Research Note

Purification of Pectate Lyase Produced by *Colletotrichum gloeosporioides* and Its Inhibition by Epicatechin: A Possible Factor Involved in the Resistance of Unripe Avocado Fruits to Anthracnose

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Received 17 June 1993. Revision received 26 November 1993. Accepted 2 December 1993.

Pectate lyase (PL) from *Colletotrichum gloeosporioides* was purified to apparent homogeneity by hydrophobic interaction chromatography followed by isoelectric focusing. The purified preparation showed one band corresponding to 40 kD on sodium dodecyl sulfate–polyacrylamide gels. The isoelectric point of the enzyme was 7.9, and the optimum pH for activity was 8.9. The purified PL efficiently macerated unripe avocado fruit wedges. *In vitro* translation of mRNA from an induced fungal culture revealed a 36-kD precursor polypeptide, which was precipitated with PL antibodies. The antibodies inhibited enzymatic activity and maceration ability on avocado wedges. Epicatechin, a flavan 3-ol present in the peel of unripe avocado fruit, had a K_i of 3.4 μM for inhibition of PL activity *in vitro*. At 20 μg/ml (68 μM), epicatechin reduced the enzyme's macerating ability by 64%. Since the flavan is present in unripe fruit at much higher concentrations (about 350 μg/g fresh weight) than the inhibitory concentrations, epicatechin may be involved in the resistance of unripe avocado fruits by inhibiting the PL activity of *C. gloeosporioides*.

Additional keywords: Persea americana, quiescent infections.

Colletotrichum gloeosporioides (Penz.) Penz. & Sacc. in Penz. causes anthracnose, the most important postharvest disease of ripening avocado fruits in the world (Binyamini and Schiffmann-Nadel 1972). Conidia of the pathogen germinate on the peel of unripe fruits and produce appressoria. The appressoria germinate, and penetration hyphae breach the cuticle, but they become latent and stop developing at the first layer of host parenchyma cells (Prusky and Plumbley 1992). Failure to develop beyond this stage appears to be due to adverse physiological conditions temporarily imposed by the host until the occurrence of fruit ripening and softening.

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MPMI Vol. 7, No. 2, 1994, pp. 293-297

Three main factors have been suggested as causes of latent fungal infections (Swinburne 1983): 1) the lack of available nutrients in unripe fruits, which become available in ripening fruits; 2) preformed compounds in unripe fruits, which decrease during ripening; and 3) the pathogen's lack of enzymatic potential to penetrate unripe fruits. Nutrient availability was found not to be a limiting factor in the development of *C. gloeosporioides* in unripe avocado fruits (Prusky *et al.* 1984). The resistance of unripe avocado fruits to attack by the pathogen was attributed largely to the presence of high concentrations of an antifungal compound, 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene, prior to infection by the fungus (Prusky *et al.* 1982). The possibility that a lack of enzymatic potential of *C. gloeosporioides* could also affect the resistance of unripe fruits was initially raised when epicatechin, a flavan 3-ol present in high concentrations in the pericarp of unripe fruits, was shown to inhibit an endopolygalacturonase produced by *C. gloeosporioides* (Prusky *et al.* 1989). This enzyme has macerating capabilities on avocado tissues, but its importance during pathogenesis has still not been determined.

C. gloeosporioides also produces pectate lyase (PL) activity (Barash and Khazzan 1970). Reports have appeared of cloned genes encoding PL and pectin lyase in *Aspergillus nidulans* (Dean and Timberlake 1989), *A. niger* (Gysler *et al.* 1990, Harmsen *et al.* 1990), and *Fusarium solani* (Gonzales-Candelas and Kolattukudy 1992). The role of PL during pathogenicity has not been critically tested. Crawford and Kolattukudy (1987) concluded that PL may be important, since antibodies against a PL from *F. solani* inhibit the development of disease symptoms on pea stems.

