Polygalacturonases Associated with Infection of Valencia Orange by *Penicillium italicum*

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ABSTRACT

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Three forms of polygalacturonase (PG), designated PG-I, PG-II, and PG-III, were purified to homogeneity from culture filtrate of *Penicillium italicum*. PG-I was characterized as an exoenzyme, whereas PG-II and PG-III were characterized as endoenzymes. All three enzymes were present in orange peel infected by *P. italicum*. Exo-PG-I was the predominant enzyme in infected tissue and constituted 69% of the total PG activity as compared with 4% in the culture filtrate. Endo-PG-II and endo-PG-III constituted only 24 and 7%, respectively, of the activity in the infected tissues, as compared with 71 and 25% in culture filtrate. The demonstration of high exo-PG activity in infected tissue suggests that it may be responsible for the excessive accumulation of D-galacturonic acid which occurs during blue mold infection.

Among postharvest diseases of citrus fruits, the blue and green molds, incited by *Penicillium italicum* Wehmer and *P. digitatum* Sacc., respectively, are categorized as soft rots (7). The modes of citrus fruit decay caused by the two molds appear to be quite similar. Histopathological studies of citrus peel tissue infected by *P. italicum* (5) or *P. digitatum* (4) 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. Barmore and Brown (4,5) attributed cell plasmolysis at the lesion front to the release of osmotically active solutes and increase in H^+ concentration in the apoplast. The initial stages of symptom development are linked to a high accumulation of D-galacturonic acid (4,5,12), which reached an average of 8.6 and 12 mg/g fresh weight in tissue infected with *P. italicum* and *P. digitatum*, respectively. Calacturonic acid accumulation and its diffusion into healthy tissue is considered an essential factor in pathogenicity of the two molds (4,5,12). This compound is capable of inducing cell wall swelling (4) and contributes to plasmolysis and other observed symptoms (5,12).

An exopolygalacturonase (exo-PG) was demonstrated to be the sole enzyme cleaving pectic chains in decayed peels of lemon (2) or orange (4) infected with *P. digitatum*. This result is closely correlated with the high accumulation of galacturonic acid in tissue infected by the green mold pathogen (4,12). In spite of the similarity in the mechanism of decay formation by the green and blue mold, only an endopolygalacturonase (endo-PG) could be detected in grapefruit peel infected by *P. italicum* (5). This finding led Barmore and Brown (5) to conclude that the type of PG produced does not cause any obvious histopathological differences during pathogenesis of the two organisms. It appeared to us that the excessive accumulation of D-galacturonic acid may not be totally explainable by the sole presence of endo-PG. The present study therefore was undertaken to examine the possibility that an exo-PG is produced during blue mold infection.

MATERIALS AND METHODS

Cultures and inoculations. A strain of *P. italicum* (I-115) was isolated from infected orange and maintained on potato-dextrose agar. For enzyme purification, *P. italicum* was cultured in a 10-L fermenter (New Brunswick Scientific, New Brunswick, NJ) on a minimal medium described by Spalding et al (13). The fungus was grown initially for 2 wk at 26 C in Roux flasks containing the same medium; 200 ml of mycelial and spore suspension was used for inoculating the fermenter.

The culture in the fermenter was incubated at 29 C with rotation at 200 rpm. After 7 days, the mycelia were removed by filtration through eight layers of cheesecloth followed by centrifugation at 10,000 *g* for 20 min. The culture fluid was used as a crude enzyme preparation. The procedure to obtain crude enzyme preparation from infected Valencia oranges (*Citrus sinensis* (L.) Osbeck) was essentially as described previously (2,3).

Enzyme purification. Eight liters of the culture fluid of *P. italicum* was filtered serially through a series of Metricell membranes (Gelman Sciences, Ann Arbor, MI) with a pore size of 5, 1.2, 0.45, and 0.22 μm. The filtrate was transferred to an ultrafiltration cell (Amicon Co., Danvers, MA) equipped with a YM-100 membrane, which retains solutes with molecular weights 10^5. The filtrate then was passed through a YM-50 membrane, which retains solutes with molecular weights 5×10^4. The pectic enzymes that remained in the filtrate fluid were concentrated to 200 ml by ultrafiltration through YM-30 membranes (excludes molecular weights $> 3 \times 10^4$). The concentrated enzymes within the ultrafiltration cell were washed twice with 200 ml of 100 mM sodium acetate buffer, pH 4.2, and dialyzed against 10 L of the same buffer for 24 hr. All of these operations were carried out at 4 C. The dialyzed enzymes were further purified by preparative isoelectric focusing. The pectic enzymes in a homogenate of orange peels infected with *P. italicum* were purified by the protocol described for culture fluid.

