Polygalacturonase from Citrus Fruit Infected with *Penicillium italicum*

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ABSTRACT

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An endo-polygalacturonase (E.C. 3.2.1.15) (endo-PG) was isolated and purified from grapefruit (*Citrus paradisi*) peel infected with *Penicillium italicum*. The estimated molecular weight of the endo-PG was 36,000 daltons and it had optimum activity at pH 5.0. Citrus pectin and sodium polypectate (NaPP) were degraded by the enzyme, but NaPP was the most readily degraded. The purified enzyme macerated citrus mesocarp in vitro. The microequivalents per gram fresh weight of diffusible free organic acid was 70.0 for decayed peel and 6.7 for healthy peel. Galacturonic acid in the decayed peel averaged 8.6 mg/g fresh weight. Plasmolysis, cell wall swelling, and degradation of the wall at the site of hyphal penetration were associated with invading hyphae. Dissolution of the middle lamella by the endo-PG is a major factor contributing to hyphal penetration.

Additional key words: pectolytic enzymes, blue mold, tissue maceration, postharvest decay, soft rot.

Pectolytic enzymes, principally endo-polygalacturonase (endo-PG) and pectin trans-eliminase (PTE) are frequently produced by various fungi and bacteria and are associated with the maceration of host tissue during pathogenesis (5,7). However, *Penicillium digitatum* Sacc., the cause of green mold of citrus, produced an exo-polygalacturonase (exo-PG) (3,4) that, in conjunction with endogenous pectinmethylesterase (PME) and galacturonic acid, causes tissue maceration. Neither PME or galacturonic acid alone cause maceration (4).

Penicillum italicum Wehmer and *P. digitatum* produce similar symptoms on citrus fruit except that *P. italicum* develops more slowly and spreads more frequently from infected to healthy fruit by contact in packed containers (9).

The objective of this investigation was to identify and characterize the pectolytic enzyme system involved in the maceration of citrus tissue infected with *P. italicum* as part of an overall study of the infection processes of these two organisms.

MATERIALS AND METHODS

Plant materials and inoculations. Mature Marsh grapefruit (*Citrus paradisi* Macf.) and Pineapple orange (*C. sinensis* Osbeck) were washed and inoculated through an injury with *P. italicum* spores dispersed in 15 ml of distilled water containing one drop of Triton X-100. The fruit were held under 92–96% relative humidity at 25 C for 6–8 days. The decayed peel was then removed and used immediately or frozen for later analysis.

Enzyme assay. Pectolytic enzyme activities were assayed by the increase in reducing groups (17), loss in viscosity (4), and by the periodate-thiobarbituric acid procedure (1). Substrates were sodium polypectate (NaPP) and citrus pectin N.F. at 7.5 and 10 mg/ml, respectively, in 50 mM sodium citrate or Tris-HCl buffer, depending on pH of the reaction mixture. The percentage methoxyl content of NaPP and citrus pectin, as determined by the procedure of Hinton (11), were 0 and 11.1, respectively. Cellulase (E.C. 3.2.1.4) activity was assayed on 0.3% carboxymethyl cellulose gum dissolved in 50 mM sodium citrate buffer at pH 6.0. All reactions were conducted at 37 C.

Alcohol-soluble pectin in the NaPP + enzyme reaction mixture was determined by a previously reported procedure (4).

Enzyme purification. An acetone powder was prepared from the decayed peel by grinding it three times in cold acetone and filtering. Residual acetone in the powder was removed with vacuum. The acetone powder was extracted with 50 mM sodium acetate buffer, pH 5.0, containing 0.01% Triton X-100. The homogenate was centrifuged (20 min at 23,000 *g*) and the supernatant was collected. Solid ammonium sulfate was added to the stirred cold (2 C) supernatant and the fraction that precipitated between 60 and 80% saturation was collected by centrifugation. The pellet was dissolved in sodium acetate buffer, and added to a Sephadex G-100 column (2.5 × 85 cm) equilibrated with 50 mM sodium acetate buffer, pH 5.0. The enzyme was eluted with the equilibration buffer at 30

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ml/hr and 6-ml fractions were collected. The fractions with polygalacturonase (PG) activity were combined and fractionated further by CM-cellulose anion exchange chromatography.

CM-cellulose (Whatman CM 23, Na^+) was equilibrated with 50 mM sodium acetate buffer, pH 5, at 4 C. The PG fraction was placed on the column (1.6 × 30 cm) and washed with the equilibration buffer. The enzyme was eluted with a linear gradient (16 hr at 30 ml/hr) of 50 mM acetate to 50 mM acetate plus 0.5 M NaCl, pH 5.0. The eluate was collected in 6-ml fractions.

