

Biochemical characterization of pectate lyases produced by fluorescent pseudomonads associated with spoilage of fresh fruits and vegetables

C.-H. LIAO, J. SULLIVAN, J. GRADY AND L.-J.C. WONG. 1997. An improved method for purification of pectate lyases (PLI and PLII) from culture fluids of *Pseudomonas fluorescens* CY091 and *Ps. viridiflava* PJ-08-6 by using a phosphocellulose cation exchanger was described. Analysis of purified PLI and PLII by sodium dodecyl sulphate-polyacrylamide and isoelectric focusing gel electrophoresis revealed that both enzymes had been purified to near homogeneity. Optimal Ca^{2+} concentration required for PLI and PLII activity was determined to be 0.5 mmol l^{-1} . The Ca^{2+} requirement could not be replaced by other metal cations such as Mg^{2+} , Cu^{2+} , Zn^{2+} , Fe^{3+} and Co^{2+} . Optimal pH for activity was determined to be between 8.5 and 9.0. The K_m values for sodium polygalacturonate were 1.28 and 1.11 mg ml^{-1} for PLI and PLII, respectively. Both PLI and PLII were stable at low temperatures (25°C or below) for at least 1 month. However, at 37°C , the activity decreased 50% in 36 h. Optimal temperatures for activity were estimated to be 46° and 52°C for PLI and PLII, respectively. Thermal stability of both enzymes at elevated temperatures (48°C or higher) increased when CaCl_2 or a positively charged molecule such as polylysine was present, but decreased when polygalacturonate or a negatively charged molecule such as heparin was present. PLI and PLII exhibit differential degrees of sensitivity to group-specific inhibitors, including iodoacetic acid and diethylpyrocarbonate. This result suggests that both sulphhydryl and imidazole groups are important for the catalytic function of PLI and PLII.

INTRODUCTION

Pectolytic fluorescent pseudomonads, mainly *Pseudomonas fluorescens* and *Pseudomonas viridiflava*, account for substantial proportions of post-harvest rot of fruits and vegetables in cold storage (Brocklehurst and Lund 1981) and at wholesale and retail markets (Liao and Wells 1987). The ability of these pseudomonads to cause maceration of plant tissues is primarily due to their ability to produce an extracellular pectate lyase (PL) capable of degrading pectic components of

plant cell walls (Liao *et al.* 1988). This enzyme cleaves the glycosidic bond of poly- α (1-4) galacturonic acid by β -trans-elimination resulting in the formation of a series of unsaturated oligouronide products, which can be readily detected by a spectrophotometric method (Collmer *et al.* 1988).

Production of PLs is common among pectolytic fluorescent pseudomonads associated with post-harvest rot of fruits and vegetables. All 15 strains of *Ps. fluorescens* and *Ps. viridiflava* previously examined in this laboratory have been shown to produce a single PL with an isoelectric point (pI) of ≈ 9.5 (Liao 1989). The simplicity of the pectic enzyme system found in soft-rotting pseudomonads is in sharp contrast to the complex PL isozyme system, found in *Erwinia chrysanthemi*, which usually produces four to five PL isozymes (PLa-e) with pIs ranging from 4.8 to 10.0 (Collmer and Keen 1986).

The alkaline PL produced by soft-rotting pseudomonads exhibits the same or similar degree of tissue-macerating ability as the alkaline PLe isozyme produced by *Erw. chrysanthemi* (Liao 1989). Alkaline PLs produced by soft-rotting bacteria including *Erwinia* and non-*Erwinia* are more efficient in causing tissue maceration than neutral and acidic PLs (Collmer and Keen 1986). The biochemical basis for this phenomenon is presently unknown. Previously, several studies have been directed to investigate the catalytic reaction mechanisms of PLs (Fuchs 1965; Nasuno and Starr 1966) and the factors affecting production of these enzymes by *Ps. fluorescens* or *Ps. viridiflava* (Liao *et al.* 1993, 1994). However, enzymological properties of PLs produced by soft-rotting pseudomonads have not yet been characterized. The objectives of this study were: (a) to develop a simple and efficient method for purification of PLs from soft-rotting pseudomonads, (b) to determine optimal pH, temperature and metal ion requirements for PL activities, and (c) to characterize the kinetic parameters of PLs from *Ps. fluorescens* and *Ps. viridiflava*.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Pseudomonas fluorescens strain CY091 and *Ps. viridiflava* strain PJ-08-6 were used throughout the study. Both strains were maintained on *Pseudomonas* agar F (Difco, Detroit, MI) for routine cultivation and grown in the MY broth medium for enzyme preparation. The MY medium (pH 7.2) containing K_2HPO_4 0.7% (w/v), KH_2PO_4 0.2% (w/v), $MgSO_4 \cdot 7H_2O$ 0.02% (w/v), $(NH_4)_2SO_4$ 0.1% (w/v), yeast extract 0.1% (w/v), $CaCl_2$ 1 mmol l^{-1} , glycerol 0.4% (w/v), was prepared as described previously (Liao *et al.* 1988). Cultures of *Ps. fluorescens* and *Ps. viridiflava* were incubated at 28°C for 48 and 60 h, respectively, prior to the enzyme extraction.

