reacting bands at a 1000-fold serum dilution. One band corresponded to PLa (pI 4.6), the other showed a pI value of 9.2, corresponding to the pI of the PLd isoenzyme (Fig. 4). Even at a 200-fold dilution of the serum, no cross-reactivity was observed with PLe, although this PL was shown to have high similarity with PLd (Van Gijssegem, 1989). The band corresponding to PLa was not visible when 1 μg total protein was loaded, but a band corresponding to PLd was detected (not shown). The specificity of our antibodies was confirmed by using concentrated supernatant of strains PMV4066 and PMV4125, which are pelA and pelA pelE mutants respectively (Fig. 4). In addition, no reacting band was detected with strain PMV4116, a Pel$^-$ mutant (data not shown). After SDS-PAGE immunoblotting, only one immunoreactive protein band of 44 kDa was detected in concentrated supernatant of strain 3937 at a 200-fold serum dilution (not shown). The size of this protein...
corresponded to that of PLa, PLd and PLe, which exhibited the same molecular mass in SDS-PAGE gels (Fig. 3a).

Discussion

The sequence of the pelA gene of E. chrysanthemi strain 3937 demonstrated that all the sequences required for the synthesis of the acidic pectate lyase, PLa, were located within a 1-6 kb DraI genomic fragment. The deduced amino acid sequences of PL preproteins indicate that their signal peptides are rather long (31 amino acids for PLa of strain 3937, and 30 amino acids for PLe of strains 3937 and EC16), since in prokaryotes, signal sequences are usually 21-23 amino acids long (Von Heijne, 1985). The length of the PLs signal sequence was confirmed by analysing the N-terminal sequence of the purified protein. A signal sequence of 43 amino acids was found for the major endoglucanase EgZ of E. chrysanthemi 3937 (Guiseppi et al., 1988). Endoglucanases and PLs are secreted by the same mechanism (Andro et al., 1984), thus a longer signal sequence could play a role in the secretion of these proteins. However, the PLb preprotein possesses a signal sequence of 22 amino acids and PLb is also secreted (Keen & Tamaki, 1986).

In strain 3937, PLa activity represented about 3% of the total PL activity, the pelA gene being transcribed at a low level compared to the other pel genes (Reverchon & Robert-Baudouy, 1987). So, to obtain an E. chrysanthemi strain which overexpresses PLa, the 1-6 kb DraI fragment was cloned behind the lacZ promoter and used to transform strain PMV4123, which produces only PLa. PLa was the major extracellular protein of the resulting strain PMV4124, which allowed purification of the protein in large amount. To obtain a PL in significant quantities, other groups (Keen & Tamaki, 1986; Schoedel & Collmer, 1986) have previously cloned a pel gene on a high-copy-number plasmid in an Escherichia coli strain, PL then being purified from the periplasm. The mechanism by which proteins cross the inner membrane seems similar in the two bacteria, but in E. coli, PLs are not secreted. Although proteins obtained from two bacterial species have very similar pl values and molecular masses, one cannot exclude slight modification of the protein after the secretion step, and we therefore chose to purify PLa from the culture supernatant of Erwinia chrysanthemi strain PMV4124. In this strain, PLa was the major secreted protein, which might explain the final purification rate of 2-21-fold (Table 2). After the first purification step, 83% of PL activity and 87% of proteins were lost. As PLa is the major supernatant protein (Fig. 2), this loss could be due to degradation of PLa during its purification, for example by loss of a cofactor.

Biochemical characterization of purified PLa from E. chrysanthemi showed that optimal conditions for PLa activity are very similar to those of other E. chrysanthemi PLs. PLa activity is stimulated by Ca2+ ions, and is very stable. After three years at 4 °C in 100 mM-Tris/HCl buffer pH 8.6, 5 × 10^-4 M-Ca2+, the activity was only reduced twofold.