Molecular weight determination. The molecular weight of PG was estimated using gel filtration with Sephadex G-100. Molecular weight standards (14,000–66,000) were obtained from Sigma (St. Louis, MO 63178).

Organic acid analysis. The organic acid content in healthy and decayed Pineapple orange peel was determined by titration by the procedure of Sinclair and Eny (21). Peel from healthy and decayed fruit was homogenized in glass-distilled water, the homogenate was centrifuged, and the supernatant was used to determine the total content of free organic acids and their salts. Diffusible organic acid content was determined by cutting 7 × 3 × 3 mm peel sections, rinsing in water for 0.5 min to remove cell contents from the cut surface, and allowing the organic acid to diffuse into glass-distilled water during continuous shaking at low speed for 1 hr. The diffusate was filtered through Whatman # 1 paper. Each extract was first titrated to pH 2.6 with 0.1 N HNO_3 to measure the content of organic acids present as salts and then back-titrated to pH 7.8 with 0.1 N NaOH to measure the free organic acid content. The organic acid content was expressed as microequivalents per gram of fresh weight of tissue. Galacturonic acid in the decayed peel was extracted and quantitated as described previously (4).

Protein determinations. Protein content was determined according to the Bio-Rad (8) and Kalb and Bernlohr (13) procedures. Crystalline bovine serum albumin was used as a standard.

Tissue maceration. Pieces of mesocarp, 7 × 3 × 2 mm, were cut from mature Marsh grapefruit and incubated in 2 ml of the PG extract (G-100 preparation) in 50 mM acetate buffer pH 5.0. Maceration was estimated by the loss in coherence of the tissue sections as described by Mount et al (18) on a scale of 0 = no maceration to 5 = complete maceration. The increase in soluble pectin during the maceration of the mesocarp sections was determined. The reaction solution was centrifuged, soluble pectin in the supernatant was precipitated with ethanol (1:4), centrifuged, and the precipitated pectin was dissolved in water and quantified with the carbazole procedure (20).

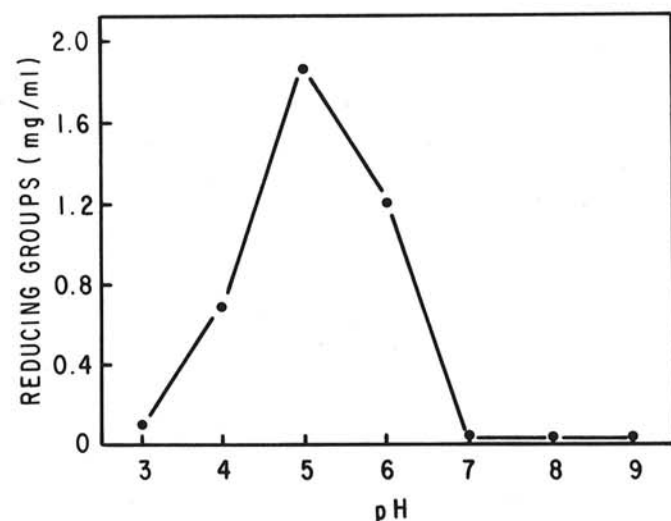

Fig. 1. Effect of pH on the degradation of sodium polypectate by endo-polygalacturonase extracted from *Penicillium italicum*-infected citrus fruit peel. Activity is expressed as milligram equivalents of reducing groups liberated after 45 min at 37 C. Buffer systems for pH 3–6 and 7–9 were sodium citrate and Tris-HCl, respectively.

Histological studies. Fresh sections of tissue immediately adjacent to the water-soaked area were prepared with a Hooker Model 1225 plant microtome (Lab-Line Instruments, Inc., Melrose Park, IL 60160). These sections were examined for the presence of hyphae and evidence of pectin demethylation (19). Cellular changes caused by ramifying hyphae of *P. italicum* were observed by fixing peel from a 1-mm strip taken across the lesion margin and containing decayed and sound tissue as described previously (4).