Enzyme purification and assay

One hundred ml of the *Ps. fluorescens* CY091 culture grown at 28°C for 40 h or 100 ml of the *Ps. viridiflava* PJ-08-6 culture grown at 28°C for 60 h in MY medium were centrifuged (10 000 g, 10 min) to remove the cells. The supernatant fluid was diluted with an equal volume of distilled water, and loaded directly onto a phosphocellulose column (2.5 × 10 cm) which had been pre-equilibrated with 10 mmol l^{-1} Tris-HCl buffer (pH 8.0). Phosphocellulose P11 was obtained from the Whatman Inc. (Clinton, NJ) and had been pretreated according to the manufacturer's instruction. After loading the sample, the column was washed with 600 ml of 10 mmol l^{-1} Tris-HCl (pH 8.0) and the enzyme was eluted with the same buffer containing 0.2 mol l^{-1} NaCl. The fractions containing the highest levels of PL activities were pooled

and further concentrated by the Amicon ultrafiltration unit (PM10 membrane, Amicon Corp., Danvers, MA). Protein concentrations were determined by the method of Lowry *et al.* (1951) or by the absorbance at 280 nm. Enzyme activities were determined by the spectrophotometric method as previously described (Collmer *et al.* 1988). One unit (U) of activity was defined as the amount of the enzyme that caused an increase of 1.73 absorbance units at 232 nm and at 25°C min^{-1} (Liao *et al.* 1988). The reaction was carried out in 1 ml volume containing 100 mmol l^{-1} Tris-HCl (pH 9.2), 0.25% (w/v) sodium polygalacturonate, 0.5 mmol l^{-1} $CaCl_2$ and an enzyme sample.

SDS-polyacrylamide and isoelectric focusing gel electrophoresis

SDS-polyacrylamide gel electrophoresis (Laemmli 1970) and ultrathin-layer polyacrylamide gel isoelectric focusing (IEF) were performed according to the procedures previously described (Liao 1989). For SDS-polyacrylamide gel electrophoresis, the gel containing 12% (w/v) acrylamide, 0.33% (w/v) bis-acrylamide and 0.1% (w/v) SDS was used. Protein M_r markers were obtained from Life Technologies Inc. (Bethesda, MD). Ultrathin layer IEF gel electrophoresis was conducted using Ampholine PAG plates (pH 3.5–9.5) from Pharmacia/LKB Biotechnologies (Piscataway, NJ). pI markers were purchased from FMC Bioproducts (Rockland, ME).

Enzyme kinetics parameters

The kinetic parameters, K_m and V_{max} were determined from double reciprocal plots of initial rates and concentrations of polygalacturonic acid. The activation energy (E_a) was calculated from the slope of a plot of $\log v$ vs $1/T$ according to the Arrhenius equation $\log v = -E_a/2.3 RT + C$, where v is the initial rate of the catalytic reaction, R is the gas constant, T is temperature (°K) and C is a constant. Thermal inactivation of PL was investigated by incubating the enzyme at a designated temperature from 37 to 52°C in 10 mmol l^{-1} Tris-HCl (pH 9.2) in the presence or absence of substrate or other effectors. At indicated times (from 0 to 100 min), aliquots were removed and enzyme activities measured as described above. The initial rate v was plotted against time t according to the first order rate equation and the rate constant of heat inactivation (k) was then calculated from the slope of the plot. The half-life, $t_{1/2}$, was calculated from k by using the first order rate equation: $t_{1/2} = 0.69/k$.

