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Comparative Analysis of the Five Major *Erwinia chrysanthemi* Pectate Lyases: Enzyme Characteristics and Potential Inhibitors

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In *Erwinia chrysanthemi* 3937, pectate lyase activity mainly results from the cumulative action of five major isoenzymes, PelA to PelE. Comparison of their amino acid sequences revealed two families, PelB-C and PelA-D-E. Molecular cloning permitted expression of the different *pel* genes in *Escherichia coli* and the isolation of each Pel independently from the other isoenzymes. We used similar experimental conditions to overproduce and purify the five Pels in a one-step chromatography method. We analyzed some of the basic enzymatic properties of these five isoenzymes. PelA has a low specific activity compared to the other four enzymes. PelB and PelC have a high affinity for their substrate: about 10-fold higher than the enzymes of the PelA-D-E group. The optimum pH is more alkaline for PelB and PelC (about 9.2) than for PelA, PelD, and PelE (from 8 to 8.8). Below pH 7, activity was negligible for PelB and PelC, while PelA, PelD, and PelE retained 25 to 30% of their activities. The temperature optima were determined to be 50°C for PelD and PelE, 55°C for PelA, and 60°C for PelB and PelC. Enzymes of the PelB-C group are more stable than those of the PelA-D-E group. Use of substrates presenting various degrees of methylation revealed that PelA, PelD, and PelE are active only for very low levels of methylation, while PelB and PelC are more active on partially methylated pectins (up to 22% for PelC and up to 45% for PelB). Pectate lyases have an absolute requirement for Ca^{2+} ions. For the five isoenzymes, maximal activity was obtained at a Ca^{2+} concentration of 0.1 mM. None of the tested cations (Ba^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Sr^{2+}, Zn^{2+}) can substitute for Ca^{2+}. At a high concentration (1 mM), most of the divalent cations inhibited pectate lyase activity. In addition, we demonstrated that two compounds present in plant tissues, epicatechin and salicylic acid, inhibit the pectate lyases at a concentration of 0.2 mM.

Pectins are important structural constituents of plant cell walls and, as such, play a key role in plant physiology and plant pathology. The general structure of pectic polymers consists of linear polysaccharide chains interspersed with ramified regions. They are essentially composed of long linear segments of D-galacturonic acid linked by α-1,4 glycosidic bonds. The carboxyl groups of the galacturonate residues are either not esterified (polygalacturonate) or present different degrees of methyl esterification (pectin) (Fig. 1). The structures of the ramified regions of pectic polymers are more complex and contain various neutral sugars, such as rhamnose, xylose, arabinose, and galactose.

Pectin degradation is accomplished by a variety of enzymes produced by microorganisms, particularly by plant pathogens. Pectinases differ in their substrate preference (polygalacturonate and low-methylated pectin or high-methylated pectin), in their reaction mechanism (β-elimination or hydrolysis), and in random or terminal attack of the polymer (endo or exo). By degrading the pectic fractions of the primary cell wall and middle lamella, pectinases facilitate pathogen penetration and colonization. The ability of purified endo pectate lyases (endo-Pels) of *Erwinia* spp. to macerate or cause cell death in plant tissue confirmed that these enzymes are of paramount importance in the soft-rot disease caused by these bacterial species (3, 11).

Erwinia chrysanthemi is characterized by its ability to secrete multiple isoenzymes of endo-Pels (EC 4.2.2.2). These enzymes randomly cleave, by β-elimination, the internal glycosidic linkages in pectic polymers, preferentially polygalacturonate or low-methylated pectin. They generate a series of oligogalacturonides, each with a 4,5-unsaturated residue at the nonreducing end (Fig. 1). In *E. chrysanthemi* 3937, most of the Pel activity results from the cumulative action of five major isoenzymes, PelA to PelE, which were first differentiated by isoelectric focusing and which present an increasing pI from PelA to PelE (5, 32). The five corresponding genes are organized in two clusters on the bacterial chromosome (*pelA*, *pelD*, and *pelE*, *pelB* and *pelC*) (17, 31). Comparison of the amino acid sequences of the five major Pels revealed two families corresponding to each genetic cluster (14), supporting the hypothesis that genes of the same family arose by duplication of an ancestral gene. As for the mature proteins, PelB and PelC show 84% identity, while PelD and PelE are 79% identical and have about 60% identity with PelA.

Deletion of the five major *pel* genes of *E. chrysanthemi* failed to totally eliminate the capacity for tissue maceration and revealed the existence of a new set of Pels (4, 20). Because of their low activity, they are described as secondary pectinases. In *E. chrysanthemi* 3937, two secondary endo-Pels, PelL and PelZ, have been characterized (23, 27a) in addition to the exo-Pel PelX (7, 35a). These Pels define new classes of enzymes, since they display no homology with the major pectinases, while PelL and PelX share low homology.

The different implication of each Pel in pathogenicity (6) led us to suppose that there are differences in the conditions of production and/or enzymatic activity. On the one hand, the regulation of transcription of each *pel* gene was analyzed in detail at both physiological and molecular levels (15) and dur-

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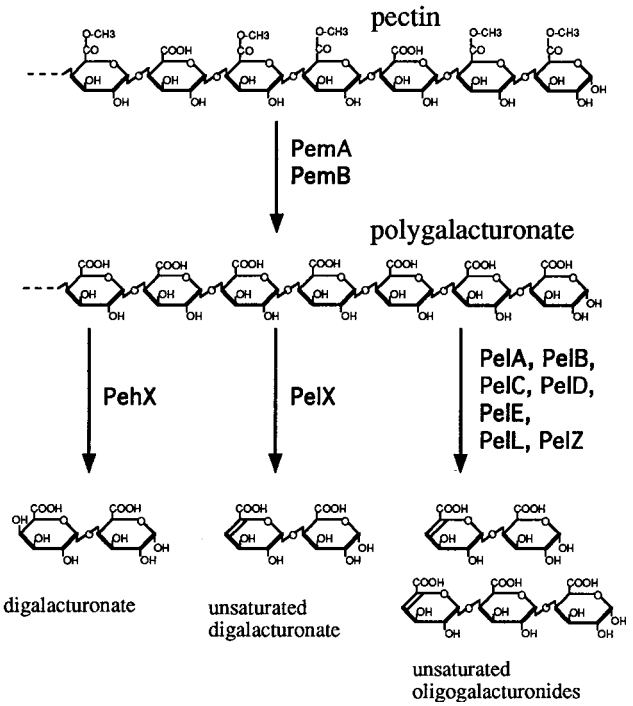