As a first study to test the possible involvement of PL in the pathogenicity of *C. gloeosporioides* on avocado and its importance during fungal attack on ripening fruits, we purified and characterized the major PL produced by the fungus and determined its sensitivity to the flavan 3-ol epicatechin.

Purification and characterization of PL.

A single-spore isolate of *C. gloeosporioides* (CG-14) from decayed avocado fruit was maintained on potato-dextrose agar containing 250 mg of chloramphenicol and 1 mg of am-

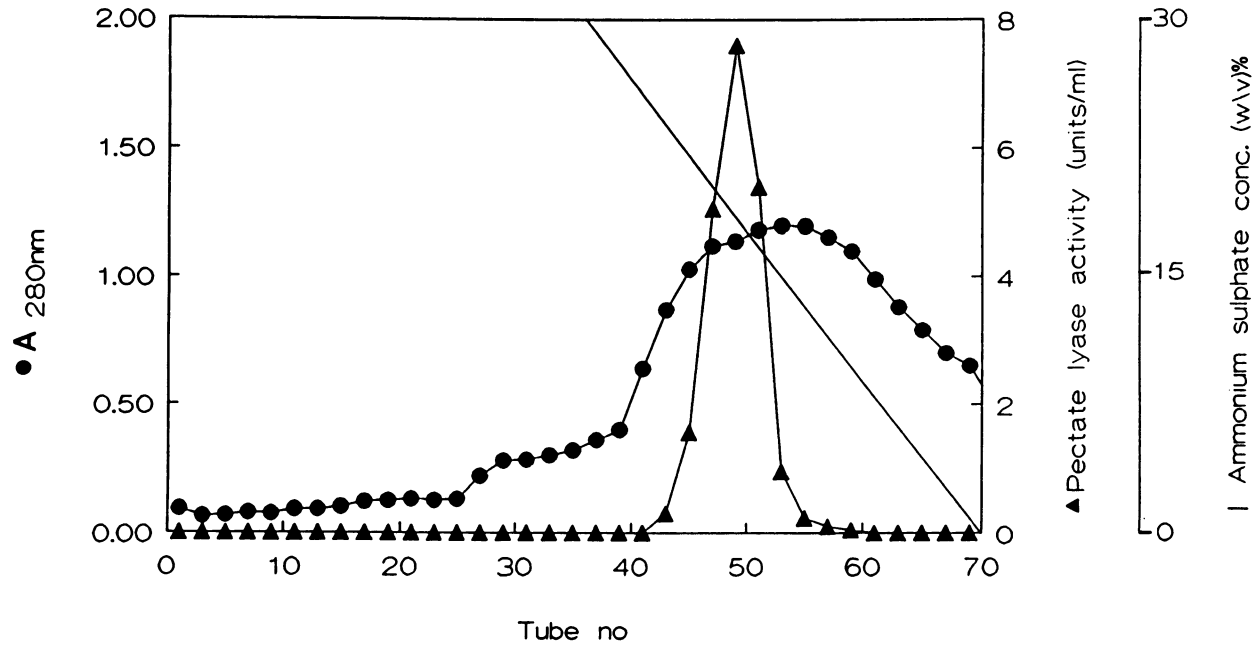

Fig. 1. Phenyl-Sepharose column chromatography of pectate lyase from *Colletotrichum gloeosporioides*. The enzyme was loaded in 30% ammonium sulfate in 25 mM Tris-HCl, pH 8.5. Unbound proteins were eluted with 300 ml of 30% ammonium sulfate, and the column was eluted with a linear gradient of ammonium sulfate (from 30 to 0%; a total volume of 120 ml) in 25 mM Tris-HCl, pH 8.5. Fractions showing pectate lyase activity were pooled and dialyzed overnight against 10 L of double-distilled water. Absorbance of fractions at 280 nm is denoted by circles, and pectate lyase activity by triangles. Fractions 17–28 were collected. Pectate lyase was assayed by monitoring the increase in absorbance at 232 nm caused by the release of 4,5-unsaturated galacturonide reaction products from sodium polypectate (Collmer *et al.* 1988).