RESULTS

Screening of the initial sodium acetate extract for cellulase, PG and PTE activity revealed only the presence of PG. The enzyme was purified by ammonium sulfate fractionation, Sephadex G-100 gel filtration, and by ion-exchange chromatography using CM-cellulose (Table 1). The molecular weight of the enzyme was estimated at 36,000 daltons, with optimum activity at pH 5.0 (Fig. 1). Methylated pectin and nonmethylated NaPP were degraded by the enzyme, but NaPP was the most readily degraded substrate. The time required for 50% decrease in viscosity of pectin and NaPP by the Sephadex-PG fraction was 75 and 21 min, respectively. The liberation of free aldehyde groups was considerably lower in comparison to the decrease in viscosity for both substrates. In addition, the alcohol soluble pectic substances in the reaction mixture after 5 hr were negligible.

The content of free organic acids and their salts from homogenized and diffusible preparations of healthy and decayed peel is presented in Table 2. In healthy peel, only 8.2% of the total organic acid content of the homogenized preparation existed as free organic acids; whereas, in decayed peel it comprised 39.9% of the total organic acid content. The organic acids in decayed tissue readily diffused into water. After 1 hr, approximately 78% of the total acid content diffused from the decayed tissue as compared to 22% for healthy tissue. The pH of the diffusible and homogenized preparations of decayed peel was also considerably lower than that from preparations of healthy peel. The galacturonic acid content in the decayed peel averaged 8.6 mg per g of fresh tissue and was absent in healthy tissue.

The coherence of the mesocarp of citrus peel was markedly reduced in vitro by PG activity and the soluble pectin content was increased during a 5 hr incubation period in the PG extract (Table 3). Sloughing of cells from the mesocarp sections and complete loss

TABLE 1. Purification of endo-polygalacturonase from citrus fruit infected with *Penicillium italicum*

Purification step	Vol. (ml)	Protein (μg/ml)	Specific activity[a]
Acetate buffer	157	205	12.9
60–80% $(NH_4)_2SO_4$ sat.	4	3,800	15.4
Sephadex G-100	43	25	48.0
CM-cellulose	24	2.9	218.3

[a] Specific activity = microgram equivalent of reducing groups liberated from sodium polypectate per μg protein per min at pH 5.0 and 37 C.

TABLE 2. Content of total and diffusible free organic acids and their salts in healthy and *Penicillium italicum*-decayed citrus peel

		Microequivalents/g tissue		
Fraction	Solution pH	Free acid	Salt of the acids	Sum
Total[a]				
healthy	5.7	10.0	112.2	122.2
decayed	3.8	75.8	113.8	189.6
Diffusible[b]				
healthy	5.5	6.7	18.7	25.4
decayed	3.9	70.0	78.3	148.3

[a] Fraction obtained by homogenization of tissue in glass-distilled water.
[b] Fraction obtained by diffusion from tissue sections for 1 hr in glass-distilled water.

of coherence occurred by 5 hr.

Sections of fresh tissue taken at the water-soaked edge of the lesion margin and treated with hydroxylamine-ferric chloride showed a distinct progression of demethylation associated with hyphal penetration (Fig. 2A). Cells surrounding the hyphae were also plasmolyzed and swollen. Cell wall dissolution occurred when the cell walls were penetrated by the hyphae (Fig. 2B).

DISCUSSION

The purified PG extracted from *P. italicum*-infected citrus fruit caused a random hydrolysis of both NaPP and citrus pectin indicative of an endo-PG. The pH optimum, molecular weight, and maceration activity of the enzyme are similar to those of an endo-PG produced by *Aspergillus* (12).

TABLE 3. Maceration of citrus mesocarp by endo-polygalacturonase from citrus fruit infected with *Penicillium italicum*[a]

Treatment time (hr)	Maceration index[b]	Pectin solubilization[c] (mg)
1	1	0.47
3	3	0.88
5	5	1.02

[a] Enzyme specific activity: 35.0; treatment conditions: sodium acetate buffer, pH 5.0 at 37 C.
[b] 0 = no maceration to 5 = complete maceration.
[c] Soluble pectin content resulting from the action of endo-polygalacturonase.

