Isolation, Purification, and Characterization of a Polygalacturonase Produced in *Penicillium solitum*-Decayed 'Golden Delicious' Apple Fruit

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ABSTRACT

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Polygalacturonase (PG) was extracted and purified from decayed 'Golden Delicious' apple fruit inoculated with *Penicillium solitum.* Ammonium sulfate, gel filtration, and cation exchange chromatography were used to purify the enzyme. Both chromatographic methods revealed a single peak corresponding to PG activity. The purified PG most likely originates from the fungus because PG activity from healthy and wounded apple tissue was undetectable. Analysis of cation exchange-purified material using sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed a single 50-kDa band. The enzyme was active over a broad pH range (3 to 7), with optimal activity between pH 4 and 5. PG was highly active at 20 and 37°C but was also detectable at 2, 50, and 75°C. Divalent cations affected PG enzyme activity; Mg and Fe increased, whereas Ca and Mn reduced activity in vitro. Thin-layer chromatographic separation of hydrolysis products and data from a PG plate activity assay based on staining with ruthenium red showed that the enzyme exhibits both exo and endo activity. Purified PG incubated with intact apple fruit tissue in vitro caused a 30% reduction in mass after 48 h, suggesting a role in *P. solitum*-mediated decay of apple fruit.

Additional keywords: maceration, postharvest decay.

Penicillium solitum Westling is a necrotrophic fungal plant pathogen that infects apple and pear fruit, primarily through wounds in storage. A closely related *Penicillium* sp., *P. expansum,* has been shown to be more aggressive than *P. solitum* (18,22). However, both pathogens are capable of causing significant economic postharvest losses (27). Investigation of decay incidence caused by *Penicillium* spp. of apple and pear showed that *P. solitum* was the most frequently isolated (145/322 = 45%) compared with the other *Penicillium* spp. collected in Victoria, Australia and was differentiated based on pectic zymograms (22). *P. solitum* has also been isolated from processed meats, cheeses, rye bread, and cereals and produces copious amounts of dark-bluish-green conidia in culture and on the surface of infected fruit (22,33). It also exhibits a brownish orange reverse in culture, which has been structurally characterized and identified as solistatin (33). Solistatin belongs to a class of polyketide-derived compounds known as compactins, which have been widely used in medicine for their cholesterol-lowering ability in humans (15). However, the role of solitstatin in the *P. solitum*–apple fruit interaction has not been examined.

The plant cell wall serves as the major physiochemical barrier to the establishment of plant pathogens (34). It contains an abundance of carbon and nitrogen in the form of polysaccharides and proteins that can be broken down into their assimilable forms via cell-wall-degrading enzymes (CWDEs), including pectinases, proteases, and cellulases. Polygalacturonases (PGs) (EC 3.2.1.15) are produced by bacterial and fungal parasites to facilitate the invasion and colonization of host tissue. They are detectable in the initial stages of plant infection and produced in copious amounts during host colonization (10). PGs have been associated with diseases characterized by tissue maceration and soft rot (3,4). Conclusive evidence that PG contributes significantly to virulence was achieved via analyses of mutants lacking PGs (11,30). PGs are also produced by plants and, in apple, have been shown to be involved in fruit ripening and softening via degradation of apple cortical cell walls (2,37).

Pectin is found in the middle lamella of primary cell walls of dicots and nongraminaceous monocots. It is complexed with hemicellulose, provides cell wall integrity, is a critical component for plant tissue organization, and adds texture to fruit and vegetables (21). Pectin is composed of polymers of D-galacturonic acid linked by α-1,4-glycosidic bonds, which are cross-linked by carboxyl groups with divalent cations such as Ca and Mg (12). Pectin lyases are another major class of hydrolases responsible for pectin breakdown and have been isolated from bacteria and fungi associated with soft rot and food spoilage (13). Carboxyl groups of polygalacturonic acid can be methyl esterified, which alters the degree of cross-linking between polypectate chains (12). PG enzyme action can be classified as endo- and or exo-activity, depending on the location of enzyme cleavage within the substrate (1,10). In this reaction, the α-1,4-glycosidic bond between adjacent polygalacturonic acid residues is hydrolyzed by a single displacement mechanism (20).

PG has been shown to play a significant role in tissue maceration, colonization, and virulence in diverse pathosystems. However, it is not clear whether reduced aggressiveness of *P. solitum* compared with *P. expansum* during host invasion and colonization results from a less efficient or "inferior" PG produced by this

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pathogen. Therefore, the objectives of this study were to (i) isolate and purify PG from *P. solitum*-decayed apple fruit, (ii) biochemically characterize the purified PG, and (iii) investigate whether or not the purified enzyme was capable of macerating apple fruit tissue in vitro. Data from this study have demonstrated for the first time that PG produced by *P. solitum* in decayed apple fruit can function over broad pH and temperature ranges, has a limited level of thermostability compared with PG from *P. expansum*, and can macerate apple fruit tissue in vitro. Results of this study provide a foundation for investigating the biological role of PG at the molecular genetic level in this pathogen.

