Role of Pectolytic Enzymes and Galacturonic Acid in Citrus Fruit Decay Caused by *Penicillium digitatum*

Charles R. Barmore and G. Eldon Brown

Associate professor, University of Florida, Institute of Food and Agricultural Sciences and research scientist III, Florida Department of Citrus, respectively, Agricultural Research and Education Center, P.O. Box 1088, Lake Alfred, 33850
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ABSTRACT

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Decay of citrus fruit by *Penicillium digitatum* was associated with content or activity of galacturonic acid, pectinmethylesterase (PME), and exopolygalacturonase (exo-PG). The pectolytic enzymes endo-polygalacturonase or pectin transeliminase were not associated with the disease. The hydrolysis of pectic acid by exo-PG resulted in an accumulation of galacturonic acid up to 12 mg/g fr wt within the lesion. Treatment of thin citrus peel sections with galacturonic acid, PME, or exo-PG did not induce cell separation. However, reduction in tissue strength was apparent with serial treatments of galacturonic acid, PME, and exo-PG. Each of these three constituents caused specific cellular changes which collectively resulted in a weakening of the cell wall. A possible role for each of these constituents in decay of citrus fruit by *P. digitatum* is discussed.

Additional key words: green mold, maceration, pectin, soft rot.

The importance of pectolytic enzymes in fungal plant diseases is well documented (3,4,6). These enzymes cause tissue maceration by degrading the pectic substances of the middle lamella. Endopolygalacturonase (endo-PG) and pectin transeliminase (PTE), which randomly cleave the α-1,4-glycosidic bond, are considered to be the primary enzymes responsible for maceration; pectinmethylesterase (PME) and exopolygalacturonase (exo-PG) apparently contribute little to this process (5).

Green mold caused on citrus fruit by *Penicillium digitatum* Sacc. is categorized as a soft rot (9). However, positive identification of an endo-PG or PTE in *P. digitatum* infected citrus fruit has not been made. Bush and Codner (5) detected PTE in culture filtrates. Barash and Angel (2) identified an exo-PG in *P. digitatum*-infected lemon fruit, but did not attempt to explain its role in maceration. An accumulation of galacturonic acid as high as 2% in lesions on *P. digitatum*-infected citrus fruits was reported by Miyakawa (13). He attributed the degradation of citrus peel to the accumulation of this acid.

In the present study, we have attempted to elucidate the interaction of pectolytic enzymes and galacturonic acid as a possible explanation of soft rot of citrus fruit peel during pathogenesis by *P. digitatum*.

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MATERIALS AND METHODS

Fruit. Mature Valencia and Hamlin oranges (*Citrus sinensis* Osbeck) were washed and inoculated with *P. digitatum*. Punctures, 4 mm deep, were made with a scalpel into the peel and an aqueous suspension of spores was added to the injury. After incubating the fruit at near 100% relative humidity and 25 C for 3–4 days, the resulting lesion, usually $\sim$4 cm in diameter, was removed from the fruit. A ring of decayed and sound tissue $\sim$1 mm in width surrounding the lesion, was discarded. Three consecutive rings ("zones") of sound peel 3, 5, and 5 mm in width then were removed and each zone was analyzed separately. The four samples were denoted as zones I (lesion), II, III and IV, respectively.

Enzyme assay and extraction. Polygalacturonase (PG) activity was assayed with 1.5% sodium polypectate (NaPP) or citrus pectin as substrates by the viscosimetric method with a Brookfield viscometer (Stoughton, MA 02072); the production of reducing groups by the dinitrosalicylic acid procedure as modified by Miller (12); the production of unsaturated polygalacturonides by the periodate-thiobarbituric acid (TBA) procedure (1) and the production of alcohol-soluble pectin (ASP) (less than four galacturonic acid units). The latter analysis was made by precipitating the NaPP polymers with 95% ethanol (1:4), filtering, and determining the galacturonic acid content in the alcohol fraction by the carbazole test (16). The pH optimum of the enzyme was determined over a pH range of 4.5–9.0, by the above

procedures. All enzyme reactions were maintained at 37 C for 45 min.

Polygalacturonase was extracted from an acetone powder of the various samples with neutral 50 mM acetate buffer containing 0.1% Triton X-100. The homogenate was centrifuged and the supernatant fluid was collected. Polygalacturonase was precipitated with the addition of cold acetone (−10 C). The precipitate was recovered by centrifugation and dissolved in 50 mM acetate buffer, pH 4.2. Temperature during extraction was maintained at 1–2 C. This initial preparation was used to measure PG activity in the four zones of infected fruit and from uninfected fruit. One unit of activity was equivalent to the amount of enzyme required to liberate 1 μmole of anhydrous galacturonic acid (AGA) per min at 37 C and pH 4.5.

