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Full Length Research Paper

Isolation, expression and comparison of a pectate lyase produced by *Fusarium oxysporum* f.sp. *cubense* race 1 and race 4

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Fusarium wilt is a devastating disease in banana production. For example, cultivar Gros Michel was destroyed by *Fusarium oxysporum* f.sp. *cubense* race 1 (FOC1) of the pathogen. Cultivar Cavendish is resistant to FOC1, but a newly occurring race 4 (FOC4) was found to be able to infect Cavendish. Studying the fungal pathogenicity is an important step towards the disease control. In this study, we cloned two *pl1* genes (723 bp and 240 aa), encoding pectate lyase 1 (PL1) from both FOC1 and FOC4, into the expression vector pPICZaA and the two genes were expressed in *Pichia pastoris* strains of SMD1168. The recombinant PL1, r-FOC1-PL1 and r-FOC4-PL1, were purified as active extracellular proteins. The optimal PL activity was observed at 50°C and pH 10. Both recombinant PL1 retained >72% activity at pH 3.0 to 11.0 and >70% activity at 10 to 70°C. Activity of the recombinant PL1 required calcium ions with the optimum activity at 0.75 mM $CaCl_2$. This is the first isolation and purification of PL1 from pathogenic FOC.

Key words: Pectate lyase, *Fusarium oxysporum* f.sp. *cubense*, Fusarium wilt, banana.

INTRODUCTION

Banana (*Musa* spp.) is a number four important crop in developing countries (Heslop-Harrison and Schwarzacher, 2007). Banana production suffers from several diseases and Fusarium wilt disease (Panama disease) is one of the most serious (Cheesman, 1962). This disease is caused by a fungal pathogen, *Fusarium oxysporum* f.sp. *cubense* (FOC). Significant yield losses in banana production have been reported from Australia, Asia, Africa and Central and South America (Ploetz, 2006). Based on pathogenicity to host cultivars under field condition, four physiological races of FOC have been identified: FOC1 infecting cultivar Gros Michel, FOC2 infecting cultivar 'Bluggoe', FOC3 infecting *Heliconia* spp. and FOC4 infecting cultivar Cavendish, and all cultivars are susceptible to FOC1 and FOC2 (Persley, 1987). Earlier last century, FOC1 destroyed nearly the world banana industry growing cultivar, Gros Michel. Consequently, Gros Michel was replaced by Cavendish cultivars, which were resistant to FOC1. However, another race of FOC, FOC4, emerged and this race is capable of attacking cultivar Cavendish. Currently, FOC4 has spread to Asia, Australia and Africa (Hwang and Ko, 2004). It is becoming a concern that if FOC4 hits the banana heartland in Latin America, it would be catastrophic for the world's banana industry (Grimm, 2008).

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Abbreviations: FOC, *Fusarium oxysporum* f.sp. *cubense*; **PLs,** pectate lyases; **PDB,** potato dextrose broth; **PGA,** polygalacturonic acid; **YPD,** yeast extract peptone dextrose; **ORFs,** open reading frames; **BLAST,** Basic Local Alignment Search Tool.

Plant pathogenic fungi produce an array of extracellular cell wall degradative enzymes that may be important in pathogenicity (Walton, 1994). Among them are pectate lyases (PLs; EC 4.2.2.2), which catalyzes the trans-elimination of pectate (Linhardt et al., 1986). For example, antibodies inhibiting the catalytic activity of PLs of *Fusarium solani* f.sp. *pisi* reduced the ability of the fungus to infect pea stems (Crawford and Kolattukudy, 1987). No study has been done on PLs from FOC. In this study, we cloned the *pl1* genes of FOC1 and FOC4 and expressed them in *Pichia pastoris*. Both enzymes retained their PL activity. This opens an avenue for further studies on the role of PL1 in FOC pathogenicity in banana cultivars.