Determination of optimal pH, temperature and Ca^{2+} concentration for PL activity

The enzyme sample was incubated in a reaction mixture containing 0.25% (w/v) polygalacturonate, 0.5 mmol l^{-1}

CaCl₂ and 100 mmol l⁻¹ Tris-HCl at pH ranging from 7.1 to 11.2. After incubation at 25°C for 2 min, the reaction was stopped by boiling for 5 min and formation of unsaturated oligouronide products in each reaction mixture was determined by the spectrophotometric method as described above. Similarly, the enzyme sample was mixed with a reaction mixture containing 0.25% (w/v) polygalacturonate, 0.5 mmol l⁻¹ CaCl₂ and 100 mmol l⁻¹ Tris-HCl (pH 8.0) and incubated for 2 min at various temperatures ranging from 15 to 70°C. The reaction was stopped by immersion of tubes in boiling water for 5 min and the amount of unsaturated oligouronides formed was measured by the spectrophotometric method. For measurement of metal ion requirements for PL activity, the reaction condition for determination of temperature optimum was used except that CaCl₂ was replaced by equal concentrations of MgCl₂, ZnCl₂, CuCl₂, CoCl₂ or FeCl₃. For measurement of optimal Ca²⁺ concentration for activity, the reaction was carried in reaction mixtures containing various concentrations of CaCl₂ ranging from 0.1 to 3.0 mmol l⁻¹.

Inactivation of PLs by group-specific reagents

PLI or PLII purified, respectively, from *Ps. fluorescens* CY091 and *Ps. viridiflava* PJ-08-6 was suspended in 10 mmol l⁻¹ Tris-HCl (pH 8.0) and incubated at 25°C with one of three group-specific reagents: iodoacetic acid (IAA), diethyl-pyrocabonate (DEPC) or iodoacetamide (IAM). At given time intervals from 2 to 10 min, aliquots were removed and enzyme activities measured. The initial rate v was plotted against time t . The first order reaction rate constant k was obtained as described above and the half-life $t_{1/2}$ was calculated from k by using the first order rate equation: $t_{1/2} = 0.69/k$ as described above.

RESULTS

Enzyme purification

We have previously reported purification of PLI and PLII from culture supernatant fluids of *Ps. fluorescens* CY091 and *Ps. viridiflava* SF312 by ammonium sulphate fractionation and DEAE ion-exchange chromatography (Liao 1989). However, PLI and PLII bound very poorly in the DEAE-cellulose column and the enzyme recovery using this method was relatively low. In this study, an improved procedure for purification of these two enzymes was developed. The new procedure eliminated the ammonium sulphate precipitation and dialysis steps. The purification was completed in a single step by directly applying the diluted PL-containing culture fluids to a phosphocellulose cation exchanger. Figure 1 illustrates the elution profile of culture supernatant fluid for *Ps. viridiflava* PJ-08-6. Only one PL activity peak was observed. The elution profile of culture supernatant fluid for *Ps. flu-*

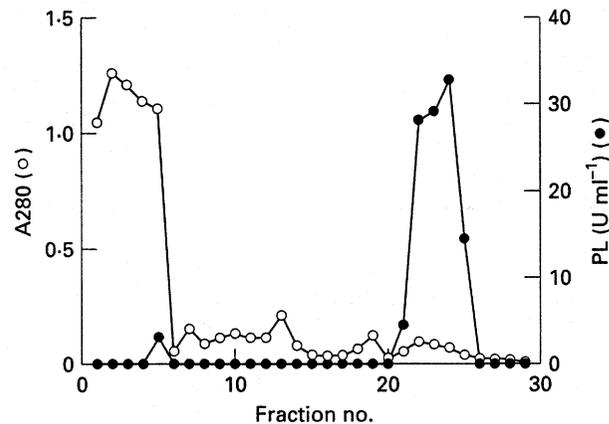


Fig. 1 Elution profile of the culture supernatant fluid of *Pseudomonas fluorescens* CY091 from a Whatman P11 phosphocellulose cation column (2.5 × 10 cm). Fractions of 15 ml were collected. The pectate lyase activity and the protein concentration of each fraction were determined as described in the Materials and Methods

orescens CY091 is also similar to that depicted in Fig. 1. The PL from *Ps. fluorescens* and from *Ps. viridiflava* designated, respectively, as PLI and PLII, was purified 115- to 135-fold with the specific activity ranging from 365 to 472 U mg⁻¹ protein. As compared to the DEAE-cellulose procedure, a 40% increase in the recovery was obtained by using the phosphocellulose procedure. Figure 2 shows the presence of a single protein band in the polyacrylamide gel stained with Coomassie blue, indicating that both PLI and PLII have been purified to near electrophoretic homogeneity. The M_r (41–42 kDa) and pI (9.5–10) of PLI and PLII are close to those predicted from the nucleotide sequences of genes coding for these two enzymes (Liao *et al.* 1996).