MATERIALS AND METHODS

Fruit, pathogen and chemicals. Mature 'Golden Delicious' apple fruit were harvested from a commercial orchard in Pennsylvania in 2007. *P. solitum* was isolated from naturally infected apple fruit (kindly supplied by Dr. Robert Spotts at Oregon State University) and maintained on potato dextrose agar. All chemicals used in this work were purchased from Sigma-Aldrich (St. Louis) unless indicated otherwise.

Fruit inoculation and PG extraction. Apple fruit were subjected to the following treatments: wound inoculation with 50 µl of conidial spore suspension of *P. solitum* (10^5 conidia/ml) as previously described (7), wound inoculation with 50 µl of sterile water; and placing 50 µl of sterile water on the surface of unwounded healthy apple fruit. After 14 days at 24°C, the peel over the lesion was removed and the decayed cortical tissue was collected for enzyme extraction. Decayed tissue was weighed and an equal amount of tissue was also harvested from the healthy and wounded apple fruit. The fruit tissue was added to an equal volume of 20 mM 2-[*N*-morpholino] ethanesulfonic acid (MES) with 1 M sodium chloride, pH 6.0, and stirred for 30 min, followed by filtering through Miracloth (Calbiochem-Behring, La Jolla, CA). The filtrates from each sample (decayed, healthy, and wounded) were centrifuged at 20,000 × *g* for 30 min and ammonium sulfate was added to the supernatant to 40% saturation while stirring. Once the ammonium sulfate dissolved, the mixtures were centrifuged for 30 min at 20,000 × *g*. Ammonium sulfate was added to the supernatants to obtain 90% saturation. These mixtures were then centrifuged for 30 min at 20,000 × *g* and the supernatants were discarded. The pellets were dissolved in 20 mM MES with 0.15 M sodium chloride (pH 6.0) and stored at 4°C. All extraction and purification procedures were carried out at 4°C.

PG purification. Approximately 5 ml of the dissolved 90% ammonium sulfate pellet was loaded onto a Sephacryl S-200 column (2.5 by 52 cm) that was equilibrated with 20 mM MES in 0.15 M sodium chloride (pH 6.0). PG was eluted using the above buffer at 30 ml/h and 60 4-ml fractions were collected. Following elution, gel filtration fractions from decayed tissue exhibiting PG activity were pooled and dialyzed overnight against 20 mM MES (pH 6.0). PG was further fractionated on a CM-Sephadex column (Pharmacia C-25 cation exchange) equilibrated with 20 mM MES, pH 6.0. The solution was placed on the column (1.6 × 9.8 cm) and washed with 20 mM MES, pH 6.0. PG was eluted with a linear gradient (30 ml/h) of 20 mM MES to 20 mM MES with 700 mM NaCl (pH 6.0) and collected in 30 4-ml fractions.

PG enzyme assay. PG activity was determined by measuring reducing sugars released from sodium polypectate, using D-galacturonic acid as the standard (38). An aliquot of the enzyme preparation was mixed with 0.1 ml of standard assay buffer (100 mM sodium acetate, pH 5.5, containing 0.4% polygalacturonic acid) and adjusted to a final volume of 0.2 ml with water in 13 × 100-mm borosilicate tubes. This mixture was incubated at 37°C for 2 h and 1 ml of 100 mM borate-borax buffer (pH 9.0) was added to stop the reaction. A 0.2-ml solution of 0.1% 2-cyanoacetamide was added and samples were placed into a boiling water bath for 10 min. Samples were then equilibrated to 20°C and absorbance at 276 nm was measured. Control reactions were carried out by adding borate-borax buffer with 2-cyanoacetamide prior to the addition of substrate buffer, and were run with each sample to determine background levels of reducing sugars. One unit of PG activity was defined as the amount of enzyme required to release 1 nanomole of reducing sugar per minute per milliliter under the assay conditions employed.

PG plate activity assay. PG activity was determined by a modified method previously described (28). A 1% (wt/vol) agarose solution was heated in a microwave until dissolved. A 0.1% solution of sodium polypectate was added to the agarose solution and adjusted to pH 5.5 with 1 N NaOH solution while stirring. Then, 25 ml of the agarose solution was poured into 100 × 15-mm petri dishes and allowed to cool. Four wells were punched in the agarose using a cork borer (2 mm in diameter) and solutions were pipetted into each of the wells. The plates were incubated at 37°C for 30 min before staining with 0.05% (wt/vol) ruthenium red (Acros) for 30 min at 20°C. Plates were destained with deionized water for 30 min and visualized on a light box.