Additional purification of the initial enzyme preparation from tissue of Zone 1 (lesion) was made for the maceration and pectin degradation studies. The PG in the initial enzyme preparation was first separated on a Sephadex G-75 column (2.6 × 60 cm) equilibrated with 50 mM acetate buffer, pH 4.2, at 4 C. Polygalacturonase was eluted from the column with the equilibration buffer. The eluate was monitored continuously at 280 nm with a Model UA-6 ISCO (Instrumentation Specialties Company, Lincoln, NB 68505) detector and collected in 5-ml fractions. Each fraction was analyzed for PG activity by the production of reducing groups. The four to five fractions containing high PG activity were combined and further purified on CM-cellulose. This fraction also was used for the maceration study.

CM-cellulose (Whatman CM23, Na^+) was equilibrated with 50 mM sodium acetate buffer, pH 4.2 at 4 C. The PG fraction was placed on the column and washed with the equilibration buffer. The enzyme was eluted with a linear gradient (16 hr at 0.8 ml/min) of 50 mM acetate to 50 mM acetate plus 0.7 M NaCl, pH 4.2. The eluate was monitored continuously at 280 nm and collected in 5-ml fractions. Each fraction was analyzed for PG activity by production of reducing groups, and the four to five fractions containing the highest PG activity were collected. Protein content was determined by the procedure of Kalb and Bernlohr (10).

Pectinmethylesterase in the four zones of infected and uninfected fruit was extracted and its activity was determined by the procedure of Rouse and Atkins (16). A concentrated PME preparation, free of endo-PG and PTE, was made from healthy Hamlin orange juice sacs according to the procedure of Evans and McHale (8). This extract was used in the maceration study.

Galacturonic acid determination. Tissue (10 g) from each of the four zones of infected fruit and from uninfected fruit was extracted with 70% ethanol, decolorized with activated charcoal, and then evaporated to 5 ml under vacuum. A 1-ml sample was added to a Dowex 1 × 8 (200- to 400-mesh) acetate column (8 × 95 mm). The column was washed with distilled water and the galacturonic acid was eluted with 4 N acetic acid. The eluate was concentrated to 1 ml under vacuum and 12 μl were spotted on Whatman No. 1 paper with a galacturonic acid standard for comparison. The material was separated by ascending chromatography in an ethanol:acetic acid:water (5:2:3,v/v)system for 12 hr. After development, the side with the standard was removed, sprayed with aniline hydrogen phthalate solution and heated for color development (11). The zone of the tissue extract corresponding to the galacturonic acid standard was cut out and placed in 3 ml of water. The water extract was analyzed for galacturonic acid by the carbazole test.

The same chromatographic procedure was used to determine the end product of NaPP degradation by the PG extract at each purification step. The NaPP solution was spotted directly on the chromatogram.

Pectin analysis. Water-soluble pectin (WSP) content in tissue from each of the four zones and uninfected tissue was determined using the procedure of Rouse and Atkins (16). The WSP was dissolved in 50 mM acetate buffer, pH 4.2, and the pectin content was determined by the carbazole test.

Degradability of each WSP fraction by the purified PG preparation was determined. Purified PG extract, 0.1 ml, was added to 5 ml of each WSP solution and incubated for 30 min at 37 C. Pectin degradation was measured by adding 95% ethanol (1:4, v/v) to the pectin solution and determining the galacturonic acid content in the alcohol-soluble fraction by the carbazole test.

Histology. Fresh sections 24–36 μm in thickness, of injured citrus peel at the margin of a mold lesion or from healthy fruit, were prepared with a Model 1225 Hooker plant microtome (Lab-Line Instruments, Inc., Melrose Park, IL 60160) and stained for pectin methylation with hydroxylamine-ferric chloride (15).

Cellular changes caused by ramifying hyphae of *P. digitatum* were observed by fixing peel from the 1-mm ring of decayed and sound tissue surrounding the lesion. The tissue was fixed in 3% glutaraldehyde in 0.1 M phosphate buffer at pH 7.0. After it was rinsed in buffer, the tissue was postfixed with 2% osmium tetroxide in additional buffer, dehydrated with increasing concentration of acetone at 4 C, and infiltrated with Spurr's resin. Sections 1–2 μm in thickness were prepared with a Sorvall JB-4 microtome (Dupont Instruments, Rockville, MD 20852) equipped with glass knives. Sections were stained with an 0.05% aqueous solution of toluidine blue.