MATERIALS AND METHODS

Fungal strains and growth condition

F. oxysporum f.sp. *cubense* race 4 (FOC4) was isolated from Guangzhou, Guangdong Province of China in 2006 and *F. oxysporum* f.sp. *cubense* race 1 (FOC1) was isolated from Nanning, Guangxi Province of China in 2003. Both fungal isolates were maintained in the Department of Plant Pathology, South China Agricultural University at Guangzhou. The pathogenicity of the fungal isolates was periodically confirmed by plant assays in a growth chamber.

For DNA extraction, mycelium was obtained from cultures grown for 5 days in potato dextrose broth (PDB) in Erlenmeyer flasks on a rotary shaker at 110 rpm at 25℃. For RNA extraction, fungal isolates were grown in SM (Di Pietro and Roncero, 1996a) supplemented with 1% (w/v) of polygalacturonic acid (PGA) sodium salt. PGA was from Sigma.

Cloning of fungal pectate lyase 1 genes

Total RNA was prepared according to the protocol of E.Z.N.A Fungal RNA Kit (OMEGA BIO TEK, USA). First-strand cDNA were synthesized from total RNA of FOC1 and FOC4 using reverse transcriptase XL (TaKaRa). Reverse transcription polymerase chain reaction (RT-PCR) was performed using the forward primer (5-GTGGAATTCATGAAGTACACTGCCATCC-3) and the reverse primer (5-CGGTCTAGACTAGCAGCTCGTGGTAACTC-3) based on the nucleotide sequence of the *F. oxysporum* f.sp. *lycopersici pl1* gene (Huertas-Gonzalez et al., 1999). The *Eco*RI and *Xba*I restriction sites are underlined in the primer sequences. The following PCR conditions were used: 35 cycles with denaturation at 94℃ for 30 s, annealing at 49℃ for 30 s and extension at 72℃ for 90 s. An initial denaturation step of 4 min at 94℃ and a final elongation step at 72℃ for 10 min were performed. The RT-PCR products were cloned into pMD18-T (TaKaRa) and verified by nucleotide sequencing analysis. Then amplicons were digested with *Eco*RI and *Xba*I and subcloned into the same enzyme sites of pPICZaA (Invitrogen) to generate recombinant eukaryotic expression vector, pPICZaA-*pl1*-FOC1 and pPICZaA-*pl1*-FOC4. The presence of the inserted *pl1*-FOC1 and *pl1*-FOC4 genes was verified by nucleotide sequencing analysis.

Genomic DNA was extracted from fungal mycelium using the E.Z.N.A Fungal DNA Kit (OMEGA) according to the instructions of the manufacturer. The DNA sequences were isolated and sequenced using DNA isolated from FOC1 and FOC4 as the template and primers as mentioned above.

The complete nucleotide sequence of *pl1* genes from FOC1 and FOC4 were deposited in the GenBank database under accession number GQ200590 and GQ200589.

Expression and purification of PL1 in *P. pastoris*

Yeast transformation was performed according to the manufacturer's instructions. The recombinant plasmid pPICZaA-*pl1*-FOC1 and pPICZaA-*pl1*-FOC4 were linearized by digestion with *Sac*I and transformed into *P. pastoris* SMD1168 strain by electroporation. The SMD1168 strains transformed with pPICZaA plasmid and SMD1168 strains without transformation served as negative controls. The cells were incubated in yeast extract peptone dextrose (YPD) plate containing 1% yeast extract, 2% peptone, 2% dextrose and 100 µg/ml of zeocin at 28℃ for 48 h. The integration of the *pl1* gene into the genome of *P. pastoris* was determined by PCR using 5'AOX1 and 3'AOX1 primers. The presence of the *pl1* gene in transformants was confirmed by gene sequencing of PCR products.

Yeast transformants were grown in 30 ml of BMGY medium (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 100 mM potassium phosphate, 4×10^{-5}% biotin and 1% glycerol) at 28℃ for 24 h. Then the cell pellet was harvested and resuspended in 200 ml BMMY (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 100 mM potassium phosphate, 4×10^{-5}% biotin and 0.5% methanol). The culture was returned to the incubator with the same conditions of growth and samples were collected at 12, 24, 36, 48, 60 and 72 h, 1ml of 100% methanol was added every 24 h to maintain the final concentration of methanol at 0.5% assuming that the methanol would have been completely utilized in 24 h.