Effect of divalent cations on PG activity. The effects of divalent cations on PG activity were determined by adding $CaCl_2$, $MgCl_2$, $MnSO_4$, and $FeCl_2$ at a 1 mM final concentration to the standard PG activity assay reaction. Reactions were conducted in duplicate and the experiment was repeated. The activity measured in the absence of cations was recorded as 100%.

Effect of temperature on PG enzyme activity. The effect of temperature on PG enzyme activity was carried out by incubating purified PG in water baths set at 2, 20, 37, 50, and 75°C for 2 h in 13 × 100-mm borosilicate tubes. Heat stability of the *P. solitum* PG was assessed by incubating purified PG in a boiling (100°C) water bath for 0, 5, 10, 15, 20, 25, and 30 s as previously described (38). PG activity was determined by detection of reducing sugars as carried out in the standard PG activity assay described earlier. Assays for each temperature and duration were conducted in triplicate and both experiments were repeated.

Effect of pH on PG activity. To determine the optimal pH for PG activity, assays were performed in 100 mM sodium acetate buffer with 0.4% polygalacturonic acid adjusted to a pH of 3.0 to 7.0 in 0.5 pH increments as described previously (38,39). Reactions were conducted in triplicate and the experiment was repeated.

Protein assay and sodium dodecyl sulfate polyacrylamide gel electrophoresis. Estimation of the purified PG protein was accomplished by using the micro-BCA protein assay kit (Pierce, Rockford, IL), based on the method described by Smith et al. (32). A standard curve was constructed using known amounts of bovine serum albumin. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of proteins was performed in a Bio-Rad Mini-protean III cell using a precast 4 to 20% gradient gel according to Leammli (16). The molecular mass of the protein was estimated using Protein Plus Precision Standards from Bio-Rad Laboratories (Hercules, CA). Protein bands were visualized using the SilverQuest staining kit (Invitrogen, Carlsbad, CA) according to the instructions provided.

Thin-layer chromatography analysis of PG products. The reaction mixture for thin-layer chromatography (TLC) analysis consisted of 475 µl of 100 mM sodium acetate, pH 5.5, with or without an ethanol-washed polygalacturonic acid substrate, plus 25 µl of cation-exchange-purified PG (0.136 U) in 20 mM MES, pH 6.0. After incubation for 2 h at 37°C, all samples were passed through Microcon centrifugal filter devices (prerinsed with 500 µl of water to remove trace amounts of glycerol) with a 10-kDa cutoff cellulose acetate membrane (Millipore Corporation, Bedford, MA) for 30 min at 20°C in a microcentrifuge at 14,000 rpm. Sample filtrates (≈450 µl) were transferred to 2-ml glass screw-cap vials. Each microfuge tube was rinsed with 0.5 ml of 80% ethanol, which was combined with the corresponding filtrate. Sample volumes were reduced to ≈0.2 ml by nitrogen evaporation

at 50°C. This was followed by the addition of another 0.5 ml of 80% ethanol and nitrogen evaporation to dryness. The filtrate residue was suspended in 100 µl of 80% ethanol, and the vials were capped and vortexed for 30 s and centrifuged at 2,000 × *g* for 2 min. The ethanolic solution was transferred to a microfuge tube and spun at 16,000 × *g* for 90 s prior to application to a 10 × 20-cm glass TLC plate precoated with 250-µm-thick silica gel 60 (EM Science, Darmstadt, Germany). Samples from the reactions and standards (mono-, di-, and trigalacturonic acid at 1 mg/ml in 80% ethanol) were applied in 1-cm-wide bands, using a stream of nitrogen to evaporate the solvent. Each sample (30 µl of 100 µl total) was used, including (i) enzyme with no substrate, (ii) substrate in buffer without enzyme, (iii) substrate with enzyme, (iv) digalacturonic acid plus enzyme, and (v) trigalacturonic acid with enzyme. Aliquots (5 µg) of the three standards (mono-, di-, and trigalacturonic acid) were applied individually to three lanes, and 5 µl of each standard was combined in a fourth lane. The TLC plate was developed in the ethyl acetate:acetic acid:formic acid:water (9:3:1:4, vol/vol/vol/vol) solvent system. After development, the plate was air dried in a fume hood for 12 h and sprayed with orcinol reagent (0.2% orcinol [wt/vol] in methanol:sulfuric acid [9:1, vol/vol]), The plate was then set on a hotplate at medium-high heat for 3 min to visualize the galacturonic acids as purple bands.