Enzyme assays. PG activity was determined by following the increase in reducing groups with dinitrosalicylic reagent, with sodium polypectate as a substrate (3). One unit of PG was defined as the amount of enzyme that liberates 1 μmol of reducing groups per minute at 30 C with D-galacturonic acid as a standard. Viscometric measurements for PG and detection of pectic lyase activity were as described previously (10).

Electrophoretic and analytical procedures. Isoelectric focusing was performed with an electrofocusing column (110 ml, LKB-8100, Ampholine, Sweden) which was prepared according to the manufacturer's instructions. Samples were electrophoresed at 4 C for 48 hr at a constant power of 4 W until the current had stabilized at 3 mA. Fractions (2 ml) were collected at the rate of 1 ml min^{-1}, and the pH was recorded immediately. Enzyme activity was measured after dialysis under the conditions described earlier. Molecular weight was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (3,10) and

TABLE 1. Purification of polygalacturonase (PG)-I, PG-II, and PG-III from a culture filtrate of *Penicillium italicum*

Purification step[a]	Volume (ml)	Activity (units·ml^{-1})	Protein (mg·ml^{-1})	Specific activity (units·mg^{-1})	Purification factor
Culture fluid	8,000	3.2	0.4	8	1
Ultrafiltration	200	73.5	0.94	78.2	9.8
Isoelectric focusing					
pH 3–10[b]					
PG-I	80	5.3	0.19	28	3.5
PG-II	80	37.9	0.09	420.6	52.5
PG-III	80	26.2	0.04	653.7	81.7
pH 2.5–5					
PG-I	10	6	0.11	54.5	6.8
pH 3.5–4.2					
PG-I	2	17.2	0.08	430	53.7
pH 6.5–9					
PG-II	16	46.6	0.09	548.3	68.5
PG-III	10	35.6	0.04	890	111.2

[a] See the Materials and Methods section.
[b] pH range of Pharmalytes incorported into the isoelectric focusing column.

gel filtration (1). Oligogalacturonides were prepared and detected by paper chromatography, as described previously (2,11). Protein was determined by the method of Lowry et al (9) with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

The purification of pectic enzymes from a culture fluid of *P. italicum* is summarized in Table 1. Selection of proteins by ultrafiltration, as described in the Materials and Methods section, resulted in approximately 10-fold purification and detection of three major protein bands by SDS-PAGE (Fig. 1). Five milliliters of the enzyme preparation after ultrafiltration with YM-100 membrane was applied to an isoelectric focusing column equilibrated with 3% Pharmalytes (Pharmacia, Uppsala, Sweden), pH range 3–10. The elution profile showed three peaks containing PG activity at pI values 3.6, 7.5, and 8.0. These were designated as PG-I, PG-II, and PG-III, respectively (Fig. 2). The two peak tubes of each PG were pooled, dialyzed, and applied to another isoelectric focusing column with a narrow pH range of 2.5–5 for PG-I and 6.5–9 for either PG-II or PG-III (Table 1). PG-I, eluted from the second column, was purified further by isoelectric focusing between pH 3.5 and 4.2. All three PG enzymes were obtained in homogeneous form as evident from the appearance of a single protein band in SDS-PAGE (Fig. 1). Attempts to detect activities of pectate lyase or pectin-methylesterase in culture fluid of *P. italicum* were unsuccessful.

The mechanism of hydrolysis of sodium polypectate by the three PG forms was followed by measuring the liberation of reducing groups and viscosity changes. The point of 50% reduction in relative viscosity was obtained when the percentage of hydrolyzed glycosidic linkages was as low as 44, 3.6, and 2.5 for PG-I, PG-II, and PG-III, respectively (Table 2). The incubation period required for reaching 50% reduction in viscosity employing 0.5 enzyme units was 230 min for PG-I as compared with 15 min or less for PG-II and PG-III. The products of hydrolysis detected by paper chromatography in the reaction mixture of PG-II or PG-III after 2.5 min contained tri-, tetra-, penta-, hexa-, and heptagalacturonic acid, whereas di- and monogalacturonic acids appeared after 5 min of incubation. Monogalacturonic acid was the sole product detected in the reaction mixture of PG-I at any stage of pectate degradation. Only PG-I was capable of hydrolyzing digalacturonic acid (Table 1). These results indicate the exo-PG nature of PG-I (poly-1,4-α-D-galacturonide-galacturonohydrolase EC 3.2.1.67) in contrast to the endo-PG nature of PG-II and PG-III (poly-1,4-α-D-galactosiduronate glycanohydrolase EC 3.2.1.15) (6). All three PG forms showed a high preference for sodium polypectate as a substrate when compared with pectin.