The culture was centrifuged at 10,000g for 20 min at 4℃ and the supernatant was filtered using membrane filter (0.22 µm, millipore). The supernatant was fractionated by salting out with solid ammonium sulfate at 0 - 80% (w/v) saturation. The precipitate formed was collected by centrifugation at 10,000 g for 20 min at 4℃, dissolved in 50 mM Tris-HCl (pH 8.0) and then dialyzed overnight against the same buffer at 4℃. After centrifugation, the recombinant r-FOC1-PL1 and r-FOC4-PL1 were further purified using a gel filtration column (Sephacryl S-100 16/60, Pharmacia) equilibrated and eluted with 50 mM Tris-HCl (pH 8.0) at a flow rate of 1 ml/min. Fractions containing PL activity were collected.

Enzyme activity and protein assays

Unless stated otherwise, the standard assay contained 1 ml of pre-warmed substrate solution (Glycine-NaOH (pH 9.0), 0.5 mM $CaCl_2$ and 0.1% PGA) and 1 µg of recombinant protein. The reaction was incubated for 1 h at 37℃, and then PL activity was measured by the periodatethiobarbituric acid (TBA) method (Nedjma et al., 2001). The activity was indicated by the formation of a red chromagen which had a maximum absorbance at 550 nm. One unit of the PL activity was defined as the amount of enzyme that increased the absorbance by 0.05 in 1 h at 550 nm.

The standard assay was modified to test the effects of varying pH and incubation temperature. To determine the optimal pH, a pH range from 3 to 12 was used with the following buffers: sodium acetate (pH 3.0 to 6.0), glycine-NaOH (pH 7.0 to 12). The optimal temperature was determined in the range of 10 to 90℃ in glycine-NaOH (pH 9.0). To estimate the pH stability, samples were incubated in buffers of different pH values at 4℃ for 24 h. To evaluate the thermostability, protein samples were incubated at different temperatures in glycine-NaOH, pH 9.0 for 2 h. The residual activity was detected according to the method previously described. To determine the optimal calcium ions of PL1 required for activity, calcium ions between 0 and 3 mM were used.

Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970) using 10% acrylamide. After electrophoresis, gels were stained with Coomassie brilliant blue G250 (Sigma). Protein was

determined according to the method of Bradford (1976) using bovine serum albumin as a standard.

Figure 1. Multiple amino acid sequence alignment of the predicted protein of *pl1* from *F. oxysporum* f. sp. *cubense* race 4 (FOC4), *F. oxysporum* f. sp. *cubense* race 1 (FOC1) and *F. oxysporum* f. sp. *lycopersici* (FOL) (AAC64368). Identical amino acids are highlighted on a shaded background.

RESULTS AND DISCUSSION

Isolation of genes encoding pectate lyase PL1 from FOC1 and FOC4

The *pl1* genes encoding pectate lyase 1 (PL1) from FOC1 and FOC4 were amplified by RT-PCR from total RNA, using specific primers derived from the *pl1* sequence of *F. oxysporum* f.sp. *lycopersici* (GenBank accession no. AF080485). The DNA fragments were cloned and sequenced.

Both *pl1*-FOC1 (GenBank accession no. GQ200590) and *pl1*-FOC4 (GenBank accession no. GQ200589) were 723 bp in length, coding for putative open reading frames (ORFs) of 240 amino acids with the predicted molecular weight of 24.84 and 24.86 kDa, the predicted pI of 7.70 and 7.41, respectively. Analysis with SignalP V2.0 detected that a putative N-terminal signal peptide sequence of 15 amino acids when cleaved would produce a mature protein. The deduced mature protein of PL1 from FOC1 (FOC1-PL1) and PL1 from FOC4 (FOC4-PL1) had the calculated molecular mass of 23.36 and 23.38 kDa and the calculated pI of 7.41 and 6.93, respectively. Similar to the deduced mature protein of *pl1* encoding a PL1 of *F. oxysporum* f.sp. *lycopersici* (FOL), there was a calculated molecular mass of 23.4 kDa and a pI of 7.4 (Huertas-Gonzalez et al., 1999)