Effects of pH, temperature and metal ions

Optimal conditions required for the activity of PLI and PLII were examined. The PLI was found to have a broad pH optimum of between 8.5 and 9.5 (Fig. 3a). However, the PLII was found to have a relatively sharp pH optimum of between 8.5 and 8.7 (Fig. 3a). When reaction mixtures were incubated for 2 min at various temperatures ranging from 15 to 70°C, maximal activities occurred at 46°C and 52°C with an Arrhenius activation energy of 13 and 15 kcal mol⁻¹ for PLI and PLII, respectively (Fig. 3b). When PLI or PLII were incubated in reaction mixtures containing various concentrations of CaCl₂, the maximal activity was found in the mixture containing 0.5 mmol l⁻¹ (Fig. 3c). The Ca²⁺ was absolutely required for the activity of PLI and PLII since no PL activity was detected in the reaction mixture in which CaCl₂ was replaced by MgCl₂, ZnCl₂, CoCl₂, CuCl₂ and FeCl₃. Addition of excessive amounts (1–3 mmol l⁻¹) of EDTA into the reac-

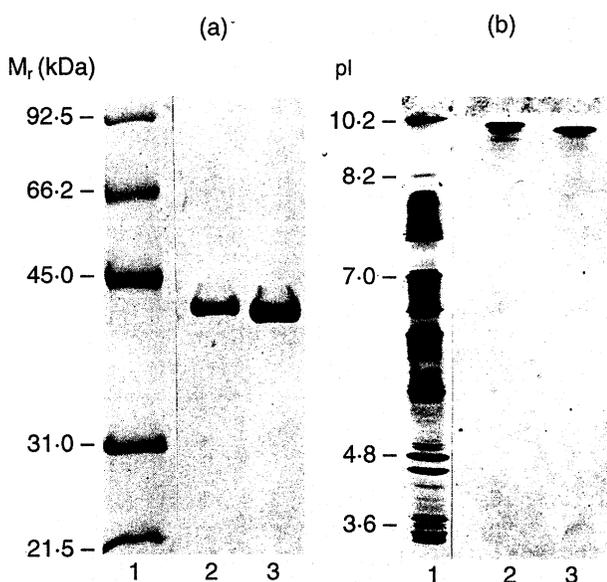


Fig. 2 (a) Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of purified pectate lyases PLI and PLII from *Pseudomonas fluorescens* CY091 (lane 2) and *Pseudomonas viridiflava* PJ-08-6 (lane 3), respectively. Molecular weight (M_r) markers (lane 1) are: phosphorylase B (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa) and soybean trypsin inhibitor (21.5 kDa). (b) Ultrathin layer isoelectric focusing polyacrylamide gel electrophoresis of purified PLI from *Pseudomonas fluorescens* CY091 (lane 2) and PLII from *Pseudomonas viridiflava* PJ-08-6 (lane 3). The pI markers (lane 1) are: cytochrome C (10.2), whale myoglobin (8.2), horse myoglobin (7.0), ovalbumin (4.8) and aminoglucosidase (3.6)

tion mixture containing CaCl_2 completely inhibited the enzymatic reaction. The kinetic parameters, K_m and V_{\max} , for PLI and PLII were determined from the double reciprocal plots shown in Fig. 3d. The K_m for PLI and PLII acting on PGA was determined to be 1.24 and 1.11 mg ml⁻¹, respectively. When the molecular weights of PLI and PLII were assumed to be 41 000 and 42 000 based on their electrophoretic mobilities in the polyacrylamide gel (Fig. 2), the turnover numbers calculated from V_{\max} were 4.2×10^4 and 2.3×10^4 per min for PLI and PLII, respectively.

Thermal stability of PLI and PLII

Both PLI and PLII appeared to be stable at lower temperatures. No loss of activity was observed when either enzyme was stored at 4°C or 25°C for over a period of 1 month. However, the enzyme was gradually inactivated at temperatures above 37°C. For PLI, 50% inactivation occurred after incubation at 37°C for 36 h, 48°C for 27 min and 52°C for 1.6 min. The thermal stability of PLI was greatly enhanced in the presence of CaCl_2 or a positively charged

molecule such as polylysine and greatly decreased in the presence of PGA or a negatively charged molecule such as heparin. The effects of CaCl_2 and PGA on the stability of PLI at 48°C is illustrated in Fig. 4. A similar pattern of effect was observed with polylysine and heparin (data not shown). The Arrhenius heat of thermal inactivation was calculated from Arrhenius plots in the presence of these effectors. The activation energy for thermal inactivation of PLI in the absence of effectors was 72 kcal mol⁻¹. The Arrhenius energy was raised to 82–91 kcal mol⁻¹ in the presence CaCl_2 or polylysine but lowered to 26–55 kcal mol⁻¹ in the presence of PGA or heparin. Glucose, a neutral (charge-free) molecule, exerted no effect on the thermal stability of the enzyme. Effects of CaCl_2 , PGA, polylysine and heparin on thermal stability of PLII were similar to those described for PLI.