TABLE 1. Extraction and purification of polygalacturonase from 'Golden Delicious' apple cortical tissue decayed by *Penicillium solitum*

Purification step	Protein (mg/ml)	Total activity (U/min/ml)	Specific activity (U/mg)	Yield (%)
Crude extract	7.7	245.7	31.9	100
90% $(NH_4)_2SO_4$ pellet	1.20	58.4	48.6	23.7
Sephacryl S-200	0.294	25.6	87.0	10.4
CM-Sephadex C-25	0.139	14.2	102.1	5.77

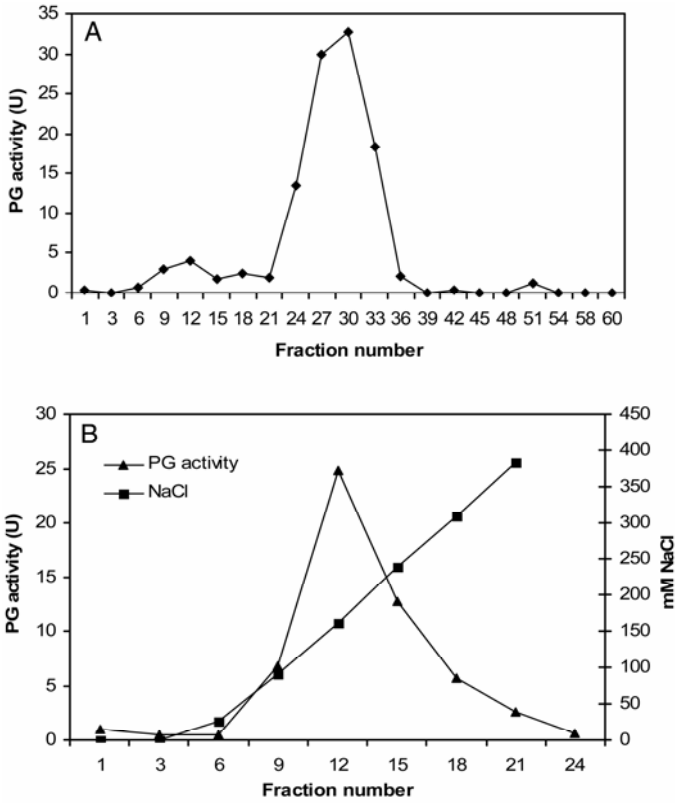

Fig. 1. A, Elution profile of a polygalacturonase (PG) sample produced by *Penicillium solitum* following fractionation on a Sephacryl S-200 gel filtration column. Proteins were eluted in 60 4-ml fractions with 20 mM 2-[*N*-morpholino]-ethanesulfonic acid (MES), pH 6.0. **B,** Elution profile of a pooled PG gel filtration peak on a CM-Sephadex cation exchange column. Proteins were eluted in 25 4-ml fractions with a 0 to 0.4 M linear sodium chloride gradient in 20 mM MES, pH 6.0.

Apple maceration assay. Apple fruit were equilibrated at 20°C prior to the maceration assay. Sterile conditions were used throughout the procedure. An apple was rinsed with soap and water, dried with a paper towel, and sprayed with 95% ethanol. After the ethanol evaporated, a 4-cm^2 area of the fruit was peeled using a sterile scalpel. A cork borer (2 mm in diameter) was used to remove plugs of apple cortical tissue, which were then cut into 1-cm pieces, weighed, and placed directly into 13 × 100-mm borosilicate tubes. Sets of three tubes containing the apple tissue were incubated with either 0.34 units of purified PG in 2 ml of 100 mM sodium acetate (pH 5.5), buffer only, or heat-denatured PG enzyme. After incubation at 20°C for 48 h, the tissue samples were removed from the test tubes, blotted for 5 s on tissue paper, and weighed.

RESULTS

Purification of PG produced by *P. soltium* in decayed apple fruit. PG produced by *P. solitum* during apple fruit colonization was purified using a multistep process that involved ammonium sulfate, gel filtration, and cation exchange chromatography. Following each step, total protein and total activity decreased, whereas specific activity increased (Table 1). Single peaks, corresponding to PG activity, are shown for gel filtration and cation exchange chromatography (Fig. 1A and B). SDS-PAGE analysis of the cation exchange purified material revealed a single band of ≈50 kDa (Fig. 2). The source of PG activity in *P. solitum*–apple fruit tissue is most likely to be fungal in origin as evidenced by the clear zone apparent from the 90% ammonium sulfate pellet collected from infected material and lack of a zone from both healthy and wounded 90% ammonium sulfate pellets (Fig. 3).