Standard protein-protein Basic Local Alignment Search Tool (BLAST) analysis revealed that both isolated PL1 shared a high amino acid sequence identity with PL from other fungi such as: PL1 of FOL (Huertas-Gonzalez et al., 1999) *pel*A (Gonzalez-Candelas and Kolattukudy 1992) *pel*B, *pel*C and *pel*D of *F.solani* f.sp. *pisi* (Guo et al., 1995a, b; Guo et al., 1996). Figure 1 shows the multiple alignments for the PL1 amino acids of FOC1, FOC4 and FOL. The FOC1-PL1 shared as high as 99.17% amino acid sequence identity with FOC4-PL1 and FOL-PL1, while FOC4-PL1 shared 98.33% amino acid sequence identity with FOL-PL1. We noted the following differences between FOC1-PL1 and FOC4-PL1: 109-V to I, 202-N to D (*Casein Kinase II phosphorylation* site). The differences between FOC1-PL1 and FOL-PL1 were: 58-T (*N-myristoylation* site) to S (*N-myristoylation* site), 193-A to T (*Protein Kinase C phosphorylation* site). The differences between FOC4-PL1 and FOL-PL1 were: 58-T (*N-myristoylation* site) to S (*N-myristoylation* site), 109- I to V, 193-A to T (*Protein Kinase C phosphorylation* site) and 202-D (*Casein Kinase II phosphorylation* site) to N.

Expression and purification of recombinant PL1

Recombinant PL1 from FOC1 and FOC4 were expressed in *P. pastoris* as secreted proteins r-FOC1-PL1 and r-FOC4-PL1, respectively. Culture samples taken at 12, 24, 36, 48, 60 and 72 h post-induction were analyzed on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 2a and b). As predicted, proteins of about 24 kDa were detected from the r-FOC1-PL1 and r-FOC4-PL1 transformant cultures, but were not observed in control *P. pastoris* transformed with pPICZαA vector.

The specific PL activity were detected from the r-FOC1-PL1 and r-FOC4-PL1 transformant cultures, but were not detected in control *P. pastoris* transformed with pPICZαA vector. Both recombinant PL1 were purified through