Tissue maceration. Sections (50 μm thick × 4 × 4 mm) of albedo from a healthy Hamlin orange were prepared with a Hooker plant microtome. Ten sections were placed in each vial containing 2 ml of either 0.3% galacturonic acid, PME (150 units per milliliter) in neutral 50 mM sodium acetate buffer, or exo-PG, in pH 4.2 sodium acetate buffer. Serial treatments with the same solutions also were made. Each solution was removed from the vial prior to the addition of the next solution. Maceration was measured by the method of Mount et al (14).

RESULTS

Enzyme purification and indentification. A 105-fold purification of the enzyme was achieved by separation of the initial extract on Sephadex G-75 and CM cellulose (Table 1). Polygalacturonase activity was contained in a single peak following separation on both columns. On the G-75 column, PG was eluted between 80 and 110 ml into the gradient and at approximately 0.18 M NaCl on the CM-cellulose column. The pH optimum of the enzyme at each purification step was 4.5. Minimal activity was observed above pH 7. The percent theoretical degradation of the NaPP solution at each purification step closely approximated the percent reduction in viscosity (Table 1). The ratio of reducing groups produced to that of the alcohol-soluble degradation product ranged from 0.94 to 1.2 which indicated that monogalacturonic acid was the primary end

TABLE 1. Purification of exopolygalacturonase from *Penicillium digitatum*-infected orange fruit

Preparation	Vol. (ml)	Viscosity[a] reduction (%)	Reducing groups (mg Eq/ml)	AGA[b] (mg/ml)	Protein (mg/ml)	Theoretical[c] degradation (%)	Specific[d] activity
Crude	10	51.7	6.2	5.7	10	47.7	.62
G-75 column	27	41.9	5.2	4.8	1.9	40.0	2.74
CMC column	21	22.1	2.3	2.5	0.035	17.6	65.71

[a]Reaction conditions: 37 C, 1.3% sodium polypectate (NaPP) in pH 4.2 acetate buffer, 45 min.
[b]Anhydrous galacturonic acid.
[c]Percent theoretical degradation = (milligram equivalent reducing groups) ÷ (NaPP content).
[d]Specific activity = milligram equivalent reducing groups per milligram of protein.

product. No indication of PTE activity based on the TBA test was found over a pH range 4.5–9.0. Chromatographic separation of the NaPP solution following incubation with PG extract exhibited a single spot, *Rf* 0.49, corresponding to galacturonic acid. These data indicate that polygalacturonase obtained from *P. digitatum*-infected fruit is an exo-form. Endopolygalacturonase was not detected in either the G-75 or CMC-purified extracts.

Exopolygalacturonase activity was greatest in the lesion (Table 2). Zones II and III, surrounding the lesion, contained an average of 0.07 and 0.02 units of PG activity, respectively. No measurable PG activity was found in peel from zone IV, or in peel of uninfected fruit.

Negligible PME activity was detected in the lesion (Table 2). Higher activity was found in peel from zone II, with comparable and highest activity observed in peel from zones III and IV and in peel from uninfected fruit.

Galacturonic acid content. Galacturonic acid was the only pectin degradation product present in infected tissue. The highest average concentration of galacturonic acid was 12mg/g fr wt found in zone I. An average of 5 mg/g fr wt galacturonic acid was found in the 3 mm of peel surrounding the lesion (zone II). Galacturonic acid was not detected in peel from zone III and IV or from peel of uninfected fruit (Table 2).

Water-soluble pectin. Water-soluble pectin was greater in peel from zone II, III, and IV than in peel from uninfected fruit (Table 3). Pectin in peel from zone I was degraded to galacturonic acid. The highest amount of WSP was found in peel fom zone II, and it also was degraded the most easily by the CMC-purified PG. Water-soluble pectin from peel of uninfected fruit was only slightly degradable.

Histology. Fresh sections taken from injured uninoculated peel showed slight demethylation in the disrupted cell walls. In infected tissue, extensive demethylation occurred in walls of cells invaded by the hyphae.

Cells in advance of invading hyphae of *P. digitatum* were plasmolyzed (Fig. 1A). Cells in close proximity of the hyphae contained walls that were extremely swollen but intact (Fig. 1A). Dissolution of the cell walls did not appear to occur until the walls were penetrated by the hyphae (Fig. 1B). Comparable swelling of cell walls and plasmolysis also was induced by treating peel with 0.5% galacturonic acid.