Inactivation of PLI and PLII by group-specific inhibitors

Effects of two sulphhydryl group-specific inhibitors (IAA and IAM) and one amino group-specific inhibitor (DEPC) on PLI and PLII activities were examined. The results (Fig. 5) show that PLI of *Ps. fluorescens* and PLII of *Ps. viridiflava* exhibit differential degrees of sensitivity to three enzyme inhibitors. At 25°C, the half-life ($t_{1/2}$) of PLI in the presence of 10 mmol l⁻¹ IAA, DEPC and IAM was calculated to be 0.7, 8 and 100 min, respectively, whereas the half-life of PLII in the presence of the IAA, DEPC and IAM was calculated to be 17, 3 and 183 min, respectively. In general, PLII appeared to be more susceptible to inhibition by DEPC than by IAA. On the contrary, PLI appeared to be more susceptible to inhibition by IAA than by DEPC. This result suggests that the critical groups in the active sites of PLI and PLII may be distinguishable. Furthermore, the data also suggest that both sulphhydryl and amino groups (possibly including imidazole groups) are all important for catalytic activity.

DISCUSSION

The pectic enzyme system of soft-rotting pseudomonads including *Ps. fluorescens* (sometimes referred to as *Ps. marginalis*) and *Ps. viridiflava* is characterized by its simplicity. While all strains of soft-rotting *Erwinia* produce an array of pectic enzymes including multiple forms of PL isozymes, hydrolases and methyl esterases, the majority of soft-rotting pseudomonads produce only one pectic enzyme identified as pectate lyase (PL) (Nasuno and Starr 1966; Liao 1989). The catalytic reaction mechanism of PLs produced by *Ps. fluorescens* (Fuchs 1965; Nasuno and Starr 1966) and the factor affecting its production (Liao *et al.* 1993, 1994) have been previously investigated, but biochemical properties of PLs produced by soft-rotting pseudomonads have not been characterized before. This paper reports some of the fundamental