FIG. 1. Degradation of pectin by *E. chrysanthemi* 3937. Pectin methylesterase (PemA, PemB) demethoxylates the pectic polymers to give polygalacturonate and methanol. Polygalacturonate is cleaved into unsaturated oligomers (dimers and longer oligomers) by the action of Pels. Strain 3937 produces five major isoenzymes of endo-Pel (PelA, PelB, PelC, PelD, PelE), a set of secondary endo-Pels (PelL, PelZ, etc.), and an exo-Pel (PelX). An exo-polygalacturonase (PehX) hydrolyzes polygalacturonate to give dimers of galacturonate.

ing plant infections (22, 24). Genetic analysis demonstrated that each *pel* gene constitutes an independent transcriptional unit and can be independently expressed (17, 31). This genetic multiplicity increases the possibilities of differential regulation either by environmental parameters or in response to signals encountered in plants. On the other hand, the variety of isoenzymes may result from individual functional adaptations of the enzymatic activities in relation to the plant conditions that obtain at the first step of infection or that evolve during tissue maceration.

The multiplicity of Pel isoenzymes has complicated their individual biochemical analysis. Molecular cloning allowed isolation of the different *pel* genes in *Escherichia coli* and permitted purification of each Pel free from the other isoenzymes. In *E. chrysanthemi*, Pels are secreted in the extracellular medium by a two-step secretion system: transfer through the cytoplasmic membrane, accompanied by maturation through the cleavage of the signal sequence, is dependent on the *sec* system, while transfer through the outer membrane is dependent on the specific *out* system (33). In *E. coli*, these proteins are retained in the periplasm because of the lack of Out secretion machinery. The periplasmic Pels produced in *E. coli* appeared to have physical and biochemical properties identical to those of the *E. chrysanthemi* proteins (1).

Previous investigations of the catalytic properties of *E. chrysanthemi* Pels (3, 8, 19, 27, 35, 40) were made with various strains, using different purification procedures, and sometimes with nonhomogeneous preparations, since the very similar molecular weights and pIs of some of the Pels render their separation difficult. Therefore, the purity of Pels that have been previously fractionated from *E. chrysanthemi* is suspect. Moreover, it is difficult to compare studies of the diverse isoenzymes, since the results have been obtained by different authors. In this paper, our purpose was to compare the enzymatic properties of the five major endo-Pels from strain 3937 of *E. chrysanthemi*. We used similar experimental conditions for overproduction and purification of the proteins from *E. coli* to study the enzymatic properties of the five Pels in a more rigorous way.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* cells were usually grown at 30°C in complete Luria-Bertani medium (26). When required, the medium was solidified with agar (15 g/liter). Antibiotics were added at the following concentrations: ampicillin (Ap), 50 μg/ml; kanamycin (Km), 20 μg/ml; chloramphenicol (Cm), 20 μg/ml. pT7 medium (NaCl, 5 g/liter; Bacto-Tryptone, 20 g/liter; yeast extract, 10 g/liter; glycerol, 2 g/liter; adjusted to pH 7.2 with 50 mM phosphate buffer) was used for overproduction of Pels (39). The recombinant plasmids were screened for pectinolytic activity either on YC medium (18) or on M63 minimal medium containing polygalacturonate (30).

TABLE 1. *E. coli* strains and plasmids

Strain or plasmid	Genotype, phenotype	Reference, origin
NM522	Δ(*lac-proAB*) Δ(*mcrB-hsdSM*)5 *supE thi* (F′ *proAB lacI*^{q} *lacZ*Δ*M15*)	Laboratory collection
BL21(DE3)	*E. coli* B, F$^-$ *dcm ompT hsdS gal* λ(DE3), T7 polymerase gene under the *lacUV5* promoter	37
K38	HfrC (λ) *phoA4 pit-10 tonA22 ompF627 relA1*	39
Plasmids		
pGP1-2	*c*I857, T7 polymerase gene under the λP$_L$ promoter, Km^r	39
pT7-5	T7Φ10, Ap^r	39
pT7-6	T7Φ10, Ap^r	39
pA612	pBluescript with 1.5-kb *Cla*I-*Dra*I fragment *pelA*$^+$, Ap^r	Laboratory collection
pNC52	pBluescript with 3.9-kb *Bam*HI fragment *pelB*$^+$, Ap^r	17
pNG1	pBluescript with 2.4-kb *Hin*dIII-*Pst*I fragment *pelC*$^+$, Cm^r	17
pND1	pBluescript with 2-kb *Bgl*II-*Fsp*I fragment *pelD*$^+$, Cm^r	Laboratory collection
pE611	pBluescript with 1.75-kb *Dra*I fragment *pelE*$^+$, Ap^r	Laboratory collection
pT7-*pelA*	pT7-5 with 1.48-kb *Cla*I-*Dra*I fragment *pelA*$^+$	This work
pT7-*pelB*	pT7-6 with 1.65-kb *Nsi*I-*Bam*HI fragment *pelB*$^+$	This work
pT7-*pelC*	pT7-6 with 1.45-kb *Bam*HI-*Sma*I fragment *pelC*$^+$	This work
pT7-*pelD*	pT7-5 with 1.65-kb *Mam*I-*Fsp*I fragment *pelD*$^+$	This work
pT7-*pelE*	pT7-5 with 1.75-kb *Dra*I fragment *pelE*$^+$	This work