In vitro maceration. Tissue strength was reduced most effectively by serial treatments with galacturonic acid, PME, and exo-PG, (treatments 7 and 8 in Table 4). Both treatments did cause some cell separation but this response was not consistent. Almost complete demethylation occurred in tissue treated with PME or exo-PG. The nontreated sections showed no demethylation whereas the galacturonic acid treated sections showed slight demethylation.

TABLE 2. Exopolygalacturonase (exo-PG) and pectinmethylesterase (PME) activity and galacturonic acid content in each of four zones of *Penicillium digitatum*-infected and in uninfected Valencia orange fruit peel

Zones[a]	exo-PG[b] units	PME[c]	Galacturonic acid (mg/g fr wt)
Zones:			
I (Lesion)	0.11	<5.0	12
II	0.07	52.1	5
III	0.02	63.3	0
IV	0.00	64.7	0
Uninfected fruit	0.00	70.4	0

[a]Lesion zones based on a "standard" lesion (usually ~4.0 cm in diameter) formed by inoculating a 4-mm-deep puncture wound with an aqueous spore suspension and incubating the fruit 3–4 days at 25 C and nearly 100% RH. The zones measured from the point of inoculation were: zone 1, 0–2 cm; zone 2 = 2.1–2.4 cm; zone 3, 2.4–2.9 cm; and zone 4, 2.9–3.4 cm.
[b]One unit of activity is the amount of exo-PG required to liberate 1 mole of anyhydrous galacturonic acid per minute at 37 C.
[c]Microequivalents of ester hydrolyzed per minute per gram of wet tissue.

TABLE 3. Water-soluble pectin in each of four zones of *Penicillium digitatum*-infected and uninfected Valencia orange peel and degradability of pectin of each zone by exopolygalacturonase

Zone[a]	Water-soluble pectin (mg/g peel)	Degradability[b] (mg AGA/ml)
Na pectate[c]	...	5.432
Zones:		
I (Lesion)	0.00	...
II	7.28	0.388
III	5.82	0.233
IV	4.61	0.194
Uninfected fruit	4.12	0.078

[a]Zones as in Table 2.
[b]Milligrams of anhydrous galacturonic acid liberated in 30 min at 37 C.
[c]Used to indicate base level of enzyme activity.