Biochemical characterization of *P. solitum* PG. The pH optimum of purified PG was determined using buffers adjusted from pH 3 to 7 with sodium polypectate in 100 mM sodium acetate buffer as the substrate. The PG enzyme was active over a broad pH range of 3.5 to 7 with an optimum occurring at pH 4 to 5 (Fig. 4). The heat stability of PG was examined by boiling aliquots of the enzyme for 0, 5, 10, 15, 20, 25, and 30 s. The purified enzyme was heat sensitive because ≈50% of the activity

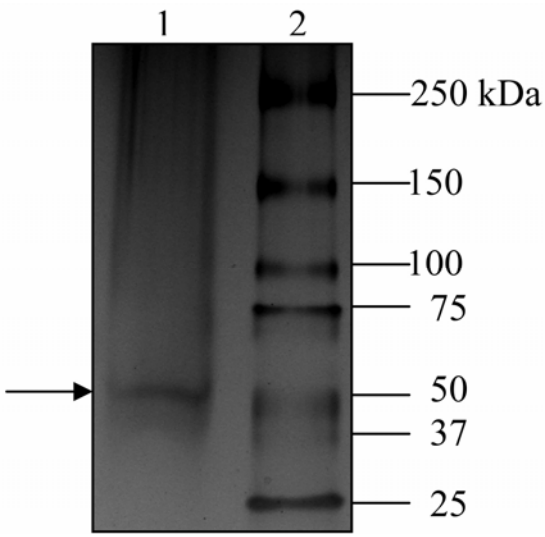

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel (4 to 20% gradient) electrophoresis that was treated with silver stain. Lane 1 contains 15 µl of CM-Sephadex cation exchange-purified polygalacturonase from *Penicillium solitum* showing a single 50-kDa band. Lane 2 contains 5 µl of molecular weight marker.

was destroyed when incubated for 10 s at 100°C. No activity was detected following incubation in boiling water for 15 s (data not shown). The effect of temperature on purified PG enzyme activity was investigated by incubating the enzyme at 2, 20, 37, 50, and 75°C for 2 h and assaying the amount of reducing sugars liberated from sodium polypectate. PG activity was detectable at all temperatures examined. However, maximum activity was detected at 20 and 37°C (Fig. 5). The purified PG hydrolyzed polygalacturonic acid in a mixed manner exhibiting both endo and exo activity according to results from PG plate assay and TLC analysis (Figs. 6 and 7). The effect of various metal ions on purified PG activity was determined using sodium polypectate (Table 2). An increase in PG enzyme activity was observed with the addition of magnesium and iron whereas a decrease in activity occurred when calcium and manganese were included in the PG assay.

Maceration of apple fruit tissue in vitro. To quantitatively measure the maceration ability of purified PG, apple tissue plugs were incubated with buffer alone, heat-denatured PG, and native PG. After 24 h, no change in the weight or visible deterioration of apple disks was detectable (data not shown). However, a significant decrease in weight was observed after 48 h for tissue disks incubated with native PG compared with those incubated in buffer only or with denatured PG (Fig. 8). A significant change in texture and slight browning of the tissue was observed for the native PG-treated apple disks.

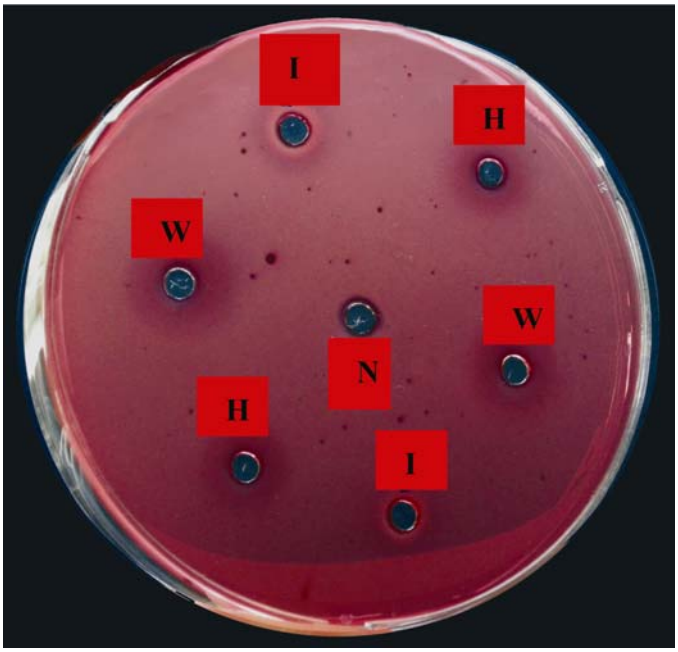

Fig. 3. Polygalacturonase plate assay of 90% ammonium sulfate pellets from 20 µg of total protein of each healthy (H), wounded (W), and *Penicillium solitum*-decayed apple fruit tissue (I). Negative control (N) contained 5 µl of 20 mM 2-[*N*-morpholino]-ethanesulfonic acid + 0.15 M sodium chloride, pH 6.0. The experiment was repeated and a representative plate is shown.