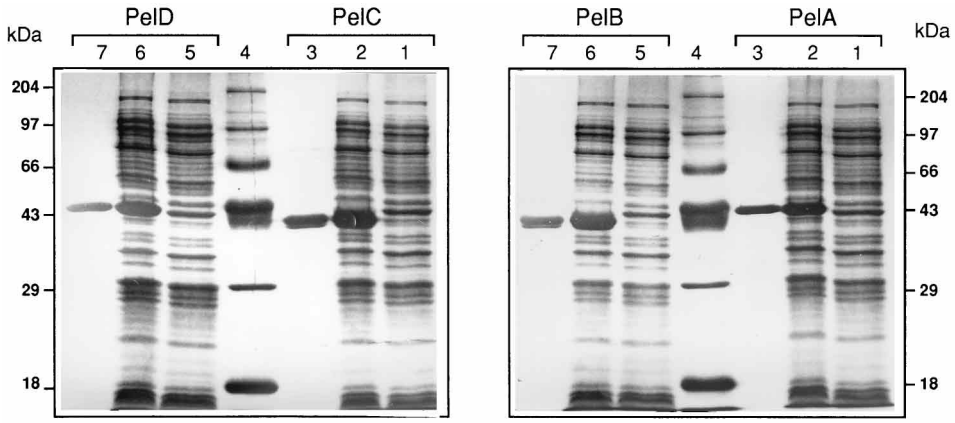

FIG. 2. SDS-PAGE of the *E. chrysanthemi* Pels purified from *E. coli*. Purification of PelA, PelB, PelC, and PelD is presented. Protein bands were detected by Coomassie blue staining. Lanes 1 and 2 and lanes 5 and 6 extracts of the recombinant *E. coli* cultures before and after IPTG induction, respectively; lanes 3 and 7, purified enzyme. The molecular sizes of the marker proteins, in kilodaltons, are indicated on both sides of the figure.

Recombinant DNA techniques. Preparation of plasmid DNA, restriction digestions, ligations, DNA electrophoresis, and bacterial transformations were carried out by the methods of Sambrook et al. (34).

Protein analysis. Protein concentrations were determined as proposed by Bradford, using the Bio-Rad protein assay with gamma globulin as a standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli on slab gels (4% stacking gel and 12% resolving gel) using the Mini-Protean II system (Bio-Rad). Protein bands were detected by Coomassie blue staining. Isoelectrofocusing was performed in a 3 to 10 pH gradient as previously described (5).

Overexpression of *pel* genes. Overexpression of the *pel* genes was obtained by using the T7 promoter/T7 RNA polymerase system (37, 39). Fragments containing the *E. chrysanthemi pel* genes were subcloned into pT7-5 or pT7-6 expression vectors (Table 1). The recombinant plasmids were first selected in *E. coli* NM522 and then screened for pectinolytic activity. The recombinant plasmids were then introduced in *E. coli* BL21(DE3), which contains a single chromosomal copy of the gene encoding T7 RNA polymerase under the control of the *lacUV5* promoter. The BL21(DE3) pT7-*pel* clones were grown in 300 ml of pT7-medium supplemented with Ap. When the optical density at 600 nm (OD_{600}) of the cultures reached 0.4 to 0.9 (depending on the Pel), the synthesis of T7 RNA polymerase was induced by the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and cells were grown for an additional 2 to 3 h at 28 or 30°C (final OD_{600}, 1 to 1.6).

Extraction and purification of the *E. chrysanthemi* Pels produced in *E. coli*. All purification steps were conducted at 4°C. After IPTG induction, the cells were collected by centrifugation, washed, and suspended in 0.2 M Tris-HCl (pH 8). The protein fractions corresponding to the different cell compartments were extracted by the protocol described by Witholt et al. (44). The Pel activity was found mainly in the periplasmic fluid. Fractions with Pel activity were adjusted to 10 mM EDTA and 1 mM dithiothreitol. The resulting extracts were subjected to ammonium sulfate fractionation. Precipitates collected at 40, 55, 70, 80, and 100% ammonium sulfate saturations were taken up in small volumes of purification buffer (50 mM Tris-HCl [pH 7], 1 mM EDTA, 1 mM dithiothreitol). The 80 or 100% ammonium sulfate fractions containing Pel activity were adjusted to 1.7 M $(NH_4)_2SO_4$. This preparation was applied to a TSK-gel Phenyl 5PW column (150 by 21.5 mm) previously equilibrated with purification buffer containing 1.7 M $(NH_4)_2SO_4$. The column was washed extensively with the same buffer, and the proteins were eluted at 4 ml/min with a decreasing linear $(NH_4)_2SO_4$ gradient from 1.7 to 0 M, using a Waters high-pressure liquid chromatography system. Fractions (2 ml each) were collected and assayed for Pel activity. The purity of active fractions was estimated by SDS-PAGE, and pure Pel-containing fractions were pooled, concentrated, and desalted with a Centricon 10 filtration device (Amicon). Purified proteins were stored at −20°C in purification buffer containing 50% (vol/vol) glycerol.

Pel assays. Pel activity was determined spectrophotometrically by the release from polygalacturonate of unsaturated oligogalacturonides that absorb at 230 nm. One unit of Pel activity is the amount of enzyme which liberates 1 μmol of product per min at 37°C. Specific activity was expressed as enzyme units per milligram of protein. Unless otherwise specified, the standard assay mixture consisted of 100 mM Tris-HCl (pH 8.5), 0.1 mM $CaCl_2$, and 0.5 g of polygalacturonate per liter in a total volume of 1 ml. The products released from polygalacturonate were continuously monitored at 37°C for 2 min. The molar extinction coefficient of unsaturated oligogalacturonides was assumed to be 5,200 (27).

The influence of Ca^{2+} was investigated by addition of $CaCl_2$ concentrations ranging from 0 to 1 mM. To verify the Ca^{2+} requirement, EDTA was added to a final concentration of 1 mM to chelate endogenous cations. The influence of other divalent cations (Ba^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Sr^{2+}, Zn^{2+}) was determined with 0.1 and 1 mM concentrations of the corresponding chloride forms.

The optimum pH was determined using the following buffers: PIPES [piperazine-*N*-*N*′-bis(2-ethanesulfonic acid]-HCl (pH 6 to 7.5), ACES [*N*-(2-acetamido)-2-aminoethanesulfonic acid]-HCl (pH 6 to 7.5), Tris-HCl (pH 6.6 to 9.5), and Bis-Tris-propane (pH 6.3 to 10.2), each at a concentration of 100 mM.