Pectic enzymes from infected orange peel also were purified as described in the Materials and Methods section. The elution

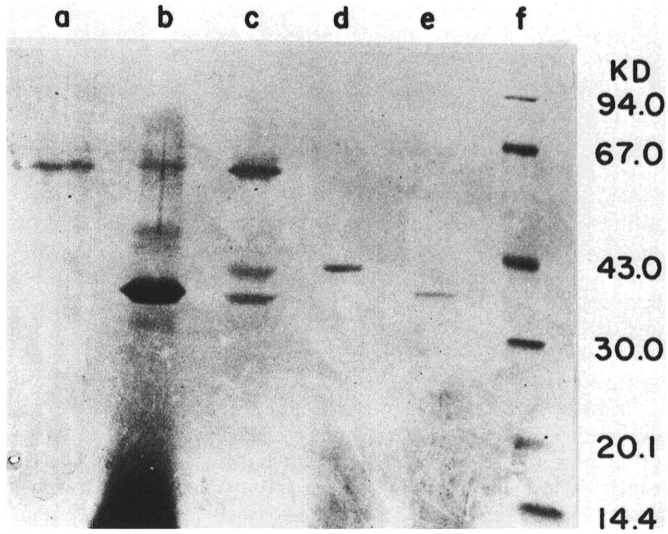

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of polygalacturonase (PG) preparations obtained during enzyme purification from culture fluid. Purification steps are described in Table 1. a, PG-I; b, crude enzyme preparation; c, enzyme preparation after ultrafiltration; d, PG-III; e, PG-II; f, molecular weight markers (top to bottom): phosphorylase b (94,000); bovine serum albumin (67,000); ovalbumin (43,000); carbonic anhydrase (30,000); soybean trypsin inhibitor (20,100); ∝-lactalbumin (14,400). Proteins were stained with Coomassie blue.

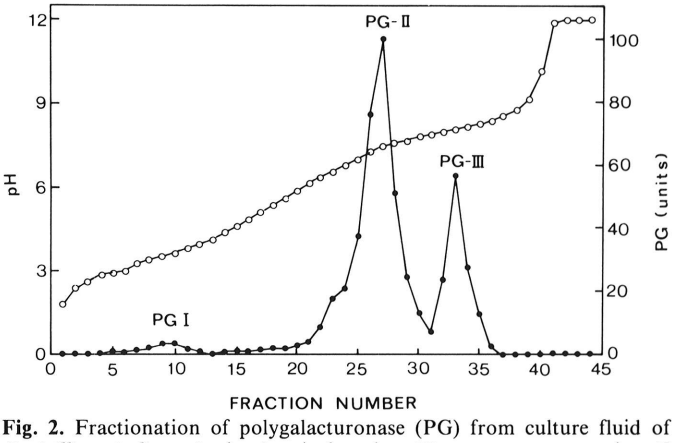

Fig. 2. Fractionation of polygalacturonase (PG) from culture fluid of *Penicillium italicum* by isoelectric focusing. The enzyme preparation (5 ml) applied to the column was obtained after ultrafiltration (Table 1). The pH gradients were formed by 3% Pharmalytes in the range of pH 3–10. Other experimental details are described in the Materials and Methods section. Fractions of 2 ml were collected. PG activity (●) signifies total units per fraction; ○ = pH.

TABLE 2. Comparative properties of pectic enzymes produced by *Penicillium italicum*

Property[a]	Exo-PG-I[b]	Endo-PG-II	Endo-PG-III
Percentage of hydrolysis of sodium polypectate at 50% reduction in viscosity	44	3.6	2.4
Hydrolysis of digalacturonate	+	—	—
Molecular weight[c]	60,000	36,000	38,000
Isoelectric point	3.8	7.5	8.0
pH optimum (at 30 C)	4.0	4.5	4.5
Optimal temperature	40	50	50

[a] Activity assays were performed with 0.5 enzyme unit as described in the Materials and Methods section.
[b] PG = polygalacturonase.
[c] The molecular weights were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