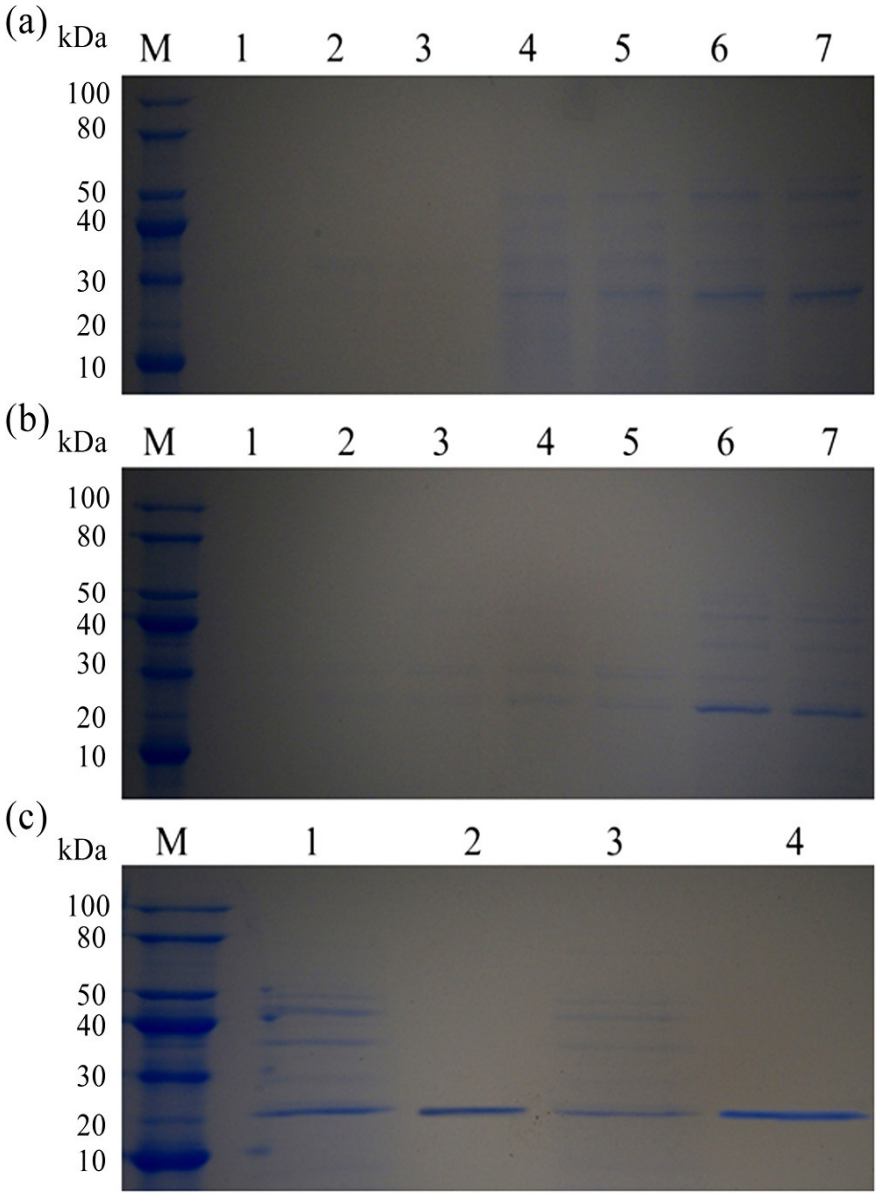

Figure 2. SDS-PAGE analysis of recombinant PL1 from (a) FOC1 and (b) FOC4 produced in *P. pastoris*. Cultures were induced for 72 h with methanol as described in materials and methods and supernatants from 12, 24, 36, 48, 60 and 72 h (Lane 2 to 7) were collected. Lane M: Protein marker; Lane 1: cultures transformed with pPICZaA. (c) SDS-PAGE analysis of purified recombinant PL1 from FOC1 and FOC4. Lane M: protein marker; Lane 1: culture supernatant from FOC1 at 72 h; Lane 2: purified r-FOC1-PL1; Lane 3: culture supernatant from FOC4 at 72 h; Lane 4: purified r-FOC4-PL1.

successive steps of $(NH_4)_2SO_4$ precipitation and gel filtration chromatography. During the purification process, the specific PL activity of r-FOC1-PL1 and r-FOC4-PL1 increased from 15.06 to 340.25 units mg protein^{-1} and 20.00 to 421.35 units mg protein^{-1}, respectively (Table 1). SDS-PAGE showed one single protein band and indicated that r-FOC1-PL1 and r-FOC4-PL1 were purified to homogeneity (Figure 2c). According to the markers, the molecular weight of both recombinant proteins was about 24 kDa.

Biochemical characterization of recombinant PL1

Both enzymes exhibited the highest activity at pH 10.0 (Figure 3a), and the highest activity at 50°C (Fig. 3b). The pH value and temperature optima for the PL1 activity from FOL were 9.0 and 42°C (Di Pietro and Roncero, 1996b). However, there was no activity of PL1 from FOL detected at pH 7.0, while the activity was also recorded with recombinant PL1 from FOC1 and FOC4 at pH 7.0.

To estimate the pH stability of recombinant PL1,

Table 1. Purification of recombinant PL1 from 200 ml of *P. pastoris* culture supernatant.

Purification step	Total protein (mg)	Total activity (unit)[a]	Yield (%)	Specific activity (units mg protein^{-1})
Supernatant r-FOC1-PL1/r-FOC4-PL1	54/50	813.24/1000	100/100	15.06/20
$(NH_4)_2SO_4$ precipitation r-FOC1-PL1/r-FOC4-PL1	23/20	660/840	81.16/84	28.7/42
Sephacryl S-100 16/60 r-FOC1-PL1/r-FOC4-PL1	1.2/1.1	408.3/463.49	50.21/46.35	340.25/421.35

[a] One unit of the PL activity was defined as the amount of enzyme that increased the absorbance by 0.05 in 1 h at 550 nm.