For determination of K_m and V_{max}, the enzymes were incubated with polygalacturonate concentrations ranging from 0.01 to 0.1 g/liter for PelB and PelC and from 0.025 to 1 g/liter for PelA, PelD, and PelE.

Activity on substrates presenting various degrees of methylation was determined by using the standard assay mixture but substituting 7, 22, 45, 60, 75, and 90% esterified citrus pectins (from Copenhagen Pectin) for polygalacturonate.

Various compounds (digalacturonate, galacturonate, galactose, salicylate, epicatechin, etc.) were tested as potential inhibitors of Pel activity. They were added to the assay mixture at concentrations ranging from 0.05 to 10 mM, and the residual activity was measured.

RESULTS

Overproduction and purification of the five Pels. Each *pel* gene of *E. chrysanthemi* 3937 was independently cloned in a pT7 expression vector (Table 1), and these constructs were introduced into *E. coli* BL21(DE3). Induction of the T7 RNA polymerase by addition of IPTG allowed for determination of the most convenient culture temperature. We noticed that at high temperatures, mainly precursor forms of recombinant Pels were obtained; overproduction was more efficient, but exportation to the periplasm and maturation of the Pels were incomplete. We therefore chose growth temperatures of 28°C for production of PelA and 30°C for production of PelB, PelC, PelD, and PelE. With these conditions, an induction factor of 10 to 25 was obtained (Fig. 2).

The first step in purification consisted of preparation of the periplasmic fraction from the recombinant cells. After this step, a purification factor of 4 to 8 was reached, and 55 to 85% of the activity was recovered. After fractionated precipitation with ammonium sulfate, Pel activity was associated with the fractions obtained at between 70 and 80% ammonium sulfate saturation for PelA, PelB, and PelC and between 80 and 100% ammonium sulfate saturation for PelD and PelE. After chromatography on a hydrophobic interaction column, PelA, PelB, and PelC were eluted at about 0.8 M ammonium sulfate, whereas PelD and PelE were eluted at about 1.2 M ammonium sulfate. In each case, a single peak with Pel activity was obtained. Finally, the Pels were purified 14- to 34-fold with 22 to 51% recovery (Table 2). The amount of Pel recovered from 300 ml of culture varied between 1 and 3 mg depending on the production level and on the final OD of the culture. SDS-

TABLE 2. Purification of the five major *E. chrysanthemi* Pels

Step	Sp act (μmol · min^{-1} · mg^{-1})[a]				
	PelA	PelB	PelC	PelD	PelE
Culture	1.6	56	18	24	50
Periplasmic fraction	5.8	465	144	98	364
$(NH_4)_2SO_4$ fractionation	8.6	1,173	321	160	962
TSK-gel Phenyl 5PW column	23	1,740	622	487	1,495
Purification factor (fold)	14	31	34	20	30

[a] Specific activities are expressed as enzyme units per mg of protein. Standard conditions were used for these assays: 100 mM Tris-HCl (pH 8.5), 0.1 mM $CaCl_2$, and 0.5 g of polygalacturonate per liter. The effect of the different steps of purification is given for the five Pels purified from *E. coli* overproducing strains.

PAGE analysis showed satisfactory purity with only weak contaminant bands (Fig. 2).

Isoelectrofocusing confirmed that the purified proteins have the same pIs as Pels present in the *E. chrysanthemi* culture supernatant, i.e., about 4.6, 7.9, 8.2, 9.8, and 10.3 for PelA, PelB, PelC, PelD, and PelE, respectively (data not shown). Similarly, separation by SDS-PAGE showed that the purified proteins have the same electrophoretic mobilities as the Pels present in the *E. chrysanthemi* culture supernatant. The apparent molecular masses were estimated to be 42.5 kDa for PelA, 39.5 kDa for PelB, 39.5 kDa for PelC, 42 kDa for PelD, and 42.5 kDa for PelE (Fig. 2). These values are slightly higher than those calculated from the nucleotide sequence of the corresponding genes. Similar divergences were observed previously (10, 40). Under standard conditions (Table 2), the specific activities of the purified proteins were 23, 1,740, 622, 487, and 1,495 U · mg^{-1} for PelA, PelB, PelC, PelD, and PelE, respectively.

Influences of pH and temperature. The pH optima appeared to be 8.5 for PelA, 9.3 for PelB, 9.2 for PelC, 8.8 for PelD, and about 8 for PelE (Fig. 3). A relatively narrow zone of optimum activity was observed. At neutral pH, activity was negligible for PelB and PelC (about 2%), while PelA, PelD, and PelE retained 25 to 50% of their activity at pH 7.

The temperature optima were determined to be approximately 50°C for PelD and PelE, 55°C for PelA, and 60°C for PelB and PelC under standard conditions (Fig. 4). In the absence of Ca^{2+} and polygalacturonate, the half-times of thermoinactivation at 60°C were 20 to 30 min for PelB and PelC. At this temperature, PelA, PelD, and PelE lost more than 80% of enzymatic activity after 2 min. The half-times of thermoinactivation at 50°C were 11, 4, and 2 min for PelA, PelD, and PelE, respectively. Therefore, PelA, PelD, and PelE are clearly less stable than PelB and PelC.

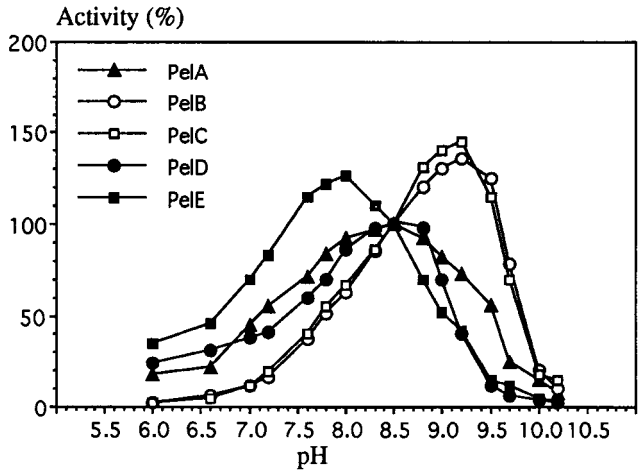

FIG. 3. Effect of pH on Pel activity. The activities of the five Pels were determined at different pHs with the following buffers: PIPES-HCl (pH 6 to 7.5), ACES-HCl (pH 6 to 7.5), Tris-HCl (pH 6.6 to 9.5), and Bis-Tris-propane (pH 6.3 to 10.2), each at a concentration of 50 mM. The activity at pH 8.5 (standard assay) was arbitrarily defined as 100. Enzymes were assayed at 37°C with 0.1 mM $CaCl_2$ and 0.5 g of polygalacturonate per liter.