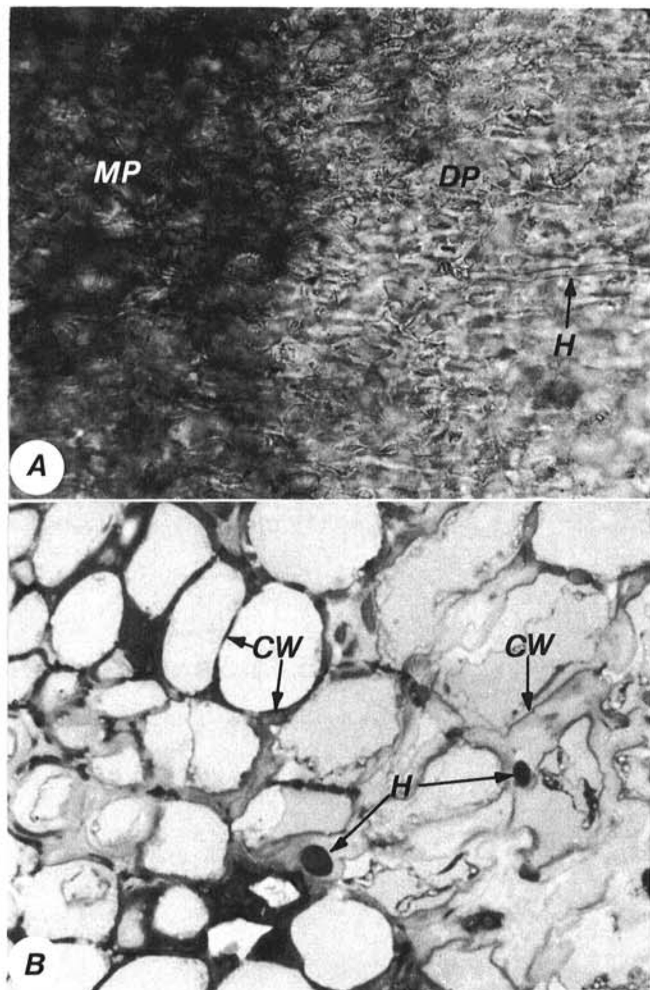

Fig. 2. Cross section of the epicarp of citrus peel infected by *Penicillium italicum*. **A,** Tissue from the lesion margin treated with hydroxylamine-ferric chloride to show demethylation of pectin. Light cell walls indicate demethylated pectin and dark cell walls indicate methylated pectin (×320). **B,** Tissue from lesion margin exhibiting plasmolyzed cells and swollen cell walls (×500). Legend: CW = cell wall, H = hypha, MP = methylated pectin, and DP = demethylated pectin.

The endo-PG produced by *P. italicum* contrasts with the exo-PG produced by *P. digitatum* (4), a similar pathogen of citrus fruit. However, the type of PG produced does not result in obvious anatomical differences during pathogenesis of the two organisms. Histological examination of tissue infected by *P. italicum* showed extensive pectin demethylation, cell wall swelling, and plasmolysis of cells in close proximity to the hyphae. Cell wall dissolution did not occur until the wall had been penetrated by the hyphae as was observed previously in tissue infected by *P. digitatum* (4). Cell plasmolysis at the lesion front could be explained by the release of osmotically active solutes in the apoplast (eg, mineral salts and other solutes from dead cells and galacturonic acid formed by the action of the endo-PG). With additional accumulation of solutes, an increase in H^+ concentration, and further degradation of the cell wall, plasmolysis would become irreversible and cell death would follow.

The overall effect of galacturonic acid accumulation and its diffusion into healthy tissue could contribute to pathogenesis like that proposed for oxalic acid produced during pathogenesis by *Sclerotium* (6) and *Cristulariella* (15). The increased electrolyte leakage from the damaged cells provides nutrients for growth of the pathogen and the low pH should favor the activity of the endo-PG (16). In addition, the release of PME from the damaged cells of the citrus rind would account for the observed progression of pectin demethylation at the lesion front. This should favor a greater hydrolysis of pectin. Green (10) also stressed the importance of organic acids in the infection of citrus fruit exocarp by both *P. digitatum* and *P. italicum*. Citric acid caused swelling and softening of the compact cells and increased the amount of soluble pectin. In our studies, swelling of cell walls was noted during the initial tissue degradation caused by both fungi. Galacturonic acid was also capable of inducing swelling (4).

The maceration of mesocarp tissue in vitro by endo-PG was not dependent upon prior treatment of the tissue with galacturonic acid or PME as was the maceration process involving exo-PG produced by *P. digitatum* (4). The random hydrolysis of pectin by endo-PG could possibly cause substantial pectin degradation without extensive demethylation.