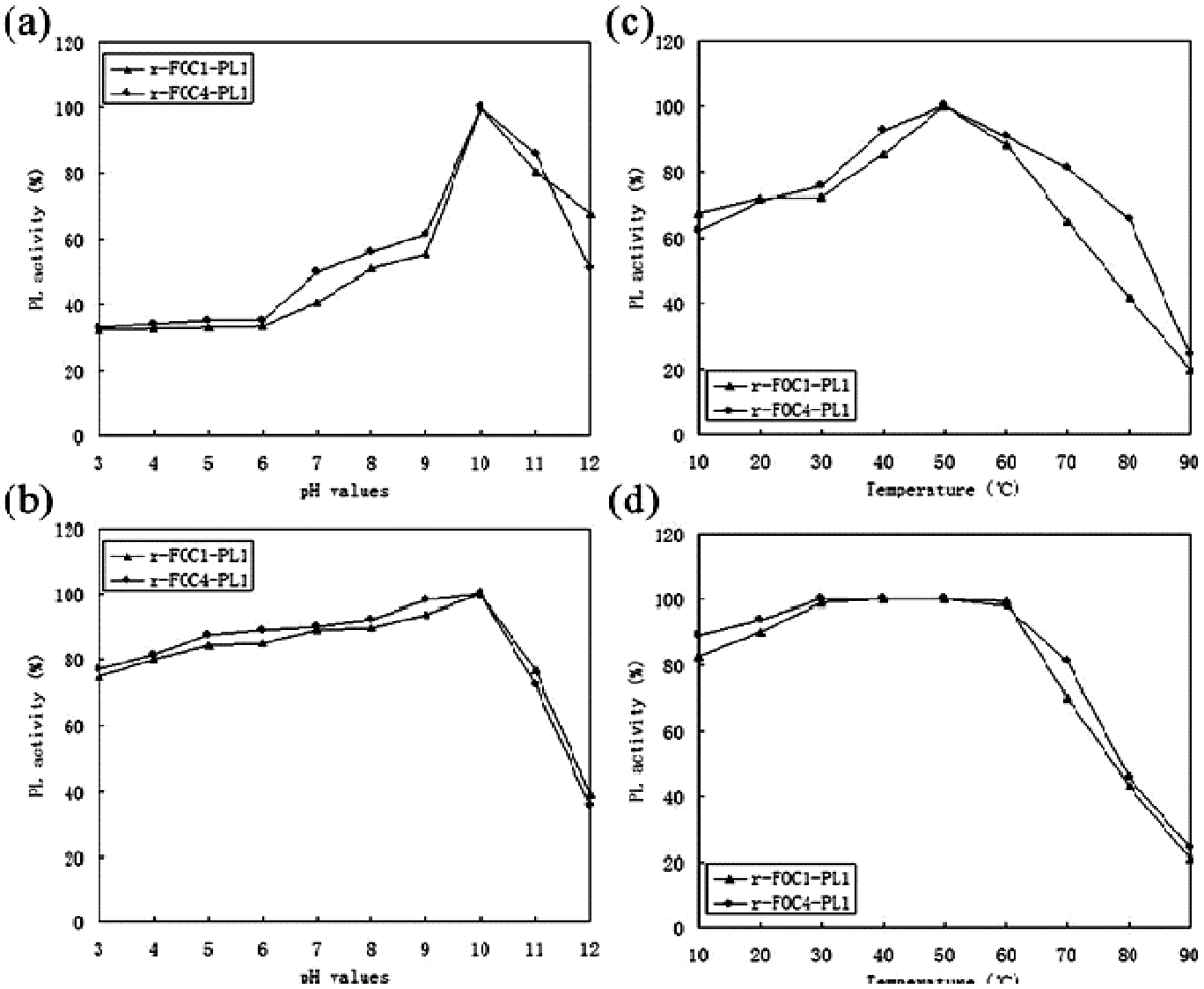

Figure 3. Enzymatic activity and stability. (a) Determination of the optimal pH; (b) enzymatic stability with respect to pH; (c) determination of the optimal temperature; (d) enzymatic stability with respect to temperature.

