Purification and Biochemical Characterization of Polygalacturonase Produced by *Penicillium expansum* During Postharvest Decay of 'Anjou' Pear

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ABSTRACT

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A polygalacturonase (PG) was extracted and purified from decayed tissue of 'Anjou' pear fruit inoculated with *Penicillium expansum.* Ammonium sulfate precipitation, gel filtration, and cation exchange chromatography were used to purify the enzyme. Both chromatographic methods revealed a single peak corresponding to PG activity. PG enzyme activity from healthy and wounded pear tissue was undetectable, which supports the claim that the purified PG is of fungal origin. The purified enzyme had a molecular mass of 41 kDa and a pI of 7.8. Activity of the PG was not associated with a glycosylated protein. The enzyme was active over a broad pH range from 3 to 6, with optimal activity at 4.5 in sodium citrate and sodium acetate buffers. The optimal temperature for activity was 37°C but the enzyme was also active at 0, 5, 10, 20, and 50°C. Thin-layer chromatographic analysis of PG hydrolysis products showed that the enzyme exhibits endo- and exo-activity. The purified enzyme macerated tissue in vitro causing ≈30% reduction in mass of pear plugs compared with ≈17% reduction for apple. Additionally, it produced 1.5-fold more soluble polyuronides on pear than apple tissue. This work shows for the first time the production of a PG by *P. expansum* during postharvest decay of pear fruit is different from the previously described PG produced in decayed apple fruit by the same pathogen.

Additional keywords: host specificity, maceration.

Penicillium expansum (Link) Thom., the causal agent of blue mold, is the most important postharvest pathogen of pome fruits (16,19,21). This pathogen infects fruit through wounds after harvest, as well as through natural openings, i.e., lenticel, stem end, and calyx (19). Blue mold is characterized by a soft, watery rot that is light brown in color, and the appearance of blue-green colored conidia on the surface at the later stages of decay development (7). Blue mold decay can lead to significant economic losses during storage, which can also impact fruit destined for processing due to the production of the carcinogenic mycotoxin patulin. Chemical control of blue mold is achieved through application of fungicides like fludioxonil and thiabendazole. However, increased use of these compounds often leads to resistance which reduces their efficacy. Therefore, a better understanding of the mechanisms of blue mold fruit rot may provide information for the development of novel innovative control strategies for this disease.

The plant cell wall serves as the major physiochemical barrier to the establishment of plant pathogens, and is composed of four major complex polysaccharides (cellulose, hemicellulose, lignin, and pectin) (25). Pectin is the major component of the middle lamella, which causes adhesion of adjacent primary cell walls in dicots and nongraminaceous monocots. In addition, pectin is complexed with hemicellulose and cellulose microfibrils, provides cell wall integrity, is a critical component for plant tissue organization, and adds texture to fruits and vegetables (17). Pectin is a polymer of D-galacturonic acid linked by β-1,4-glycosidic bonds, which are cross-linked by carboxyl groups with divalent cations such as Ca and Mg (10).

Polygalacturonases (PGs) (EC 3.2.1.15) are produced by bacterial and fungal plant pathogens to hydrolyze pectins and thereby facilitate the invasion and colonization of host tissue. PGs cleave the β-1,4-glycosidic bond between adjacent polygalacturonic acid residues by a single displacement mechanism (15). They are detectable in the initial stages of plant infection and produced in copious amounts during host colonization (6). PGs have been associated with diseases characterized by tissue maceration and soft rot (2,3). Genetic evidence that PG contributes significantly to virulence has been achieved via analyses of PG genes in *Botrytis cinerea* (*Bcpg1* gene) and in *Aspergillus flavus* (*P2c* gene) (8,23). PGs are also produced by plants, and in pear have been shown to be involved in fruit ripening and softening via degradation of cortical cell walls (9,22).

PGs play a significant role in tissue maceration, colonization, and virulence. However, it is not known if different PGs are produced in different hosts during postharvest decay. *P. expansum* produces at least five PG enzymes in culture (C. L. Yao, *unpublished data*). However, only one PG has previously been isolated from *P. expansum*-decayed apple fruit (26). Therefore, the objectives of this study were (i) to isolate and purify PG from *P. expansum*-decayed pear fruit, (ii) to biochemically characterize the purified PG, (iii) to determine if the purified enzyme was

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capable of macerating pear and apple fruit tissue in vitro, (iv) and to compare biochemical characteristics between PGs produced in apple and pear fruit by the same isolate of *P. expansum*. Data from this study indicates that PG produced by *P. expansum* in decayed pear fruit can function over a broad pH and temperature range, can macerate pear and apple fruit tissue in vitro, and is different from the PG produced by this pathogen during apple fruit decay.