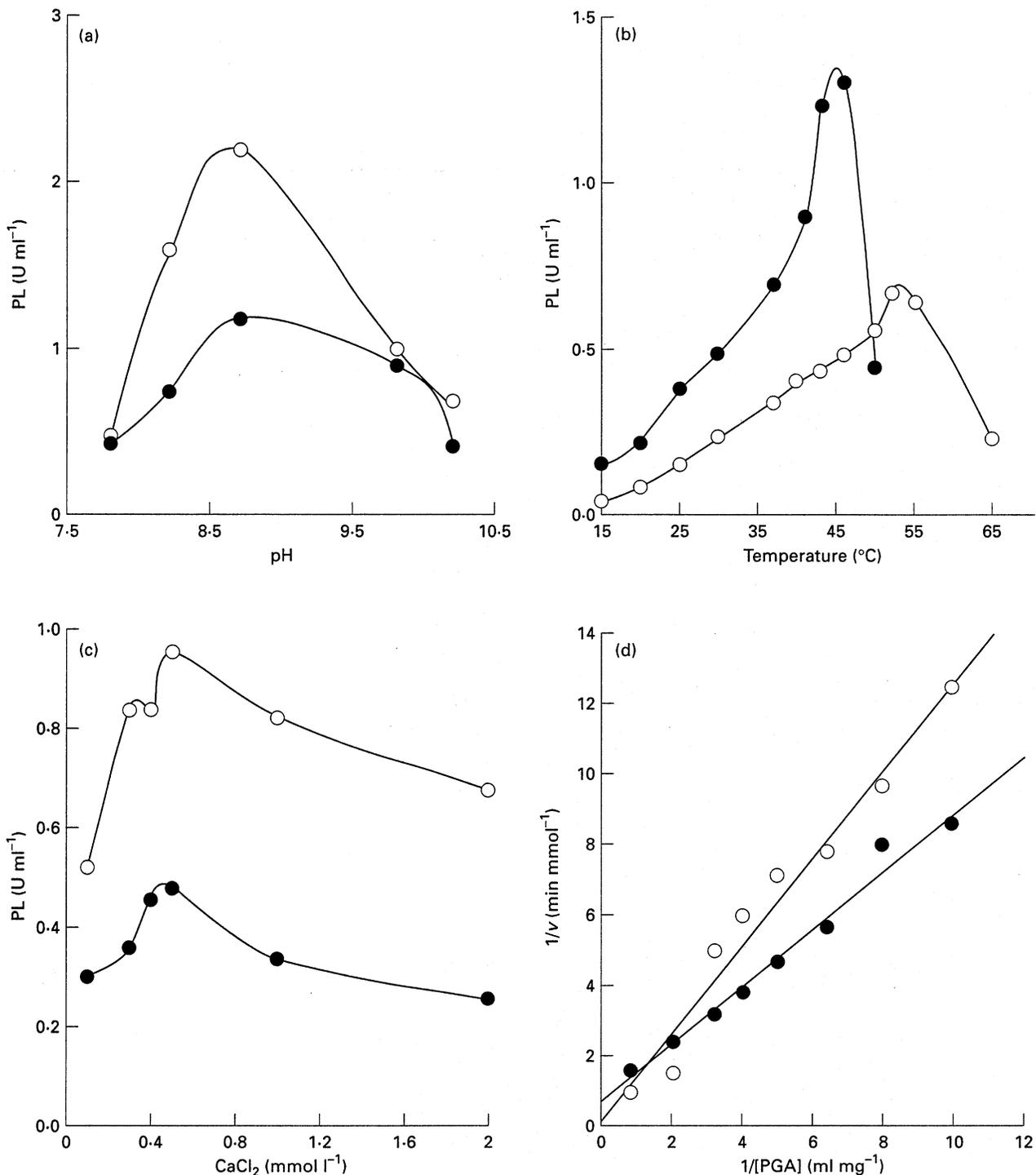


Fig. 3 Effects of (a) pH, (b) temperature and (c) CaCl₂ concentrations on activities of pectate lyases PLI and PLII from *Pseudomonas fluorescens* CY091 and *Pseudomonas viridiflava* PJ-08-6, respectively. (d) Double reciprocal plot of initial rates vs concentrations of sodium polygalacturonate. ●, PLI of *Ps. fluorescens* CY091; ○, PLII of *Ps. viridiflava* PJ-08-6

properties of PLs produced by *Ps. fluorescens* and *Ps. viridiflava*, which include metal ion requirements, pH and temperature optima, enzyme kinetics parameters, and effects of

various enzyme inhibitors and effectors on enzyme activity or stability. Such new biochemical information, in combination with knowledge obtained from recent advances in cloning

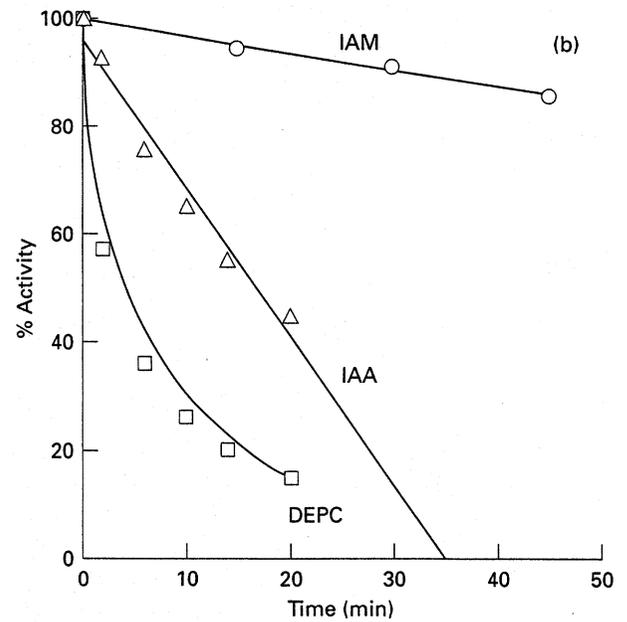
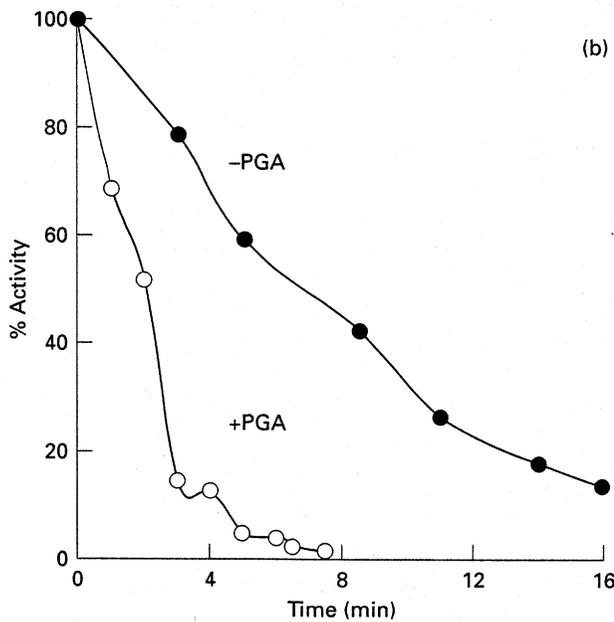
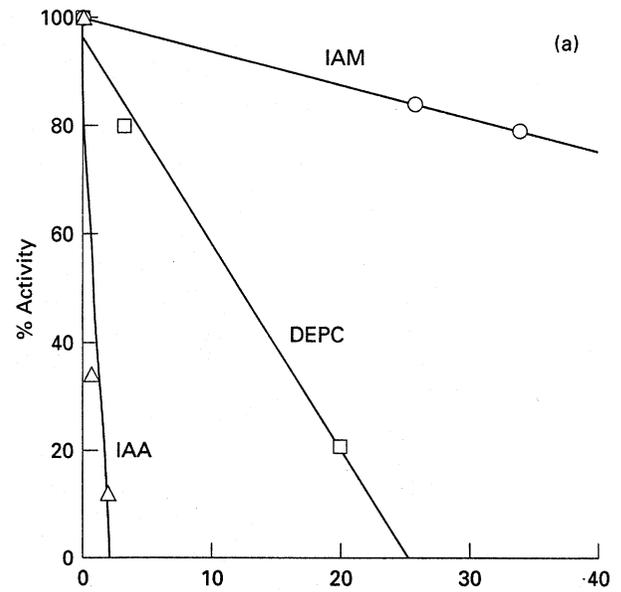
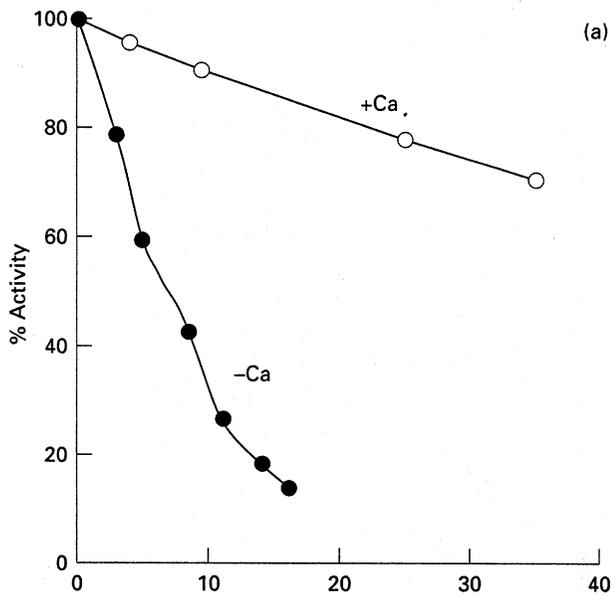