samples were incubated in buffers of different pH values at 4℃ for 24 h. The remaining PL activity was assayed. Both recombinant PL1 retained >72% activity at pH 3.0 to 11.0. At basic pH 12.0, r-FOC1-PL1 retained 39.2% activity, whereas 35.5% activity of r-FOC4-PL1 remained (Figure 3c).

To investigate the thermostability of recombinant PL1, both enzymes were incubated at different temperatures in

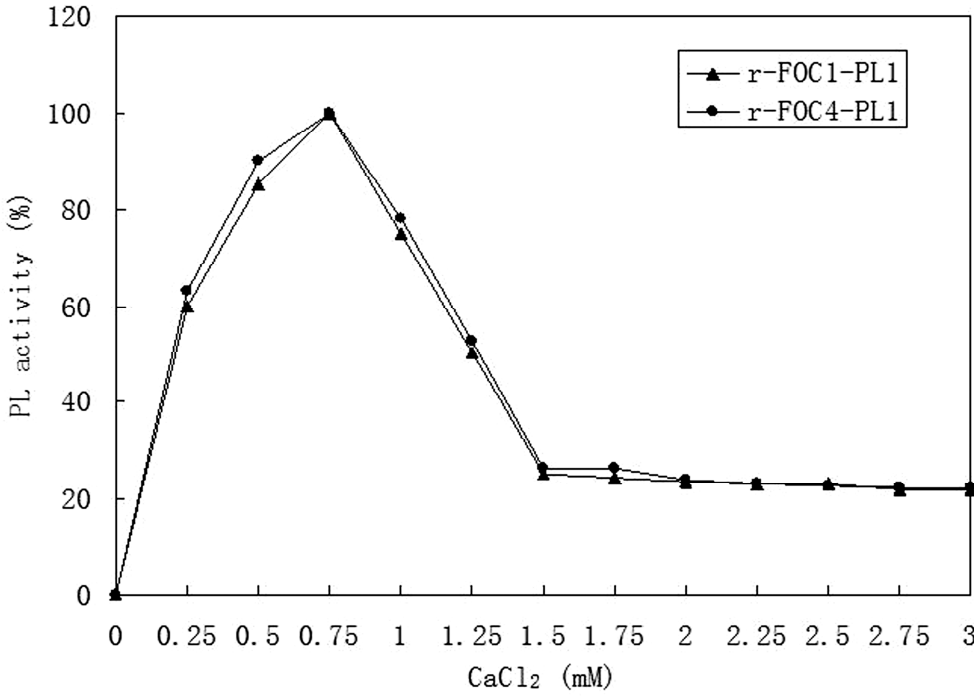

Figure 4. Effect of $CaCl_2$ concentration on recombinant PL1 activity. The activity in the standard assay containing 0.75 mM $CaCl_2$ was arbitrarily set as 100%. The enzymatic activity was tested by the periodatethiobarbituric acid (TBA) method (Nedjma et al., 2001).

glycine-NaOH, pH 9.0, for 2 h. The residual activity was detected. Both recombinant PL1 retained >70% activity at 10 to 70°C. At 80 and 90°C, r-FOC1-PL1 retained 43.19 and 21.08% activity, whereas 46.00 and 24.35% activity of r-FOC4-PL1 remained (Figure 3d). Figure 4 demonstrates that recombinant PL1 requires calcium ions for activity and that optimum activity was noted with 0.75 mM $CaCl_2$.

In conclusion, we demonstrated that both *pl* gene could express in the *P. pastoris*, and had their PL activity. The two PLs have similar molecular basis and highly similar characters. Further studies are required to obtain insight into the role of PL1 in FOC pathogenicity in banana cultivars.

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