MATERIALS AND METHODS

Fruit, pathogen and chemicals. Mature 'Anjou' pear fruit were purchased from a local grocery store in Beltsville, MD. The *P. expansum* isolate used in this study is the same one described by Yao et al. (26), which was isolated from naturally infected apple fruit and maintained on potato dextrose agar. All chemicals used in this work were purchased from Sigma Chemical Co. (St. Louis, MO) unless indicated otherwise.

Pear fruit inoculation and enzyme extraction. Pear fruit was washed with soap and water, sprayed with 70% ethanol, and dried with a paper towel. Pears were then subjected to the following treatments: wound inoculation with 50 µl of conidial spore suspension of *P. expansum* (10^5 conidia/ml) as previously described (5); wound inoculation with 50 µl of sterile water; and addition of 50 µl of sterile water on the fruit surface of unwounded healthy pears. After 14 days of storage 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 intact and wounded pears. 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 intact, and wounded) were centrifuged at $20,000 \times g$ 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 $20,000 \times g$. Ammonium sulfate was again added to the supernatants to obtain 90% saturation. These mixtures were then centrifuged for 30 min at $20,000 \times g$ 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.

Enzyme purification. Approximately 5 ml of the dissolved 90% ammonium sulfate pellet was loaded onto a Sephacryl S-200 column (2.5×52 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 was eluted with a linear gradient (30 ml/h) of 20 mM MES to 20 mM MES with 1 M NaCl (pH 6.0). Thirty 4-ml fractions were collected and stored at 4°C for further analysis.

Reducing sugar assay for PG activity. PG activity was determined by measuring reducing sugars released from sodium polypectate, using D-galacturonic acid as the standard as described by Yao et al. (26). An aliquot of the enzyme preparation was mixed with 0.1 ml of 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 borosilicate tubes (13×100 mm). This mixture was incubated at 37°C for 20 min and 1 ml of 100 mM borate-borax buffer (pH 9.0) was added to stop the reaction. Solutions of 0.1% 2-cyanoacetamide (0.2 ml) were 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 assayed for 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.

Plate assay for PG activity. PG activity was determined by a modified method previously described by Saad et al. (20). A 1% (wt/vol) agarose solution was heated 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, 15 ml of the agarose solution was poured into petri dishes (100×15 mm) and allowed to cool. Four wells were punched in the agarose using a cork borer (2 mm diameter) and solutions were pipetted into each of the wells. The plates were incubated at 37°C for 14 h before incubating with 0.05% (wt/vol) ruthenium red (Acros) for 1 h at 20°C. Plates were destained with deionized water for 30 min and visualized on a light box.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins was performed in a Bio-Rad Mini-protean III cell using a precast 12% gel according to Leammli (12). The molecular mass of the protein was estimated using Protein Plus Precision Standards (Bio-Rad Laboratories, Hercules, CA). Protein bands were visualized using Bio-Safe Coomassie stain (Bio-Rad Laboratories) according to the manufacturer's protocol.

Isoelectric focusing-polyacrylamide gel electrophoresis. Isoelectric focusing-polyacrylamide gel electrophoresis (IEF-PAGE) of purified PG was performed using a pre-cast Ampholine IEF gel (pH 3.5 to 9.5, GE Life Sciences) in a Pharmacia Biotech Multiphor II system following the manufacturer's instructions (Amersham-Pharmacia). The electrode strips were soaked with 1 M phosphoric acid (anode) and 1 M sodium hydroxide (cathode) and were placed on opposing sides of the gel. Protein
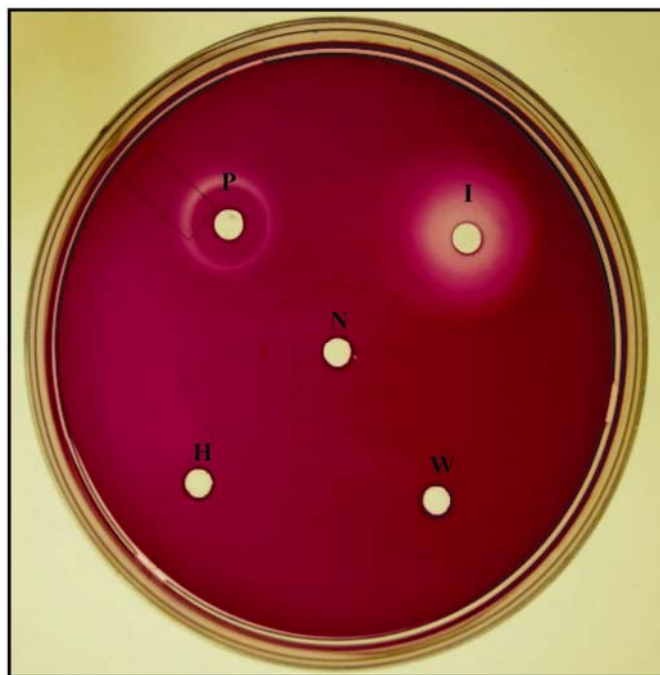