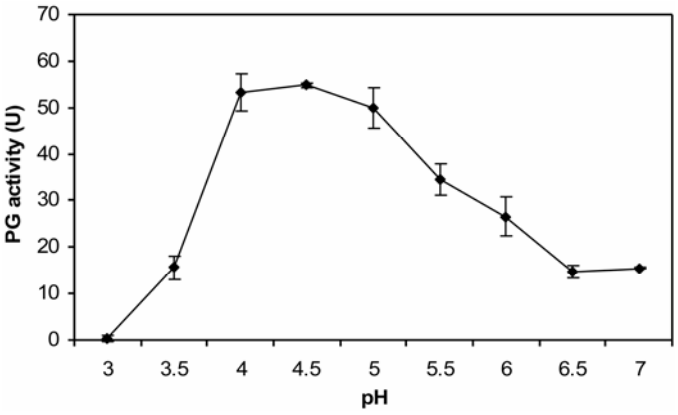

Fig. 4. Activity profile of purified polygalacturonase produced by *Penicillium solitum* in apple fruit at different pHs. Each data point represents the mean and standard deviation of samples assayed in triplicate.

DISCUSSION

Gel filtration and cation exchange chromatography resulted in purification of PG produced by *P. solitum* to homogeneity. The molecular mass of the protein was estimated at 50 kDa, which is consistent with reports for other fungal PGs of 20 to 78 kDa (20). For example, PG1 isolated from *Phomopsis cucurbitae* during

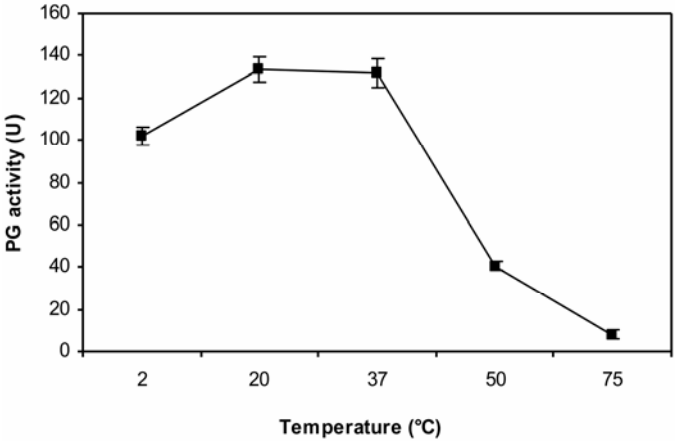

Fig. 5. Activity profile of purified polygalacturonase produced by *Penicillium solitum* in apple fruit at various temperatures. Each data point represents the mean and standard deviation of samples assayed in triplicate.

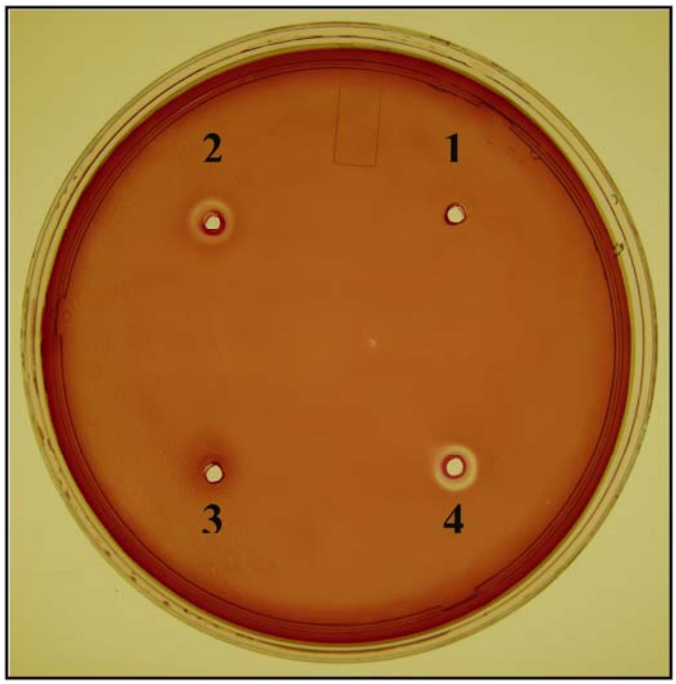

Fig. 6. Assay of polygalacturonase (PG) activity after staining 1% agarose plates containing 0.1% polygalacturonic acid with 0.05% ruthenium red solution. The wells were loaded with: 1, 20 mM 2-[*N*-morpholino]-ethanesulfonic acid in 0.15 M sodium chloride pH 6.0; 2, 5 U of native *Penicillium solitum* PG from cation exchange; 3, 5 U of denatured *P. solitum* PG from cation exchange; and 4, 5 U of native *P. expansum* PG from cation exchange. Three plates were assayed together and the experiment was repeated. The results of one plate from a representative experiment are shown.