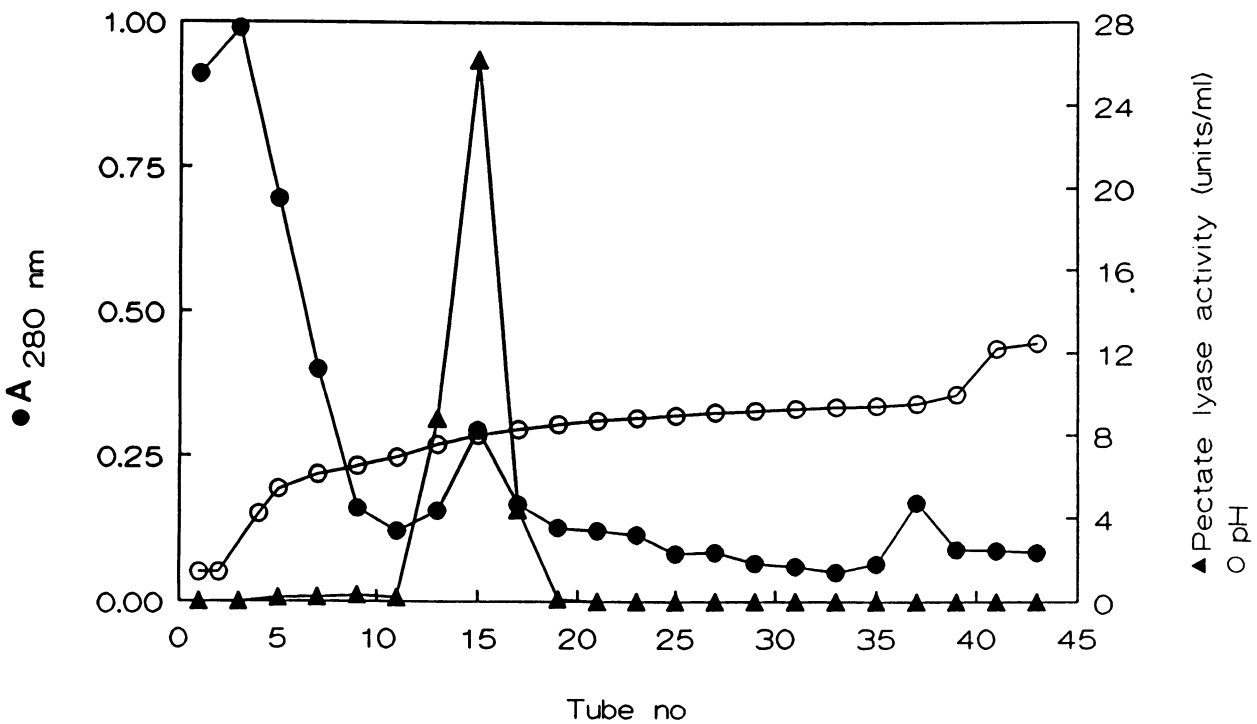

Fig. 2. Column electrofocusing of *Colletotrichum gloeosporioides* pectate lyase from the phenyl Sepharose column. The dialyzed solution was subjected to preparative isoelectric focusing by the use of an LKB 8100-1 column with 1% carrier ampholytes (pH 3.0–10). The sample was applied in a linear sucrose gradient from 50 to 5% (w/v) and electrophoresed at constant power of 7 W for 48 hr to a final 2,520 V. Fractions of 2.0 ml were collected. Fraction pH is indicated by open circles, absorbance at 280 nm by closed circles, and pectate lyase activity by triangles.

