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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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 Ki 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 Schifmann-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.

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-heneicos-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 niger (Dean and Timberlake 1989), A. niger (Gysler et al. 1990, Harmsen et al. 1990), and Fusarium solani (Gonzalez-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 galacturonic reaction products from sodium polypectate (Collmer *et al.* 1986).

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

<table>
<thead>
<tr>
<th></th>
<th>Total protein (mg)</th>
<th>Total activity (µmol/min)</th>
<th>Specific activity (µmol·min⁻¹·mg⁻¹)</th>
<th>Recovery (%)</th>
<th>Enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture filtrate</td>
<td>2.960.0</td>
<td>580.2</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenyl-Sepharose 1</td>
<td>16.4</td>
<td>283.2</td>
<td>17.3</td>
<td>49</td>
<td>104-fold</td>
</tr>
<tr>
<td>Phenyl-Sepharose 2</td>
<td>3.2</td>
<td>218.4</td>
<td>68.3</td>
<td>37</td>
<td>356-fold</td>
</tr>
<tr>
<td>Isoelectric focusing</td>
<td>0.6</td>
<td>148.8</td>
<td>248.0</td>
<td>26</td>
<td>1,256-fold</td>
</tr>
</tbody>
</table>

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 Kᵜₐ of 0.134 mg of polygalacturonic acid per milliliter and a Vₘₐₓ of 212 U·min⁻¹·mg⁻¹. These values are very much like those published for F. solani PL: a Kᵜₐ of 0.130 mg/ml and a Vₘₐₓ of 190 U·min⁻¹·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 CaCl₂ 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). Electrophoresis from SDS gels 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 bionted from an SDS gel (AAIAXDTVG), was similar to the sequence of the 14-kD peptide obtained after CNBr digestion (AAIAXDTVG), 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 kDa 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-kDa precursor polyepitide, while the molecular mass of the purified product was 39 kDa, suggests glycosylation 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.

**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 peels 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 Kᵦ 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-

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**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 [35S]methionine (New England). An aliquot of the translation products was also subjected to immunoprecipitation. The product was adsorbed to normal acetic acid fluid and pelleted, and the supernatant was collected; antibodies were added to a final concentration of 2 μg/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 autoradiography with Enhance (New England Nuclear). The arrow in lane A indicates the precipitate at 34–36 kDa.

**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 x 0.4 cm and 0.5–0.6 cm thick, consisting of exocarp 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 $3.4$ sM (1.0 sM/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.

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LITERATURE CITED