The production of PG by *P. italicum* and *P. digitatum* in the decay process should aid hyphal penetration of the cell walls as has been suggested by Ayers et al (2) for *Aphanomyces euteiches*. Infection by *P. italicum* and *P. digitatum* requires an injury into the spongy parenchymatous mesocarp cells. Anatomical studies of citrus peel infected by these two fungi have shown that hyphae penetrate the spongy mesocarp cells much more rapidly than the more compact cells of the exocarp. Once either of these fungi begin to degrade the mesocarp, penetration of the hyphae into the exocarp is not impaired (14). Additionally, the type of PG produced by the two *Penicillia* apparently does not determine the rate of decay development. *Penicillium italicum* produces a much more effective macerating enzyme than does *P. digitatum*, but lesions produced by *P. italicum* spread considerably more slowly (9).

LITERATURE CITED

1. Ayers, W. A., Papavizas, G. C., and Diem, A. F. 1966. Polygalacturonase trans-eliminase and polygalacturonase production by *Rhizoctonia solani*. Phytopathology 56:1006-1011.
2. Ayers, W. A., Papavizas, G. C., and Lumsden, R. D. 1969. Purification and properties of the endopolygalacturonase of *Amphanomyces euteiches*. Phytopathology 59:925-930.
3. Barash, I., and Angel, E. 1970. Isolation and properties of an exopoly-galacturonase produced by *Pencillium digitatum* during infection of lemon fruits. Israel J. Bot. 19:599-608.
4. Barmore, C. R., and Brown, G. E. 1979. Role of pectolytic enzymes and galacturonic acid in citrus fruit decay caused by *Penicillium digitatum*. Phytopathology 69:675-678.
5. Bateman, D. F. 1976. Plant cell wall hydrolysis by pathogens. Pages 79-99 in: J. Friend and D. R. Threlfall, eds. Biochemical Aspects of Plant-Parasite Relationships. Annu. Proc. Phytochem. Soc. Academic Press. New York. 354 pp.

6. Bateman, D. F., and Beer, S. V. 1965. Simultaneous production and synergistic action of oxalic acid and polygalacturonase during pathogenesis by *Sclerotium rolfsii*. Phytopathology 55:204-211.
7. Bateman, D. F., and Millar, R. L. 1966. Pectic enzymes in tissue degradation. Annu. Rev. Phytopathol. 4:119-146.
8. Bio-Rad Laboratories. 1977. Bio-Rad protein assay. Tech. Bull. 1051. 4 pp.
9. Fawcett, H. S. 1927. Relation of temperature to growth of *Penicillium italicum* and *P. digitatum* and to citrus fruit decay produced by these fungi. J. Agric. Res. 35:925-931.
10. Green, F. M. 1932. The infection of oranges by *Penicillium*. J. Pomol. Hortic. Sci. 10:184-215.
11. Hinton, G. L. 1939. Fruit Pectins: Their Chemical Behavior and Jellying Properties. Department of Scientific and Industrial Research. Food Investigation. Special Report 48. His Majesty's Stationery Office, London. 96 pp.
12. Ishii, S., and Yokotsuka, T. 1972. Purification and properties of endopolygalacturonase from *Aspergillus japonicus*. Agric. Biol. Chem. 36:1885-1893.
13. Kalb, V. F., Jr., and Bernlohr, R. W. 1977. A new spectrometric assay for protein in cell extracts. Anal. Biochem. 82:362-371.
14. Klotz, L. J. 1930. Some microscopical studies on *Penicillium* decay of citrus. Phytopathology 20:251-256.
15. Kurian, P., and Stelzig, D. A. 1979. The synergistic role of oxalic acid and endopolygalacturonase in bean leaves infected with *Cristulariella pyramialis*. Phytopathology 69:1301-1304.
16. Marloth, R. H. 1931. The influence of hydrogen-ion concentration and sodium bicarbonate and related substances on *Penicillium italicum* and *P. digitatum*. Phytopathology 21:169-198.
17. Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal. Chem. 31:426-428.
18. Mount, M. S., Bateman, D. F., and Basham, H. G. 1970. Induction of electrolyte loss, tissue maceration, and cellular death of potato tissue by an endopolygalacturonase trans-eliminase. Phytopathology 60:924-931.
19. Reeves, R. M. 1959. A specific hydroxylamine-ferric chloride reaction for histochemical localization of pectin. Stain Technol. 34:201-211.
20. Rouse, A. H., and Atkins, C. D. 1955. Pectinesterase and pectin in commercial citrus juices as determined by methods at the Citrus Experiment Station. Fla. Agric. Exp. Sta. Bull. 570. 19 pp.
21. Sinclair, W. B., and Eny, D. M. 1947. Ether-soluble organic acids and buffer properties of citrus peel. Bot. Gaz. 108:398-407.