Table 1. Purification of pectate lyase from *Colletotrichum gloeosporioides*

	Total protein (mg)	Total activity (μmol/min)	Specific activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)	Recovery (%)	Enrichment
Culture filtrate	2,960.0	580.2	0.2	...	...
Phenyl-Sepharose 1	16.4	283.2	17.3	49	104-fold
Phenyl-Sepharose 2	3.2	218.4	68.3	37	356-fold
Isoelectric focusing	0.6	148.8	248.0	26	1,256-fold

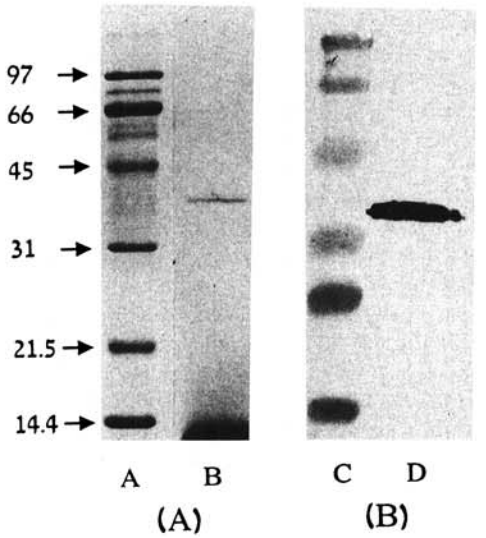

Fig. 3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (**A**) and western blot analysis (**B**) of purified *Colletotrichum gloeosporioides* pectate lyase recovered from an electrofocusing column. **A,** Lane A, size standards: phosphorylase b (97 kD), bovine serum albumin (66 kD), ovalbumin (45 kD), carbonic anhydrase (31 kD), trypsin inhibitor (21.5 kD), and lysozyme (14.4 kD); lane B, 2 μg of purified pectate lyase. **B,** Lane C, prestained SDS-PAGE size markers; lane D, 2 μg of purified pectate lyase (Bradford 1976). Proteins were analyzed on denaturing SDS-polyacrylamide gels (12.5%) according to the method of Laemmli (1970) by the use of a Mini Protean II device (Bio-Rad). Electroblotting from SDS gel to nitrocellulose for immunoblotting analysis was performed in Bio-Rad transfer cells at a constant 60 V in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid buffer, pH 11, in 10% methanol at room temperature.

picillin per liter of medium. *C. gloeosporioides* was grown at 28° C with continuous shaking, in 1-L Erlenmeyer flasks containing 500 ml of medium. The medium consisted of citrus pectin (5 g/L), KNO_3 (5 g/L), KH_2PO_4 (4 g/L), $MgSO_4 \cdot 7H_2O$ (2 g/L), $CaCl_2 \cdot 7H_2O$ (0.3 g/L), and $FeCl_3$ (0.01 g/L). PL activity was detected in the culture medium of *C. gloeosporioides* after 4–5 days growth in a mineral medium with pectin as the only carbon source, when the pH of the medium reached 6.5–7.0. Adjusting the initial pH of the medium to 7.0 advanced the production of the enzyme by 2–3 days. The maximal PL activity was detected when the pH of the medium reached 8.5–8.9. Culture filtrate was collected and concentrated by rotary evaporation.

Ammonium sulfate was added to the dialyzed and concentrated culture filtrate to a concentration of 30% (w/v), and the mixture was passed through a phenyl-Sepharose column. The main peak of the adsorbed PL was eluted at approximately 25% (w/v) ammonium sulfate during stepwise gradient. A second peak of activity was obtained with 20% salt. The major peak was run again through a phenyl-Sepharose column that was gradient-eluted. The adsorbed PL was eluted at approximately 18% (w/v) ammonium sulfate (Fig. 1). Active fractions were pooled and applied to a preparative isoelectric focusing column. This yielded a major peak of PL activity with a pI of 7.9 (Fig. 2). The specific activity of the enzyme was enriched 1,256-fold at the end of the purification process (Table 1).