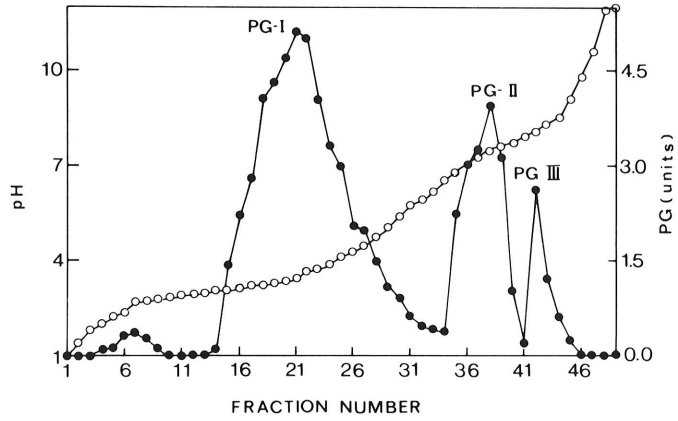

Fig. 3. Fractionation of polygalacturonase (PG) from orange peel infected by *Penicillium italicum* by isoelectric focusing. The enzyme preparation (5 ml) applied to the column was obtained after ultrafiltration (Table 1). The pH gradients were formed by 3% Pharmalytes in the range of pH 3–10. Purification procedures are described in the Materials and Methods section. ● = PG activity; ○ = pH.

profile of PG activity from the isoelectric focusing column indicated the presence of four peaks, three of which possess isoelectric points identical to exo-PG-I, endo-PG-II, and endo-PG-III (Fig. 3). However, the proportion of each of the enzyme forms in infected tissue and culture was different. The percentage of total PG activity attributable to exo-PG-I, endo-PG-II, and endo-PG-III was 69, 24, and 7, respectively, in infected tissue as compared with 4, 71, and 25, respectively, in culture filtrate. The three enzymes from infected tissue exhibited identical mechanisms of hydrolysis, pH optima, and molecular weights, as determined by gel filtration (1), to those of the corresponding enzymes from culture filtrate. An additional PG isozyme with a pI of approximately 2.4 was detected only in the infected tissue and could account for approximately 2% of the PG activity. This additional PG isozyme was not investigated further. The specific activities for exo-PG-I, endo-PG-II, and endo-PG-III from infected tissue were 164, 156, and 205 units of protein mg^{-1}, respectively, after isoelectric focusing.

The presence of exo-PG as the predominant PG form in orange peel infected with *P. italicum* contrasts with the results of Barmore and Brown (5), which indicated only an endo-PG in infected grapefruit peel. Their inability to detect exo-PG may have resulted from contamination with endo-PG in their partially purified enzyme preparation. The presence of endo-PG can interfere readily with the identification of an exo-PG unless its unique ability to hydrolyze digalacturonic acid is tested. Alternatively, it is possible that the production of the PG forms produced by *P. italicum* during grapefruit infection differs from that of orange. Chemical composition and pH of the medium were indeed reported to affect the type of pectic enzymes secreted by pathogenic fungi (6). In our study, for example, the relative amount of the exo-PG was 4% in culture medium, as compared with 69% in the infected orange peel. However, it might be difficult to assume that the differences in chemical composition or pH that may exist between orange and grapefruit peels are sufficient to account for complete suppression of the exo-PG. Moreover, it is difficult to envisage the excessive accumulation of galacturonic acid during blue mold infection of grapefruit (5) without the presence of exo-PG. In the present study, none of the endo-PGs of *P. italicum* could break down even trigalacturonic acid when incubated for 2 hr at 30 C with a high enzyme concentration (2 units/ml). Furthermore, the relative rates of hydrolysis catalyzed by endo-PG-II and endo-PG-III decreased with a shortening of the oligogalacturonide chain length. Thus, the rate of hydrolysis of hepta-, hexa-, penta-, and tetragalacturonide by endo-PG-III was 35, 29, 20, and 3.5%, respectively, as compared with sodium polypectate as a substrate. As indicated earlier, galacturonic acid appeared only during later stages of sodium polypectate hydrolysis by the endo-PGs, whereas only oligogalacturonides were detected during the initial stages. Oligogalacturonides have been reported to act as competitive inhibitors of endo-PGs (11) and thus may further slow the release of free galacturonic acid from citrus pectin.

Additional properties of the polygalacturonases produced by *P. italicum* are summarized in Table 2. The exo-PG-I generally exhibited properties similar to those of the exo-PGs reported for *P. digitatum* (2) and *Aspergillus niger* (8), but the optimal pH was more acidic (pH 4) than any of the above-mentioned enzymes. The lower pH optimum indicates that it may function effectively in the acidic environment of the infected peel. Based primarily on the molecular weight and pH optimum, the endo-PG characterized previously by Barmore and Brown (5) corresponds to endo-PG-II.

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