Fig. 4 Effects of (a) CaCl_2 and (b) polygalacturonate (PGA) on thermal stability of pectate lyases from *Pseudomonas fluorescens* CY091. The reaction was carried out at 48°C in a mixture containing or lacking CaCl_2 (as indicated by + or - Ca) or in a mixture containing or lacking polygalacturonate (as indicated by + or - PGA)

Fig. 5 Inhibition of pectate lyases PLI and PLII from (a) *Pseudomonas fluorescens* CY091 and (b) *Pseudomonas viridiflava* PJ-08-6 by group-specific inhibitors, iodoacetic acid (IAA), iodoacetamide (IAM) and diethylpyrocarbonate (DEPC)

and characterization of these genes coding for these enzymes (Liao *et al.* 1996), will enable us to better understand the catalytic and biological functions of these enzymes. In addition, this information is useful for consideration of practical application of PL enzymes for food and pulp processing

(Kurowski and Dunleavy 1976) and for development of new strategies for preventing fruit and vegetable spoilage.

PLI and PLII are fairly stable at low temperatures (25°C or below). Thermal stability of both enzymes is greatly enhanced in the presence of Ca^{2+} or a positively charged molecule such as polylysine. The requirement of Ca^{2+} for maintaining the structural integrity of PLs produced by *Erw. chrysanthemi*

(Henrissat *et al.* 1995) and for prolonging the thermal stability of proteases produced by *Ps. fluorescens* (McKellar and Cholette 1986) have been previously demonstrated. Recently, we have also shown that Ca^{2+} is absolutely required for the production of PLI by *Ps. fluorescens* strain CY091 (Liao *et al.* 1994). It is possible that Ca^{2+} may not be required for maintaining the structural integrity and thermal stability of the enzyme protein. The data presented here suggest that the increase in thermal stability is in part due to the decrease in heat inactivation by raising the Arrhenius energy from 72 kcal mol⁻¹ in the absence of Ca^{2+} to 82 kcal mol⁻¹ in the presence of Ca^{2+} . We suspect that the positively charged polylysine probably binds to the enzyme at a site different from that required by Ca^{2+} and results in the formation of a stable conformation more resistant to heat inactivation. On the other hand, the binding of polygalacturonate or the polyanionic heparin to the enzyme probably leads to the formation of a less stable conformation, that is more susceptible to heat denaturation. The molecular mechanism by which the binding of the effector to the enzyme leads to the decrease in the Arrhenius activation energy and the decrease in the thermal stability of PLI and PLII is not understood.