The active fraction showed one Coomassie Blue staining band on sodium dodecyl sulfate (SDS)–polyacrylamide gels, corresponding to an apparent molecular mass of 40 kD (Fig. 3). This was confirmed by gel filtration chromatography, in which the PL showed a molecular mass of 39 kD, and by CNBr digestion, in which three bands (18, 14, and 7 kD) were obtained. The PL of *C. gloeosporioides* is larger than that produced by *F. solani* (28 kD) (Crawford and Kolattukudy 1987), smaller than that produced by *Rhizoctonia solani* (45 kD) (Marcus *et al.* 1986), and approximately the same mass as those produced by *A. nidulans* and an *Erwinia* sp. (40 kD) (Dean and Timberlake 1989; Keen and Tamaki 1986). The purified PL had a K_m of 0.134 mg of polygalacturonic acid per milliliter and a V_{max} of 212 $U \cdot min^{-1} \cdot mg^{-1}$. These values are very much like those published for *F. solani* PL: a K_m of 0.130 mg/ml and a V_{max} of 190 $U \cdot min^{-1} \cdot mg^{-1}$ were obtained by Crawford and Kolattukudy (1987). PL of *C. gloeosporioides* has an alkaline isoelectric point (pI 7.9) and an activity optimum at pH 8.9; these values are different from those of *F. solani* (pI 8.3 and activity optimum at pH 9.4) (Crawford and Kolattukudy 1987) and *A. nidulans* (pI 4.2) (Dean and Timberlake 1989). The PLs of *F. solani* and *C. gloeosporioides* both show an absolute requirement for calcium and are completely inhibited by 5 mM EDTA. Adjusting the $CaCl_2$ concentration to 5 mM restored the lyase activity of *C. gloeosporioides*.

The PL of *C. gloeosporioides* macerated avocado wedges twice as efficiently as the endopolygalacturonase produced by the fungus (Prusky *et al.* 1989). Electroblotting from SDS gel to Immobilon transfer membranes (Millipore) for sequencing was performed in Bio-Rad transfer cells at a constant 60 V in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid buffer, pH 11, in 10% methanol at room temperature. The N-terminal sequence of the 40-kD protein blotted from an SDS gel (AAIXDATDVG), was similar to the sequence of the 14-kD peptide obtained after CNBr digestion (AAIXDATDVGY), and it was accordingly concluded that the 14-kD peptide was located at the N terminus. No similarity could be detected between the N terminus of *C. gloeosporioides* PL and that of *F. solani* PL (Gonzalez-Candelas and Kolattukudy 1992) or that of *Aspergillus* PL (Kusters–Van Someren *et al.* 1991).

Antibodies were prepared against the PL in balb mice, and they detected as little as 10 ng of PL at a titer of 1:10,000. Incubation of 25 μg of normal ascitic fluid with 5 U of PL re-

sulted in normal maceration of avocado wedges. However incubation of 25 μg of anti-PL with avocado wedges reduced maceration activity by 80%. Immunoprecipitation of *in vitro* translation products resulting from RNA recovered from *C. gloeosporioides* grown on pectin revealed the presence of a major band of 36 kD that cross-reacted with the PL antibodies (Fig. 4). This band was absent when RNA was isolated from the fungus grown in the presence of 10 mM glucose. The presence of a 36-kD precursor polypeptide, while the molecular mass of the purified product was 39 kD, suggests glycosilation of the PL by the fungus.

Previous investigators have suggested that fungal PLs may be important virulence factors. Crawford and Kolattukudy (1987) reported that antibodies raised against PL of *F. solani* inhibited infection of pea stems. Furthermore, hypovirulent isolates of *R. solani* showed very little PL activity, while virulent isolates showed significantly more (Marcus *et al.* 1986). Our results show that antibodies raised against the *C. gloeosporioides* PL inhibited activity on wedges of avocado mesocarp tissue both *in vitro* and *in vivo*. These results support the possible importance of the enzyme during *C. gloeosporioides* attack in avocado.