decay of muskmelon was determined to be 54 kDa (39). However, the PG produced by *P. solitum* is larger than the 34-kDa enzyme that was isolated and characterized from *P. expansum*-decayed apple fruit (38).

The optimal PG activity at pH 4 to 5 is consistent with data from other studies that have shown that pH optima for various fungal PGs are pH 3 to 5 (19,24,31). A narrow pH range of 4.5 to 6.5 with an optimum of 5.5 was determined for PG activity of *P. expansum* (38). A broader pH range of PG activity may provide *P. solitum* an ecological advantage because the fungus could utilize nutrients as a saprophyte while also being capable of degrading pectin from apple cell walls at a pH (3.95 to 4.54) that is common for apple fruit (23).

Temperature activity studies of the purified enzyme from *P. solitum* revealed that PG is active at temperatures as low as 2°C. Activity at low temperature may facilitate host invasion and colonization during cold storage (35). Lack of *P. solitum* PG enzyme stability at 100°C contrasts with that reported for PG produced by *P. expasum* in decayed apple fruit (38). *P. expansum* PG activity was detectable following 15 min of incubation in boiling water whereas the PG purified from *P. solitum* was inactive following 15 s at 100°C (data not shown).

The increase in PG enzyme activity when Mg and Fe were added to the enzyme assay is in accord with an earlier report wherein Mg stimulated activity of a purified endoglucanase from the wood-decaying fungus *Daldinia eschscholzii*, which was hypothesized to be the result of stabilization of the overall enzyme structure (14). A decrease in PG activity was observed with the addition of Ca and Mn, which have also been shown to inhibit other fungal PGs in vitro (6,17,19). Several studies have shown that increased Ca in the fruit inhibits *P. expansum*-mediated decay of apple fruit after harvest (7–9). High Ca levels are thought to increase cell wall integrity by cross-linking pectic acids and thereby making pectin less accessible for breakdown by PG. However, it is not clear from data contained in this study how Ca decreases PG enzyme activity in vitro. Ca-mediated inhibition observed in vitro may be through binding of Ca to the polypectate in the enzyme assay buffer or binding to the purified PG enzyme and acting as a negative allosteric effector.

The purified PG hydrolyzed sodium polypectate in a mixed manner exhibiting both endo and exo activity. Mixed activity has also been reported for the purified PG from *P. expansum* (38). In the current study, the hydrolysis products were composed mostly of monomeric galacturonic acid but small amounts of digalacturonate and trigalacturonate were also detected in the enzyme reaction. Endo cleavage of polypectate was further substantiated by the PG plate assay because this class of hydrolase contributes more to the rapid reduction of the polypectate substrate (in 30 min), thus yielding a clear zone because internal residues are cleaved on a random basis. The purified PG enzyme was unable to hydrolyze di- and trigalacturonic acid when they were included as a substrate in place of polypectate. This result suggests that the enzyme requires a minimum polymer length >3 residues for enzymatic cleavage. This is consistent with data from other fungal PGs exhibiting endo action because they also were unable to cleave dimers and trimers of galacturonic acid (5,25).

A 30% reduction in mass resulted when apple tissue plugs were incubated with native PG from *P. solitum*. The reduction in mass represents the ability of the purified PG to degrade apple fruit tissue in vitro and substantiates the role of this enzyme in postharvest decay of apple fruit tissue by *P. solitum*. Interestingly, a 30% reduction in mass of muskmelon fruit plugs resulted from incubation with a purified PG from *Phomopsis cucurbitae* in vitro (39). The authors concluded that PG may be involved in Phomopsis fruit rot of muskmelon based on the ability of the enzyme to macerate muskmelon fruit tissue in vitro. Molecular genetic evidence has shown that PG is a virulence factor in both fungal and bacterial pathogens. *Pseudomonas solanacerum* and *Agrobacterium tumefaciens* biovar 3 mutants lacking PG genes were less virulent on their respective hosts (26,29). Functional analysis of the *Bcpg*1 gene encoding an endopolygalacturonase in *Botrytis cinerea* showed that PG-null strains were less virulent on tomato