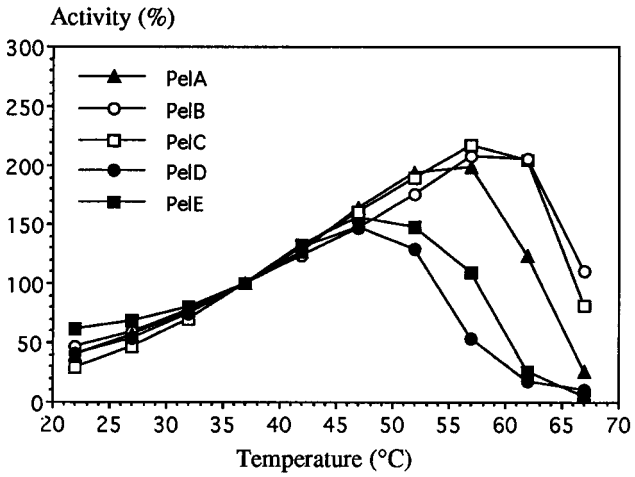

FIG. 4. Effect of temperature on Pel activity. The five Pels were assayed at different temperatures in 100 mM Tris-HCl (pH 8.5)–0.1 mM $CaCl_2$–0.5 g of polygalacturonate per liter. The activity at 37°C (standard assay) was arbitrarily defined as 100.

Effect of divalent cations. Pels are known to have an absolute requirement for Ca^{2+} ions. To verify this requirement, EDTA was added to chelate endogenous cations because the substrate always contains traces of Ca^{2+}. No activity was detected in the presence of EDTA at a final concentration of 1 mM (Table 3). The influence of Ca^{2+} was investigated by adding $CaCl_2$ concentrations ranging from 0 to 1 mM at pH 8.5 in the presence of polygalacturonate as substrate (Fig. 5). Similar results were obtained for the five isoenzymes. The maximal activity was observed for concentrations of about 0.1 mM, with more than 80% of activity at concentrations ranging from 0.05 to 0.2 mM. The activity drops sharply for concentrations below these values, except for PelE and PelA, which retained 68 and 50% of their activities, respectively, without Ca^{2+} addition. Above the optimal concentration, the activity gradually decreased. At 1 mM Ca^{2+}, the activity of PelE was strongly inhibited, while PelA, PelB, PelC, and PelD retained 24 to 37% of their maximal activities. The Ca^{2+} requirement appeared to be slightly dependent on the pH of the medium. While the optimal Ca^{2+} concentration was 0.100 mM at pHs 7.8 and 8.5, a concentration of 0.025 mM gave the highest activity at pH 9.2 (Fig. 5). When 45% methylated pectin was used as substrate, the inhibition due to high Ca^{2+} concentrations decreased and the maximal activity was observed in a large range of Ca^{2+} concentrations (from 0.1 to 1 mM) for the five isoenzymes (Fig. 5).

The influence of other divalent cations (Ba^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Sr^{2+}, Zn^{2+}) was investigated using 0.1 and 1 mM of the corresponding chloride salts (Table 3). In contrast to Ca^{2+}, none of these cations can activate Pels. Cu^{2+} and Mg^{2+} did not affect Pel activity. Mn^{2+} and Sr^{2+} weakly inhibited the five isoenzymes, mainly at a concentration of 1 mM. A stronger inhibition was observed with Ba^{2+} and Co^{2+}. However, among

TABLE 3. Effect of divalent cations on activities of Pel isoenzymes

Addition	Concn (mM)	Enzyme activity (%)[a]				
		PelA	PelB	PelC	PelD	PelE
None		100	100	100	100	100
EDTA	1	0	0	0	0	0
Ions						
Ca^{2+}	0.1	205	280	420	540	145
	1	76	69	102	165	13
Ba^{2+}	0.1	29	51	50	42	66
	1	0	0	0	0	0
Co^{2+}	0.1	96	89	83	95	94
	1	0	0	0	0	0
Cu^{2+}	0.1	88	83	85	108	106
	1	102	67	79	100	95
Mg^{2+}	0.1	106	81	88	97	91
	1	92	97	82	85	86
Mn^{2+}	0.1	95	53	50	63	54
	1	49	18	23	45	34
Sr^{2+}	0.1	98	52	42	75	53
	1	55	11	12	30	36
Zn^{2+}	0.1	0	25	28	13	8
	1	0	0	0	0	0

[a] Specific activities are expressed as a percentage of the value obtained in an assay mixture consisting of 100 mM Tris-HCl (pH 8.5) and 0.5 g of polygalacturonate per liter without ion addition. The influence of each divalent cation was determined by using the corresponding chloride salt. Each result reported is the average of three independent experiments, and the standard deviation was, in each case, less than 10%. A zero indicates that the activity found was lower than 5%.

TABLE 4. K_m and V_{max} values for each Pel with polygalacturonate as substrate[a]

Pel	K_m (g · liter^{-1})	V_{max} (μmol · min^{-1} · mg^{-1})
PelA	0.30	46
PelB	0.03	2,448
PelC	0.02	762
PelD	0.28	670
PelE	0.42	3,803

[a] The initial velocity for the reactions was determined at different polygalacturonate concentrations under standard conditions. Lineweaver-Burk transformation of the data gave K_m and V_{max} values for each isoenzyme.

these cations, Zn^{2+} was the strongest inhibitor of Pel activity, since effective inhibition was observed at a low concentration (0.1 mM).