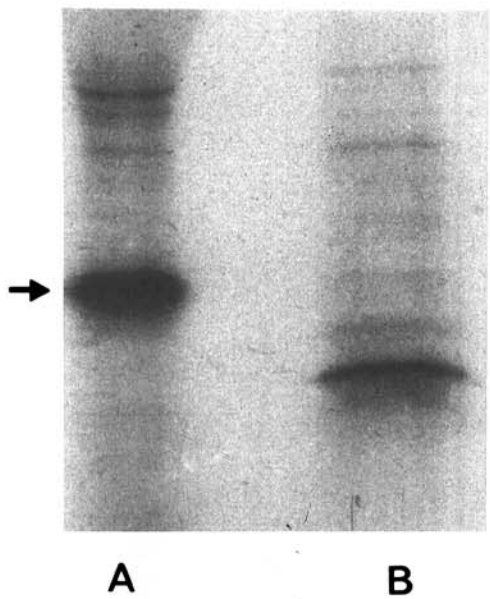

Fig. 4. Autoradiogram of the primary translation product of pectate lyase mRNA precipitated by antibodies following *in vitro* translation of total mRNA extracted from *Colletotrichum gloeosporioides* grown in the presence of pectin (lane A) and pectin + glucose (lane B). Total RNA was extracted from pectin-induced or 10 mM glucose-repressed mycelium of *C. gloeosporioides*, using the hot sodium dodecyl sulfate (SDS)–phenol method for plant RNA described by Ausubel *et al.* (1991). mRNA was isolated by the use of the PolyATtract mRNA isolation system III (Promega) and translated by the rabbit reticulocyte lysate system (Promega) with [^{35}S]methionine (New England). An aliquot of the translation products was also subjected to immunoprecipitation. The product was adsorbed to normal ascitic fluid and pelleted, and the supernatant was collected; antibodies were added to a final concentration of 2 mg/ml and adsorbed overnight at 4° C with continuous shaking; immunoglobulin G anti-mouse serum (Bio-Rad) was added, and the preparation was incubated for 2 hr at room temperature. The immunoprecipitated products were pelleted, resuspended in 25 μl of SDS loading buffer, electrophoresed on a 12% SDS-polyacrylamide gel, and analyzed by autofluorography with Enhance (New England Nuclear). The arrow in lane A indicates the precipitate at 34–36 kD.

PL inhibitors.

Natural protein inhibitors of PL were extracted from ripe and unripe avocado fruits. Acetone powders were prepared and extracted in 50 mM Tris-HCl, pH 8.5. Aliquots were dialyzed by Centricon 10 (Amicon Co.) and added to standard PL enzyme and maceration assay mixtures. Undialyzed extracts of acetone powder from avocado peel reduced PL activity by 14–33%, while dialyzed extracts had no effect on PL activity. The greatest inhibition was obtained with extracts from unripe immature fruits at the beginning of the harvest season, while less inhibition was observed in peel extract from unripe overmature fruits. Extracts of peels of ripe fruits reduced PL activity by only 5–8%. We could not detect significant protein inhibitors of PL in unripe avocado fruit extracts, which is consistent with the lack of literature reports from other plants. Dialyzed acetone extracts had no effect on PL activity, which suggests the presence of a low molecular weight inhibitor. Epicatechin has been shown to affect disease resistance by modulating levels of preformed antifungal compounds in avocado peel (Prusky *et al.* 1985), but its effect on PL was not observed. Epicatechin (Sigma) at concentrations of 5, 10, and 15 μg/ml reduced PL activity by 28, 65, and 95%, respectively, and double reciprocal plots showed a K_i of 3.4 μM for epicatechin. Epicatechin inhibited the maceration ability of the enzyme at concentrations higher than 5 μg/ml; the maximal inhibition was observed at 30 μg/ml (Fig. 5). Epicatechin also inhibited maceration symptoms on the flesh of whole avocado fruits. Maceration activity on the flesh of freshly harvested, peeled fruits was observed after spotting 10 μl of purified enzyme extract (200 μg/ml) and incubating at 25° C. Maceration was clearly visible on the flesh after 48 hr, but no symptoms occurred when epicatechin (350 μg/ml) was added. This concentration of epicatechin is near the lowest found in the peel of resistant unripe fruits (Prusky *et al.* 1988). It has been reported that the epicatechin concentration in the peel of unripe fruit ranges from 450 to 2,000 μg/g fresh weight and decreases to 12–15 μg/g fresh weight in ripe fruit expressing symptoms. Symptom expression occurs in ripe fruit only when epicatechin decreases to about 25 μg/g fresh weight (Prusky *et al.* 1985). Prusky and co-