The polyclonal antibodies that we obtained against PLa cross-reacted only with PLd (Fig. 4). Similarly no cross-reactivity was observed between these two iso-enzymes by Thurn et al. (1987) using polyclonal antibodies raised against PLe from strain EC16, which corresponds to the PLd of strain 3937, or by Vergnet-Ballas et al. (1986) using monoclonal antibodies from strain 3937, which recognized solely PLd and PLe of all tested E. chrysanthemi strains. It is surprising that the PLe of E. chrysanthemi 3937 did not react with our polyclonal antiserum, since PLd is very similar at the sequence level to PLe in the very closely related E. chrysanthemi strain B374 (Boccarda et al., 1991; Van Gijsegem et al., 1989), and since the three proteins belong to the same family, according to the homologies observed by Hinton et al. (1989). The cross-reactivity observed could be explained by comparing polypeptide sequences, the PLa protein of E. chrysanthemi 3937 being slightly more similar to PLd than PLe of strain B374. Alternatively, the three-dimensional structure of PLe could be sufficiently different from those of PLd and PLa to explain these results.

Immunolocalization of PLs secreted by the bacteria in Saintpaulia plants infected by E. chrysanthemi 3937 has been performed using monoclonal antibodies respectively specific for PLb and PLC (Temsah et al., 1991). The results showed that PLb and PLC were preferentially located in the middle lamella and cell junction, whereas preliminary results indicated that PLd and PLe were preferentially located in the cell wall area close to the plasmalemma (M. Temsah, personal communication). Does PLa act differently in planta from the other PLs? Study of the localization of PLa in planta, using antibodies, could illuminate the importance of this isoenzyme in pathogenesis. However, monoclonal antibodies seem necessary, since our polyclonal antiserum cross-reacted with PLd. Alternately, a pelD mutant could be used.

Different studies (Hinton et al., 1989; Tamaki et al., 1988; Van Gijsegem, 1989; A. Darrasse & Y. Bertheau, unpublished) have shown that the PLa, PLd, PLe family, determined by sequence homology or by PCR amplification, is not present in Erwinia carotovora strains. Analysis of the PL profile of a large number of E. chrysanthemi strains of different geographical origins has
been performed (Bocca et al., 1991). PLA was present in most of the strains but not in a very homogeneous group of strains isolated in temperate countries. Studies of PL synthesis of strain 3937 at different temperatures showed an eightfold decrease at 37°C compared to 30°C (data not shown). However, strain PMV4122, which secrected only PLs, exhibited the same activity at both temperatures. Taken together, these results might suggest that PLA could contribute to the ability of E. chrysanthemi to cause symptoms at supraoptimal temperatures.

The PLA isoenzyme of E. chrysanthemi EC16 is a much less efficient macerating factor of plant tissue than PLe (Garibaldi & Bateman, 1971; Tamaki et al., 1988). It was suggested that the difference in pl between these two proteins may explain this observation. However, results obtained by Tamaki et al. (1988) for several recombinant pelA/pelE genes suggest that other factors, in addition to the low pl value of PLA, may contribute to its poor macerating efficiency. Despite the poor ability of PLA to macerate potato tubers, Bocca et al. (1988) showed that PLA was essential for full pathogenicity of E. chrysanthemi 3937 on Saintpaulia plants. PLs with little plant-macerating activity on potato tubers and low pl values, similar to PLA, have also been described for the non-plant-pathogenic bacteria Klebsiella pneumoniae and Yersinia pseudotuberculosis (Chatterjee et al., 1979; Manulis et al., 1988). The fact that these bacteria were often encountered in the soil raises the possibility that the acidic PLs may have alternative, as yet unknown, physiological functions for the survival of these bacteria in the soil.

Like all the genes of E. chrysanthemi involved in pectin degradation that have been identified, pelA is regulated by KdgR (C. Bourson, unpublished). Analysis of the pelA nucleotide sequence has shown the presence of two putative KdgR-binding sites, one 17 bp long and the other 15 bp long, similar to the KdgR-box consensus sequence A(AT)AAAA(AT)GAAA…TGTTTCA-T( AT)(AT)T determined by Condemine & Robert-Baudouy (1991). In PL-inducing medium, pelA expression was shown to be very weak compared with that of the other pel genes. However, preliminary results suggested that pelA is induced by total soluble plant extracts (C. Bourson and others, unpublished). Pupillo et al. (1976) have already shown that unlike the other PLs, the induction of PLA was stimulated in planta. Studies are in progress to determine whether the conditions for PLA induction differ from those for other PLs; these should give information on the possible role of PLA in planta.

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References