Influence of substrate. The initial velocity for the reaction was determined at different polygalacturonate concentrations under standard conditions. Pel activity was inhibited when substrate concentration exceeded 0.5 g/liter for PelB and PelC and 1 g/liter for PelA, PelD, and PelE. Apparent K_m and V_{max} values are given in Table 4. The affinity for polygalacturonate of the neutral isoenzymes PelB and PelC was about 10-fold higher than that of the three other isoenzymes. The highest V_{max} values were observed for PelB and PelE, while PelA showed the lowest V_{max}.

Activity on substrates presenting various degrees of methyl-

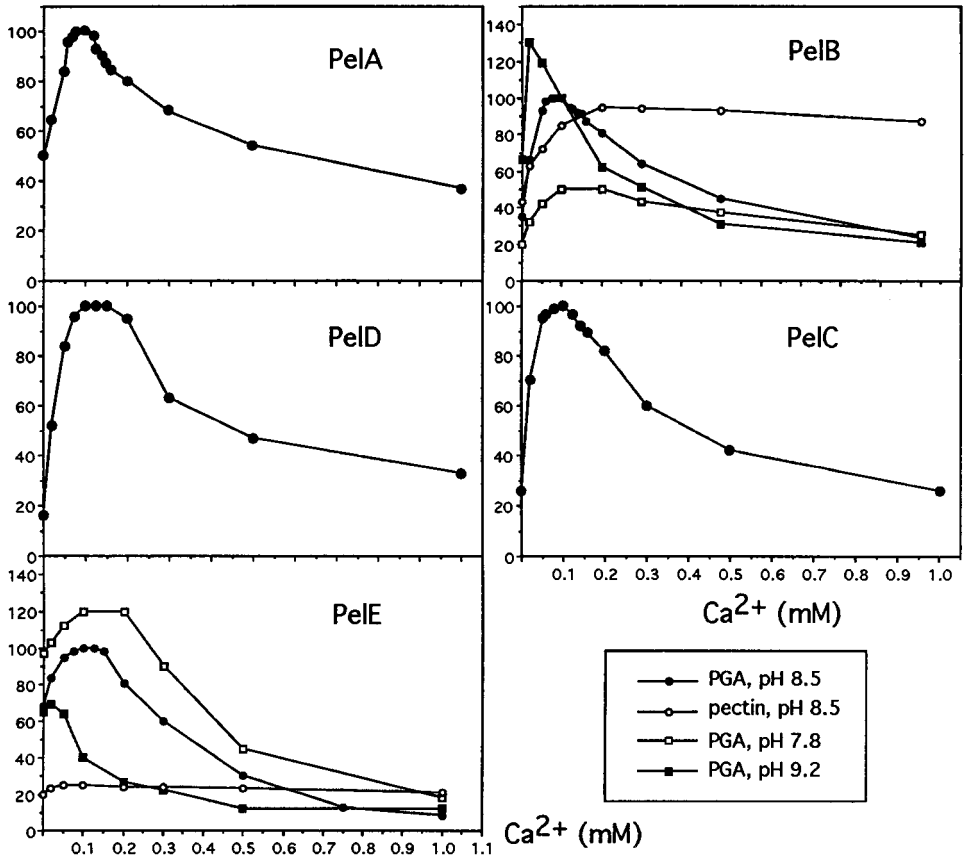

FIG. 5. Influence of Ca^{2+} concentration on Pel activity. The five Pels were assayed with or without $CaCl_2$ at various concentrations. Measurements were done at 37°C; in 100 mM Tris-HCl at pH 7.8, 8.5, or 9.2; and in the presence of substrate (0.5 g of either polygalacturonate [PGA] or 45% methylated pectin per ml). The value of 100 corresponds to a 0.1 mM Ca^{2+} concentration at pH 8.5 in the presence of polygalacturonate.

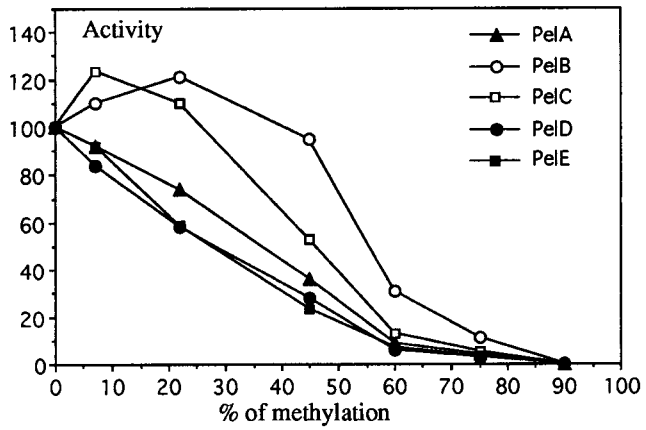

FIG. 6. Effect of the degree of pectin methylation on Pel activity. Activities of the five Pels toward pectins, presenting various degrees of methylation, were expressed relative to that toward nonmethylated substrate, which was arbitrarily defined as 100. Assays were performed at 37°C in 100 mM Tris-HCl (pH 8.5)–0.1 mM $CaCl_2$–0.5 g of substrate per liter.

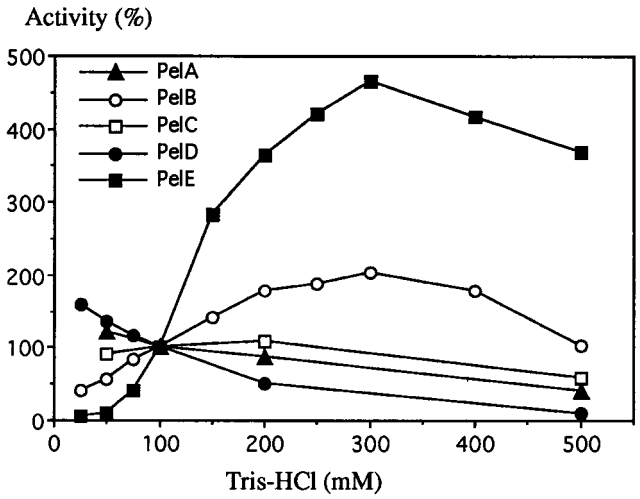

FIG. 7. Influence of buffer concentration on Pel activity. The activities of the five Pels were determined at different Tris-HCl concentrations. Measurements were done at 37°C at pH 8.5 in the presence of 0.1 mM $CaCl_2$ and 0.5 g of polygalacturonate per liter. The activity with 100 mM Tris-HCl was arbitrarily defined as 100.