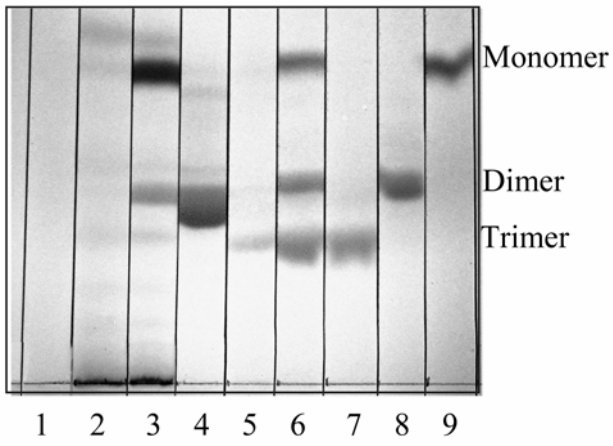

Fig. 7. Thin-layer chromatographic analysis of purified polygalacturonase (PG) reaction products with various polygalacturonic acid substrates. Standard assay buffer included 0.4% (wt/vol) Na polypectate. Lane 1, purified PG only; lane 2, standard assay buffer only; lane 3, 3.4 U of purified PG in standard assay buffer; lane 4, 3.4 U of purified PG in assay buffer containing polygalacturonic acid dimer; lane 5, 3.4 U of purified PG in assay buffer containing polygalacturonic acid trimer; lane 6, polygalacturonate monomer, dimer, and trimer standards; lane 7, polygalacturonate trimer; lane 8, polygalacturonate dimer; lane 9, polygalacturonate monomer.

TABLE 2. Effect of divalent cations on purified polygalacturonase activity produced by *Penicillium solitum*

Compound[a]	Percent relative activity (SD)[b]
Control	100.0
$CaCl_2$	85.1 (5.03)
$MgCl_2$	111.0 (2.51)
$MnSO_4$	87.3 (6.15)
$FeCl_2$	130.0 (5.34)

[a] Assays were carried out in the presence of 1 mM concentration of each compound.

[b] Relative activity was determined by assaying polygalacturonase at 37°C in 100 mM Na acetate buffer pH 5.5 for 2 h. Mean values from samples assayed in duplicate are shown from a representative experiment. Standard deviation (SD) of the mean is shown in parentheses.

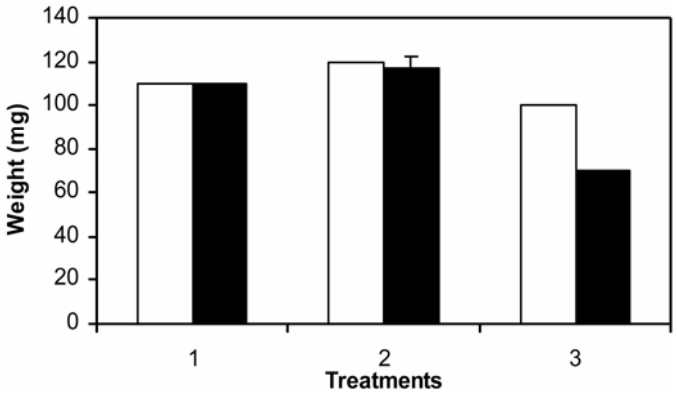

Fig. 8. Fresh weight changes in apple cortical tissue disks after different treatments: 1, 100 mM sodium acetate buffer (pH 5.5); 2, heat-denatured *Penicillium solitum* polygalacturonase (PG) in buffer; and 3, native *P. solitum* PG in buffer. Weight before treatment, white columns and weight after 48 h, black columns. Vertical bars represent the means of treatments conducted in triplicate. Error bars represent the standard deviation of the mean.

fruit and leaves and apple fruit compared with wild-type strains (11). A recent study of 10 PG genes from *Phytophthora parasictica* showed that each of the gene products plays a distinct role in decomposition of the plant cell wall (36).

The PG that was purified from *P. solitum*-decayed apple fruit was almost certainly produced by the fungus. This conclusion is based on the inability to detect PG activity from either wounded or intact apple fruit tissue in the plate assay after ammonium sulfate precipitation or fractionation. However, definitive proof will require sequence homology comparisons between the host (apple) and the pathogen (*P. solitum*) PG genes. Although such work is beyond the scope of this study, it will be considered in the future.

This is the first report on the isolation, purification, and biochemical characterization of PG from *P. solitum*-decayed tissue. This work provides a foundation for future genetic study of the role of PG in *P. solitum*–apple fruit interactions. The elucidation of its role by molecular cloning and functional analysis will provide further insight into the mechanisms of fungal invasion and colonization and will lead to the development of more specific and effective control strategies for postharvest fungal plant pathogens.

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