Fig. 1. Polygalacturonase plate assay of 90% ammonium sulfate pellets from intact healthy (H), wounded healthy (W), and *Penicillium expansum*-decayed 'Anjou' pear fruit (I). The negative control (N) contained 20 mM MES + 0.15 M sodium chloride (pH 6.0). The positive control (P) contained a commercial preparation of pectinase obtained from *Aspergillus niger.* The experiment was repeated and a representative plate is shown.

samples were applied to application strips that were then placed directly on the gel. IEF protein standards (pI range 3.5 to 10.7, SERVA) were diluted 1:5 with water prior to loading. Isoelectric focusing was then conducted at 1,500 v, 25 ma, 25 w for 1.5 h. The gel was stained with silver using the SilverQuest staining kit (Invitrogen) according to the manufacturer's instructions.

Glycosylation analysis. A Qproteome total glycoprotein isolation kit, containing lectin affinity spin columns, was purchased from Qiagen. Purified PG (1.2 U) was fractionated according to the manufacturer's protocol. Fractions eluted from the column; flow through, wash, mannose elution and sialic acid elution were collected and concentrated by acetone precipitation and analyzed for activity using the PG plate activity assay. A well characterized glycoprotein (Ribonuclease b) was purchased from Sigma and served as a positive control for the lectin columns. Fractions from the positive control were concentrated by acetone precipitation and analyzed by SDS-PAGE. Protein bands were visualized using Bio-Safe Coomassie stain (Bio-Rad Laboratories) according to the instructions provided.

Temperature optima. The optimal temperature for PG enzyme activity was determined using the reducing sugar assay by incubating 1.2 U of purified PG with 100 mM sodium acetate buffer, pH 5.5, in borosilicate tubes (13 × 100 mm) placed in water baths at 0, 5, 10, 20, 37, 50, and 75°C for 20 min. Assays for each temperature were conducted in triplicate and the experiment was repeated.

Thermal stability. Thermostability of the purified enzyme was determined by boiling aliquots of the enzyme (1.2 U) over time (0, 5, 10, 15, 20, 25, and 30 min) and assaying activity in 100 mM sodium acetate buffer (pH 4.5) at 37°C. Reactions were conducted in triplicate and the experiment was repeated.

pH optima. To determine the optimal pH for PG activity, assays were performed in 100 mM sodium acetate and 100 mM sodium citrate with 0.4% polygalacturonic acid adjusted to a pH ranging from 3.0 to 7.0 in 0.5 pH increments as described (26,27). Reactions were conducted in triplicate and the experiment was repeated.

Analysis of PG hydrolysis products by thin-layer chromatography. Purified PG (6 U) was used to determine the hydrolysis products of polygalacturonic acid after incubation in 100 mM sodium acetate buffer (pH 4.5) for 0, 5, 10, 15, 20, 25, 30, and 60 min and 24 h at 37°C. From the 0.5 ml reaction volume, a 50 µl aliquot was removed at each time interval and heated at 100°C for 30 min. After boiling, the samples were immediately placed at –80°C until analyzed. Thawed samples were then nitrogen-evaporated to dryness and resuspended in 20 µl of 50% ethanol. A five-microliter aliquot from each time point was separated on a 10 × 20 cm glass thin-layer chromatography (TLC) plate precoated with 250 µm thick silica gel 60 (EM Science, Darmstadt, Germany). The monomer, dimer, and trimer of galacturonic acid were used as standards. 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 plates were 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 placed on a hotplate at medium high heat for 3 min to visualize the galacturonic acids.