ation was determined by using the standard assay mixture but substituting 7, 22, 45, 60, 75, and 90% esterified citrus pectins (from Copenhagen Pectin) for polygalacturonate (Fig. 6). PelA, PelD, and PelE showed activity only with substrates presenting a low degree of methylation. They showed higher activity with polygalacturonate and retained more than 80% of their activity with methylation of up to 7%. In contrast, the neutral isoenzymes PelB and PelC were most active toward partially methylated substrates. PelC showed good activity for pectins with 7 to 22% methylation. PelB showed optimal activity on 22% methylated substrate and retained more than 80% activity with methylation of up to 45%.

Other factors affecting Pel activity. We examined the sensitivity of Pel activity to the end reaction product digalacturonate and to various monomeric sugars, such as galacturonate, glucuronate, galactose, and glucose. For the five Pels, no significant inhibition was observed in the presence of any of these compounds at concentrations ranging from 0.1 to 10 mM. A low activation (up to 15%) was observed in the presence of galacturonate or glucuronate at concentrations between 0.5 and 5 mM. Such activation was previously observed for *Erwinia carotovora* Pels (25). Inhibition was observed with digalacturonate, galacturonate, or glucuronate at concentrations higher than 50 mM (data not shown).

In the different buffers tested (glycin-NaOH, glycyl-glycin, etc.), used at 100 mM and pH 8.5, the Pel activity was lower than in the classic Tris-HCl buffer, except for Bis-Tris-propane, which gave slightly higher activities (data not shown). However, the buffer concentration affects the activity (Fig. 7). Increasing Tris-HCl concentration strongly activated PelE and weakly activated PelB and PelC but inhibited PelA and PelD. This effect is linked to the ionic strength of the medium, since the presence of NaCl also affects Pel activity. With 100 mM Tris-HCl, the addition of 50 mM NaCl provoked an inhibition of about 80% for PelD or PelA without significantly affecting PelB or PelC, while PelE was activated.

Epicatechin, a flavan 3-ol present in high concentrations in the pericarp of unripe fruit, is a natural inhibitor of some fungal Pels (43). The five Pels from *E. chrysanthemi* 3937 were also inhibited by this compound, with a K_i ranging between 0.1 and 0.2 mM. We observed that salicylic acid, another plant compound involved in the plant defense response, was able to inhibit the activity of the five *E. chrysanthemi* Pels with a K_i of 0.2 to 0.4 mM.

DISCUSSION

E. chrysanthemi 3937 secretes five major Pels: PelA, PelB, PelC, PelD, and PelE. The nomenclature of these isoenzymes is related to their pI, from the most acidic, PelA, to the most basic, PelE. Through comparison of their amino acid sequences, two families have been identified among these *E. chrysanthemi* Pels: the PelA-D-E class and the PelB-C class (14). These two classes were confirmed by immunological cross-reactivities. Indeed, monoclonal antibodies obtained from the supernatant of strain 3937 recognized either solely PelD and PelE or solely PelB and PelC (42). In addition, polyclonal antibodies raised against PelA cross-reacted with PelD (10). The alkaline isoenzymes seem to attack pectin more randomly than the neutral ones do, as evidenced by analysis of reaction products and by viscosimetry (1, 11, 28). Analysis of the lengths of the end products generated by each isoenzyme was carried out with the Pels of *E. chrysanthemi* EC16 (28). PelA produces a large set of oligomers, from dimers to dodecamers. PelB and PelC produce a majority of trimers, with some dimers and tetramers. The major end product of PelE is the dimer. Early investigations revealed other differences among the isoenzymes in their capacities to macerate plant tissues (1, 3, 11). The basic isoenzymes, PelD and PelE, are the most effective in macerating tissues. PelB and PelC are also capable of maceration, while PelA is unable to efficiently macerate plant tissue. Concerning their level of production by bacteria, PelB and PelC appeared to be synthesized at similar levels in laboratory cultures and in planta (16, 22, 24). In contrast, less uniformity was found for the production of enzymes of the PelA-D-E group; different isoenzymes are synthesized depending on the growth conditions or on the infected plant (16, 22, 24). Infection of various plants also revealed the main influence of enzymes of the PelA-D-E group in pathogenicity (4, 6).

We have analyzed some of the basic enzymatic properties of the five major Pels of *E. chrysanthemi* 3937 by using proteins purified from *E. coli* recombinant cells and thus devoid of

contamination by any other pectinolytic activity. This study of Pel properties also revealed the existence of the two families and further differences between the PelB-C and the PelA-D-E groups. The first difference concerns the pH suitable for activity. The optimum pH is more alkaline for PelB and PelC (about 9.2) than for PelA, PelD, and PelE (from 8 to 8.8). In addition, PelB and PelC present a narrower zone of optimum activity than PelA, PelD, and PelE. These latest enzymes retained 20 to 30% of activity at neutral pH. In consequence, the enzymes of the PelA-D-E group are probably more active in plant tissues, which present an acidic pH, than are those of the PelB-C group. This result could, at least partially, explain why PelB and PelC appeared to be less important for virulence than PelA, PelD, and PelE (6).

At the level of thermal stability, PelB and PelC are clearly more stable than PelA, PelD, and PelE, as shown by analysis of the optimum temperatures and of the half-times of inactivation. Differences in the thermal stabilities of isoenzymes from the two families may result from their tertiary structures. It was shown that PelB and PelC contain two intramolecular disulfide bonds that can stabilize the proteins, while PelA, PelD, and PelE have only one intramolecular disulfide bond (45, 46).