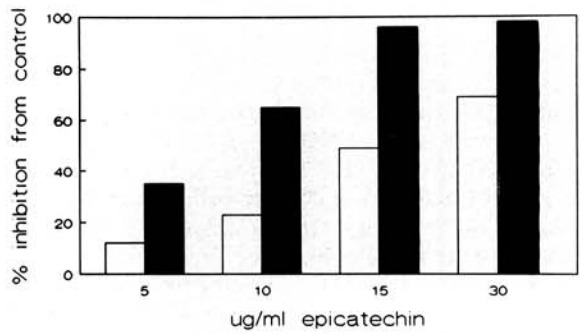

Fig. 5. Inhibition of pectate lyase (PL) activity (solid bars) and maceration activity (open bars) on avocado wedges by epicatechin. Different concentrations of epicatechin were added to the standard PL assay mixture. The PL reaction mixture in the absence of epicatechin was used as a control. Maceration activity was determined by incubating five wedges, approximately 0.2 × 0.4 cm and 0.5–0.6 cm thick, consisting of exo- and mesocarp of freshly harvested avocado fruit (cultivar Fuerte) in 2.5 ml of 25 mM Tris-HCl, pH 8.0, at 30° C with 10 U of enzyme. Maceration activity was determined by measuring the increase in the turbidity of the bathing solution at 590 nm.

workers (1988) concluded that cultivar resistance is related to the amount of epicatechin in the avocado peel and noted that fruit become susceptible to *C. gloeosporioides* during fruit ripening, when epicatechin levels decrease. Prusky *et al.* (1989) reported that epicatechin inhibits endopolygalacturonase produced by *C. gloeosporioides* (K_i = 0.29 mM) and suggested that it might affect the development of disease due to the fungus. In this study, epicatechin was found to be a more efficient inhibitor of the *C. gloeosporioides* PL, with a K_i of 3.4 μM (1.0 μg/ml). Epicatechin therefore is the first PL inhibitor found in plants. The susceptibility of other PLs to epicatechin has not been tested, but PLs from an *Erwinia* sp. were not inhibited at concentrations that affected the *C. gloeosporioides* enzyme (N. T. Keen and D. Prusky, unpublished data).

Since epicatechin inhibits the polygalacturonase (Prusky *et al.* 1989) and PL produced by *C. gloeosporioides*, its contribution to resistance may also involve these functions.

ACKNOWLEDGMENTS

We are grateful to David Kobiler for antibody preparations and to N. T. Keen for reviewing this manuscript.

LITERATURE CITED

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. 1991. Current Protocols in Molecular Biology. Wiley Interscience, New York.

Barash, I., and Khazzan, S. 1970. The relationship and properties of pectic glycosidases produced by host and pathogen during anthracnose disease of avocado. Phytochemistry 9:1189-1197.

Binyamini, N., and Schiffmann-Nadel, M. 1972. Latent infection in avocado fruit due to *Colletotrichum gloeosporioides*. Phytopathology 62: 592-594.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Collmer, A., Ried, J. L., and Mount, S. M. 1988. Assay methods for pectic enzymes. Methods Enzymol. 161:329-335.