Fruit tissue maceration assay. Pear fruit were removed from cold storage (1.4°C) and set out on a laboratory bench top at 20°C for 1 h. The fruit were then rinsed with soap and water, sprayed with 70% ethanol, and dried with a paper towel. Wounds (3 × 3 mm) were made in the fruit using a nail embedded in a piece of cork, and 50 µl of sterile water or 5 U of PG enzyme were added to each wound. Pear fruit were kept at 37°C for 24 h after which tissue maceration was evaluated by slicing through the wound with a scalpel. For in vitro tissue maceration tests both pear and 'Golden Delicious' apple fruit were removed directly from 1.4°C and rinsed with soap and water. The fruit were then sprayed with 70% ethanol and wiped dry. A sterilized hand held vegetable peeler was used to aseptically remove the peel from the fruit. Apple and pear tissue plugs were collected from the peeled area using a No. 2 cork borer. All plugs were trimmed to 1 cm in length using a sterile scalpel. Each plug was then placed into 50 ml of 100 mM Na-acetate buffer pH 4.5 in a beaker and incubated for 1 h at 37°C. The plugs were then removed from the beakers, blotted for 5 s and the initial weight (0 time) was recorded. One tissue plug was placed into each borosilicate tube (13 × 100 mm) and 2 ml of Na-acetate buffer, pH 4.5, was added. Then, either 12.8 µl of buffer, pH 4.5, 12.8 µl (1.2 U) of denatured PG, or 12.8 µl (1.2 U) of native PG enzyme was added to each tube. The samples were then incubated at 37°C for 48 h. The plugs were removed from each tube, blotted on tissue paper for 5 s and the weight was recorded. Tissue plugs were then discarded and the remaining buffer in the tubes was placed at 4°C for future analysis of total soluble polyuronides.

Total soluble polyuronide assay. Total soluble polyuronides were determined according to the method previously described (4). Briefly, 100 µl of buffer sample from each maceration assay was removed and placed in microfuge tubes. Tubes were then vortexed briefly and centrifuged at 14,000 × g for 1 min. Ten microliters of the pooled buffer solution was added to 90 µl of water in a borosilicate tube (13 × 100 mm) and briefly vortexed to mix. Samples were placed in an ice bath and 600 µl of sulfuric acid tetra borate solution (12.5 mM tetra borate in concentrated sulfuric acid) was added. All samples were vortexed briefly and placed in a boiling water bath at 100°C for 5 min. Samples were then cooled immediately on ice to room temperature and 10 µl of m-phenylphenol reagent (0.15% m-phenylphenol in 0.5% sodium hydroxide) was added and mixed by vortexing. Absorbance (520 nm) of the samples was determined using a spectrophotometer (SmartSpec, Bio-Rad Laboratories). Concentration of polyuronides in buffer solutions from the maceration assay was determined according to a standard curve developed for galacturonic acid. All samples were assayed in triplicate and the experiment was repeated.

RESULTS

Purification of PG produced by *P. expansum* in decayed pear fruit. PG produced by *P. expansum* during pear fruit colonization was purified using a multistep process. For each step, total protein decreased and specific activity increased (Table 1). The source of PG activity in *P. expansum*–pear fruit tissue is fungal in origin as evidenced by the clear zone from the 90% ammonium sulfate pellet collected from infected material and lack of a clear zone from both intact and wounded healthy tissue using 90% ammonium sulfate pellets (Fig. 1). Other protein fractions from healthy and wounded pear fruit including: crude extract, 40% ammonium sulfate pellet and supernatant, and 90% ammonium sulfate pellet and supernatant had undetectable PG activity using the reducing sugar assay (data not shown).

Biochemical characterization of *P. expansum* PG. Single peaks, corresponding to PG activity, were detectable for both gel filtration and cation exchange chromatography (Fig. 2A and B). SDS-PAGE analysis of the cation exchange purified material

TABLE 1. Purity and activity of the purified polygalacturonase from 'Anjou' pear fruit decayed by *Penicillium expansum* at various stages of enzyme purification[a]

Step	Total activity (U/min/ml)	Protein (mg/ml)	Specific activity (U/mg)
90% pellet	137.0	25.6	5.36
Gel filtration	27.9	0.22	127
Dialysis	15.8	0.20	79
Cation exchange	128.6	0.01	12,860

[a] Values represent the means from samples assayed in triplicate.

revealed a single band of approximately 41 kDa (Fig. 3A). A pI of 7.8 for the purified PG was determined by analytical isoelectric focusing (Fig. 3B). Fractionation of the enzyme using a glyco-protein affinity column showed that PG activity was detected only in the flow through and wash fractions and was undetectable in the mannose and sialic acid buffer elution steps (Fig. 4). Optimal reaction temperature was determined to be 37°C. However, PG activity was also detectable at 0, 5, 10, 20, and 50°C (Fig. 5A) The PG was active over a broad pH range from 3 to 6 with the optimum at pH 4.5 in both buffer systems (Fig. 5B). The purified enzyme was heat labile as boiling for 5 min reduced approximately 80% of PG activity (Fig. 5C). The PG hydrolyzed poly-galacturonic acid in a mixed manner exhibiting both endo and exo activity according to TLC separation of enzymatic hydrolysis products (Fig. 6).