In this study, we found that PLI and PLII responded somewhat differently to three group-specific inhibitors. Although the enzyme is inhibited by both sulphhydryl-group specific inhibitors (IAA and IAM), IAA is a much stronger inhibitor than IAM. Furthermore, PLI appears to be more susceptible to the inhibition by IAA than by DEPC. On the contrary, PLII appears to be more susceptible to the inhibition by DEPC than by IAA. We suspect that the inhibition was caused by DEPC in a mechanism similar to that caused by pyridoxal phosphate, which had been shown to induce the formation of Schiff base with the amino groups in the protein. Although the results presented in the study suggest that both sulphhydryl and amino groups are critical for catalytic activities of PLI and PLII, this hypothesis needs to be further confirmed by studying the PL mutant proteins generated by site-specific mutagenesis. Amino acid sequences of PLI and PLII proteins have recently been inferred from the PLI and PLII genes cloned (Liao *et al.* 1996). The availability of gene and protein sequence information in combination with the biochemical properties presented here would greatly facilitate the study of the molecular mechanisms involved in the catalytic function and tissue-macerating ability of PLI and PLII in the future.

ACKNOWLEDGEMENTS

The authors would like to thank Dr S.-S. Wong and Drs J. S. Huang, Dan McCallus and Kevin Hicks for critical review of this manuscript before submission.

REFERENCES

- Brocklehurst, T.F. and Lund, B.M. (1981) Properties of pseudomonads causing spoilage of vegetables stored at low temperatures. *Journal of Applied Bacteriology* **50**, 259–266.
- Collmer, A. and Keen, N.T. (1986) The role of pectic enzymes in plant pathogenesis. *Annual Review of Phytopathology* **24**, 383–409.
- Collmer, A., Ried, J.L. and Mount, M.S. (1988) Assay methods for pectic enzymes. *Methods in Enzymology* **161**, 329–335.
- Fuchs, A. (1965) The *trans*-eliminative breakdown of Na-polygalacturonate by *Pseudomonas fluorescens*. *Antonie van Leeuwenhoek* **31**, 323–340.
- Henrissat, B., Hefferon, S.E., Yoder, M.D., Lietzke, S.E. and Journak, F. (1995) Structural implication of structure-based sequence alignment of proteins in the pectate lyase superfamily. *Plant Physiology* **107**, 963–976.
- Kurowski, W.M. and Dunleavy, J.A. (1976) Pectinase production by bacteria associated with improved preservative permeability in Sietke spruce: synthesis and secretion of polygalacturonate lyase by *Cytophaga johnsonae*. *Journal of Applied Bacteriology* **41**, 119–128.
- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–687.
- Liao, C.-H. (1989) Analysis of pectate lyases produced by soft rot bacteria associated with spoilage of vegetables. *Applied and Environmental Microbiology* **55**, 1677–1683.
- Liao, C.-H. and Wells, J.M. (1987) Diversity of pectolytic, fluorescent pseudomonads causing soft rots of fresh vegetables at produce markets. *Phytopathology* **77**, 673–677.
- Liao, C.-H., Hung, H.Y. and Chatterjee, A.K. (1988) An extracellular pectate lyase is the pathogenicity factor of the soft-rotting bacterium *Pseudomonas viridiflava*. *Molecular Plant-Microbe Interactions* **1**, 199–206.
- Liao, C.-H., McCallus, D.E. and Wells, J.M. (1993) Calcium-dependent pectate lyase production in the soft-rotting bacterium *Pseudomonas fluorescens*. *Phytopathology* **83**, 813–818.
- Liao, C.-H., McCallus, D.E. and Fett, W.F. (1994) Molecular characterization of two gene loci required for production of the key pathogenicity factor pectate lyase in *Pseudomonas viridiflava*. *Molecular Plant-Microbe Interactions* **7**, 391–400.
- Liao, C.-H., Gaffney, T.D., Bradley, S.P. and Wong, L.C. (1996) Cloning of a pectate lyase gene from *Xanthomonas campestris* pv. *malvacearum* and comparison of its sequence relationship with *pel* genes of soft-rot *Erwinia* and *Pseudomonas*. *Molecular Plant-Microbe Interactions* **9**, 14–21.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.
- McKellar, R.C. and Cholette, H. (1986) Possible role of calcium in the formation of active extracellular proteinase by *Pseudomonas fluorescens*. *Journal of Applied Bacteriology* **60**, 37–44.
- Nasuno, S. and Starr, M.P. (1966) Pectic enzymes of *Pseudomonas marginalis*. *Phytopathology* **56**, 1414–1415.