Crawford, M. S., and Kolattukudy, P. E. 1987. Pectate lyase from *Fusarium solani* sp. *pisi:* Purification characterization, *in vitro* translation of the mRNA, and involvement in pathogenicity. Arch. Biochem. Biophys. 258(1):196-205.

Dean, R. A., and Timberlake, W. A. 1989. Regulation of the *Aspergillus nidulans* pectate gene (pel A). Plant Cell 1:275-284.

Gonzalez-Candelas, L., and Kolattukudy, P. E. 1992. Isolation and analysis of a novel inducible pectate lyase gene from the phytopathogenic fungus *Fusarium solani* f sp. *pisi* (*Nectria haematococca*, mating population VI). J. Bacteriol. 174:6343-6349.

Gysler, C., Harmsen, J. A. M., Kester, H. C. M., Visser, J., and Heim, J. 1990. Isolation and structure of pectin lyase D-encoding gene from *Aspergillus niger*. Gene 89:101-108.

Harmsen, J. A. M., Kusters–Van Someren, M. A., and Visser, J. 1990. Cloning and expression of a second *Aspergillus niger* pectin lyase gene (pelA): Indications of a pectin lyase gene family in *A. niger*. Curr. Genet. 18:161-166.

Keen, N. T., and Tamaki, S. 1986. Structure of two pectate lyase genes from *Erwinia chrysanthemi* EC16 and their high-level expression in *Escherichia coli*. J. Bacteriol. 168:595-606.

Kusters–Van Someren, M. A., Harmsen, A. M., Kester, H. C. M., and Visser, J. 1991. Structure of the *Aspergillus niger* pelA gene and its expression in *Aspergillus niger* and *Aspergillus nidulans*. Curr. Genet. 20:293-299.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680-685.

Marcus, L., Barash, I., Sneh, B., Koltin, Y., and Finkler, A. 1986. Purification and characterization of pectolytic enzymes produced by virulent and hypovirulent isolates of *Rhizoctonia solani* Kuhn. Physiol. Mol. Plant Pathol. 29:325-336.

Prusky, D., Ascarelli, A., and Jacoby, B. 1984. Lack of involvement of nutrients in the latency of *Colletotrichum gloeosporioides* in unripe avocado fruits. J. Phytopathol. 110:106-109.

Prusky, D., Gold, S., and Keen, N. T. 1989. Purification and characterization of an endopolygalacturonase produced by *Colletotrichum gloeosporioides*. Physiol. Mol. Plant Pathol. 35:121-133.

Prusky, D., Keen, N. T., Sims, J. J., and Midland, S. L. 1982. Possible involvement of an antifungal compound in the latency of *Colletotrichum gloeosporioides* on avocado fruits. Phytopathology 72:1578-1582.

Prusky, D., Kobiler, I., and Jacoby, B. 1988. Involvement of epicatechin in cultivar susceptibility of avocado fruits to *Colletotrichum gloeosporiodes* after harvest. J. Phytopathol. 123:140-146.

Prusky, D., Kobiler, I., Jacoby, B., Sims, J. J., and Midland, S. L. 1985. Effect of inhibitors of lipoxygenase activity and its possible relation to latency of *Colletotrichum gloeosporioides*. Physiol. Mol. Plant Pathol. 27:269-279.

Prusky, D., and Plumbley, R. A. 1992. Quiescent infections of *Colletotrichum* in tropical and subtropical fruits. Pages 289-307 in: *Colletotrichum:* Biology, Pathology and Control. J. A. Bailey and M. J. Jeger, eds. C.A.B. International, Wallingford, UK.

Swinburne, T. R. 1983. Quiescent infections in post-harvest diseases. Pages 1-21 in: Post-Harvest Pathology of Fruits and Vegetables. C. Dennis, ed. Academic Press, London.