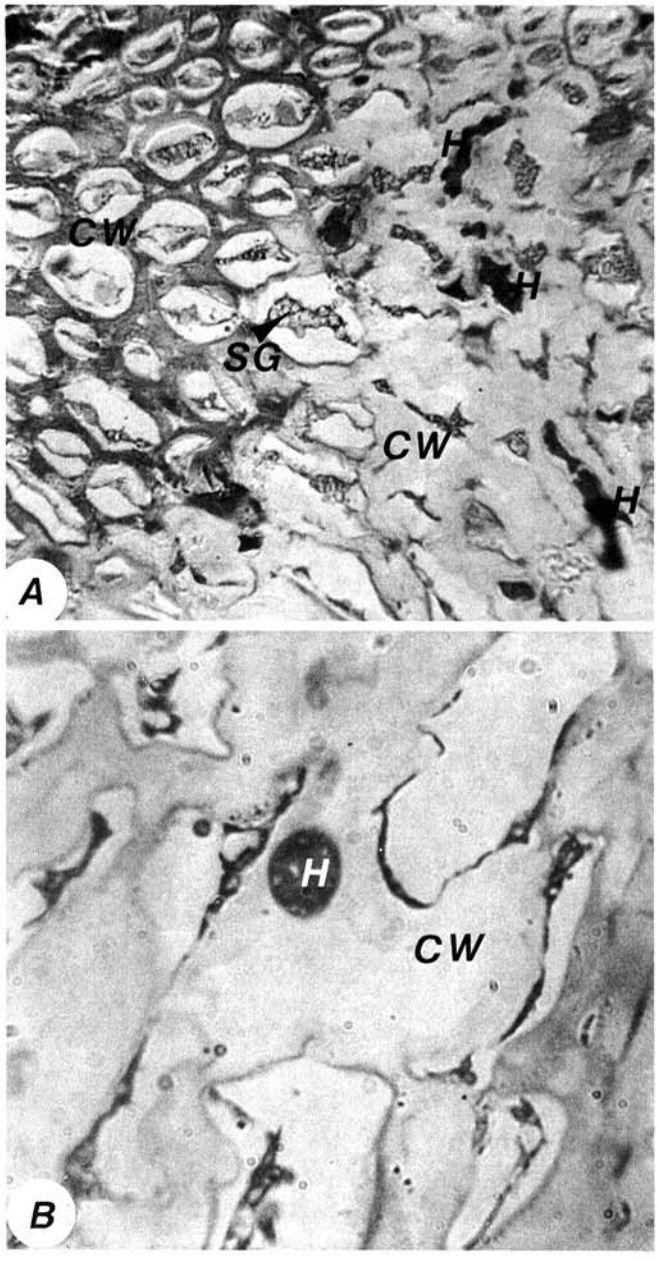

Fig. 1. Flavedo of citrus peel infected by *Penicillium digitatum*. **A**, Tissue taken from lesion margin exhibiting plasmolyzed cells in advance of hyphae and plasmolyzed cells with swollen cell walls near and associated with invading hyphae (×500). **B**, Dissolution of swollen cell wall penetrated by a hypha of *Penicillium digitatum* (×1,125). Legend: CW = cell wall, H = hypha, SG = starch grains.

DISCUSSION

The only pectolytic enzymes we could associate with the maceration of citrus fruit infected with *P. digitatum* were exo-PG and PME. Exopolygalacturonase is produced by the fungus and PME is a natural constituent of the fruit. These two enzymes are not considered important in tissue maceration. However, these two enzymes can alter the pectin components of the middle lamella, via demethylation and terminal degradation of the pectic material.

The development of maceration in *P. digitatum* infected citrus fruit can be divided into three phases: collapse of the cytoplasm, swelling of the cell walls, and cell separation. The collapse of the cytoplasm and cell wall swelling occur in advance of hyphal penetration, whereas, cell separation is associated with hyphal penetration into the cell wall. Cell walls in these areas also are characterized by extensive pectin demethylation. Collapse of the cytoplasm and cell wall swelling may be attributed to the accumulation of galacturonic acid formed by the action of exo-PG. An average of 5 mg/g fr wt galacturonic acid was found in tissue adjacent to the lesion and 12 mg/g fr wt in the lesion, concentrations which are more than sufficient to cause this damage. As reported by Miyakawa (13), galacturonic acid did not cause maceration.

The demethylation response is attributed to a release of endogenous PME from the damaged cell. The demethylation reaction is very important in the generation of pectic acid, the substrate for exo-PG. Pectic acid is insoluble unless NA^+ and K^+ pectates are formed. The increase in WSP and its degradability in zones II and III is attributed to this combined effect. Water-soluble pectin from healthy tissue is highly methylated and is only slightly degraded by exo-PG.

Swelling of the cell walls and increased solubilization of pectin are probably important in causing an initial weakening of the cell wall. Similar changes have been shown by Doesburg (7) to be important in the softening of apple tissue during ripening. The final step in the maceration process may involve a continual degradation of pectin by exo-PG and a physical disruption of the cell wall by the hyphae. This is based on the observations that cell separation always was in close proximity to the hyphae and that exo-PG also was very active in this zone.

It was not possible to show by in vitro tests the importance of PME in the proposed maceration process since substantial demethylation of pectin was observed in all exo.-PG treatments. The demethylation is attributed to endogenous PME being released by the cell as a result of the treatment. Pectinmethylesterase would be expected to be an important component since demethylation of pectin is a prerequisite for exo-PG activity.

TABLE 4. Effect of pectinmethylesterase (PME), exopolygalacturonase (exo-PG), and galacturonic acid (GA) on maceration of Hamlin orange peel albedo tissue sections

No.	Treatment[a,b] Description	Maceration[c,d]
1	Control (21 hr)	0.6
2	PME (21 hr)	2.0
3	GA (21 hr)	1.6
4	GA (6 hr)⇒PME (15 hr)	1.6
5	Exo-PG (21 hr)	2.5
6	PME (6 hr)⇒exo-PG (15 hr)	2.2
7	GA (6 hr)⇒exo-PG (15 hr)	4.7
8	GA (6 hr)⇒PME (6 hr)⇒exo-PG (9 hr)	3.3

[a]Treatment time in parentheses.
[b]Arrow indicates transfer to next treatment.
[c]Tissue strength rating: 0 = most resistant, and 5 = least resistant to separation with dissecting needles.
[d]Average of three tests each replicated three times.

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