When pectins with different degrees of methylation were used, PelA, PelD, and PelE showed activity only for substrates with very low levels of methylation. In contrast, PelB and PelC were more active on partially methylated pectins than on the nonmethylated polymer polygalacturonate. They show high activity (more than 80%) on pectins methylated up to 22% for PelC and 45% for PelB. The degree of pectin methylation varies between plant species, between tissues, and between domains within a single wall. Gold labeling and microscope analysis demonstrated that PelB and PelC are preferentially located in some domains of the middle lamella and cell junctions (41), whereas PelD and PelE are found along the plasmalemma of the cell wall (5a). These results suggest a possible specialization of each class of isoenzymes toward different pectic polymers. For the PelB-C group, substrate recognition and/or cleavage is not affected by the presence of some methylated galacturonate residues. In contrast, for enzymes of the PelA-D-E group, preliminary action of the bacterial secreted pectin methyl esterase appears necessary to favor Pel activity.

The activity of the major *E. chrysanthemi* Pels shows an absolute requirement for Ca^{2+} ions. The optimal concentration, 0.1 mM, is similar for the five isoenzymes, and the five Pels are inhibited by a high Ca^{2+} concentration. However, some differences were noticed between the isoenzymes. For instance, PelE retains a very high activity without Ca^{2+} addition (the substrate always contains traces of Ca^{2+}), but the inhibition of PelE at a high Ca^{2+} concentration is more pronounced. In addition, the optimal Ca^{2+} concentration varied with the methylation of substrate and with the pH of the medium. These results reflect the complex effects of Ca^{2+} in the reaction. These cations act on at least two levels, since they can interact with the carboxyl groups of the substrate and since binding of Ca^{2+} in the enzyme active site is necessary for catalysis.

None of the other cations tested (Ba^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Sr^{2+}, Zn^{2+}) can substitute for Ca^{2+} to activate the five Pels. However, most of these cations can inhibit Pel activity both in the absence and in the presence of Ca^{2+}. Zn^{2+} has the strongest inhibitory effects, while Cu^{2+} and Mg^{2+} do not significantly affect the activity. Ba^{2+}, Co^{2+}, Mn^{2+}, and Sr^{2+} are inhibitors when used at a concentration of 1 mM. The ionic strength of the medium also affects the activity, but the effect depends on the isoenzyme. For instance, the addition of 50 mM NaCl activated PelE but inhibited PelD or PelA. Many compounds, such as acidic sugars (galacturonate, glucuronate, digalacturonate) at concentrations higher than 50 mM, decrease Pel activity. However, no differences between the five isoenzymes were noticed for these inhibitions, which are observed only at high concentrations and do not display specificity. In addition, we demonstrated that two compounds present in plant tissues, epicatechin and salicylic acid, at concentrations of about 0.2 mM inhibit Pel activity. The affinity of *E. chrysanthemi* isoenzymes for epicatechin is about 50-fold lower than that previously reported for a pectate lyase of the fungus *Colletotrichum gloeosporioides* (43) but is similar to that reported for an endopolygalacturonase of the same organism (29). The epicatechin concentration in the peel of ripe fruit is about 80 μM compared to 1 to 6 mM in unripe fruits (29). Such concentrations are sufficient for inhibition of *E. chrysanthemi* Pels. Epicatechin was the first reported inhibitor of Pel activity isolated from plants. Salicylic acid is an important compound produced by plant cells during the defense response against pathogens (9). Therefore, different plant compounds are able to inhibit the Pels produced by the pathogens.

It was relatively difficult to compare the kinetic results obtained in this work with previously published results. The K_m previously reported for PelA of *E. chrysanthemi* 3937 (10) is similar to that obtained in our study. In contrast, the K_m reported for the PelB and PelC isoenzymes of *E. chrysanthemi* EC1237 (8) are 10-fold higher than those obtained for the corresponding enzymes of strain 3937. This apparent divergence between the two strains may result from the different processes of purification used, leading in some cases to a partial inactivation of the enzymes. Shevchik et al. (35) reported a comparative analysis of Pels from different *Erwinia* species, including *E. chrysanthemi* ENA49. Since the genetic analyses were not performed simultaneously, it is difficult to establish a correspondence between the isoenzymes produced by different *E. chrysanthemi* strains. The properties of Pel1, Pel2, and Pel4 of *E. chrysanthemi* ENA49 are quite similar to those of PelB, PelC, and PelD, respectively, of *E. chrysanthemi* 3937. In contrast, significant differences are observed in the case of the most basic isoenzyme, Pel5, which presents higher optimum pH and temperature than PelE of strain 3937. Several Pels of *E. carotovora* have also been characterized (2, 35, 36, 38). At the genetic level, all the extracellular Pels of *E. carotovora* belong to the PelB-C family. Indeed, their properties (alkaline optimum pH, thermal stability, activity on methylated substrates) are similar to those observed for PelB-C of *E. chrysanthemi* 3937.

In conclusion, the two families of *E. chrysanthemi* major Pels established after genetic and immunological studies are clearly found again at the biochemical level. PelB and PelC look very similar to each other: they have a high affinity for substrates, a very alkaline optimum pH, a good thermal stability, and significant activity on partially methylated pectins. PelA, PelD, and PelE also share some similarities, but each element of this family reveals particularities. PelE has a high specific activity, a low optimum pH, and a low Ca^{2+} requirement. PelA has a very low specific activity that may reflect its type of action. PelA probably recognizes and cleaves rather long fragments of pectic polymers, since its end reaction products are clearly longer than those obtained for the other isoenzymes.

Crystallography analysis of one Pel from each class, PelC and PelE, demonstrated that these proteins consist of an unusual backbone of parallel β strands coiled in a large right-handed helix (21, 45, 46). Comparison between PelC and PelE revealed similarities in the global structures but differences in the localization of charges at the surfaces of the proteins and variations in the lateral chains. Identification of the structurally

conserved amino acids led to a realignment of the sequences of Pels with different origins (12, 13). These studies led to the proposal of two putative distinct active sites: residues involved in the Ca^{2+}-binding site and residues that fold in the parallel β helix or constitute the Asn ladder. However, the identification of amino acids directly involved in Pel activity is not yet completed. The biochemical data reported in this paper and the differences in biochemical properties observed between the isoenzymes of the two classes provide a new view for exploring the differences which exist at the structural level.

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