Maceration of pear and apple fruit tissue. PG purified from *P. expansum*-decayed pear fruit was added to wounds made in intact pear fruit to qualitatively evaluate its tissue macerating ability. After incubation at 37°C for 48 h, both the diameter and depth of the wound increased. There were no visible changes in the wounded only and water only controls (data not shown). To quantitatively measure the maceration ability of purified PG, pear and apple tissue plugs were incubated with buffer alone and buffer with purified PG from *P. expansum*-decayed pear tissue. There was a difference in the decrease in fruit tissue weight that occurred in pear (≈30%) compared with apple (≈17%) samples after 48 h incubation (Fig. 7A). A change in texture and a slight change in color of the tissue were observed for the native PG-treated pear tissue, but not for apple tissue (data not shown).

Polyuronides from pear and apple fruit in vitro. Buffer solutions from macerated apple and pear tissue plugs were assayed for total soluble polyuronide content. Pear tissue incubated with the enzyme for 48 h at 37°C produced 32.6 µg of soluble polyuronides compared with 22.3 µg for apple fruit tissue. Basal levels of soluble polyuronides in control treatments containing pear or apple fruit tissue in buffer solutions without enzyme were 6.3 and 12 µg, respectively (Fig. 7B).

DISCUSSION

Multiple lines of biochemical evidence (MW, pI, and pH optima) suggest that the PG produced by *P. expansum* during apple decay is different from the PG produced during pear decay.
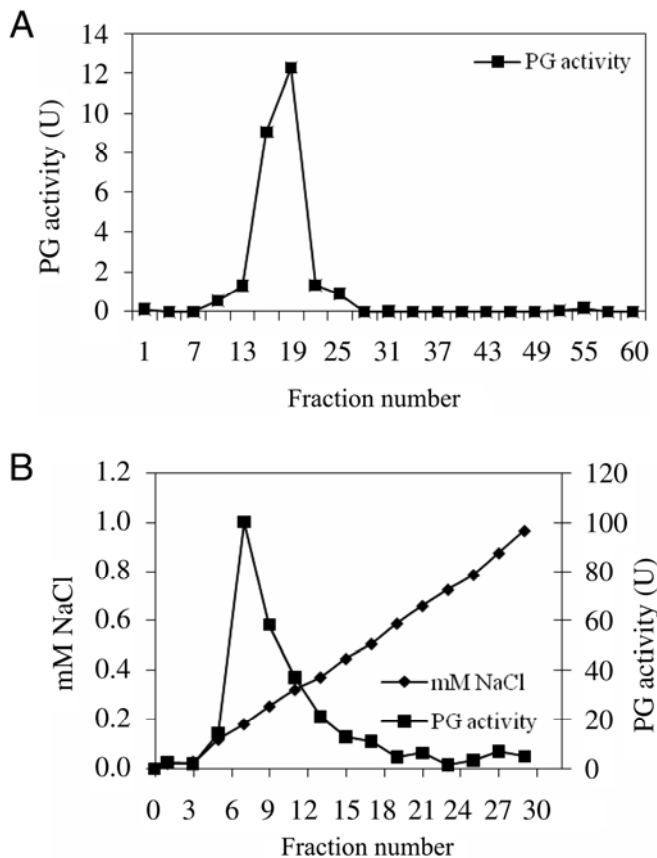
Fig. 2. A, Elution profile of a polygalacturonase (PG) produced by *Penicillium expansum* following fractionation on a Sephacryl S-200 gel filtration column. Proteins were eluted in 60 4-ml fractions with 20 mM 2-[*N*-morpholino]-ethanesulfonic acid (pH 6.0) with 150 mM NaCl. B, PG activity in gradient elution of 10 pooled and dialyzed gel filtration peaks that were fractionated on a CM-Sephadex cation exchange column. Proteins were eluted in 30 4-ml fractions with a linear 0 to 1.0 M NaCl gradient in 20 mM MES (pH 6.0).
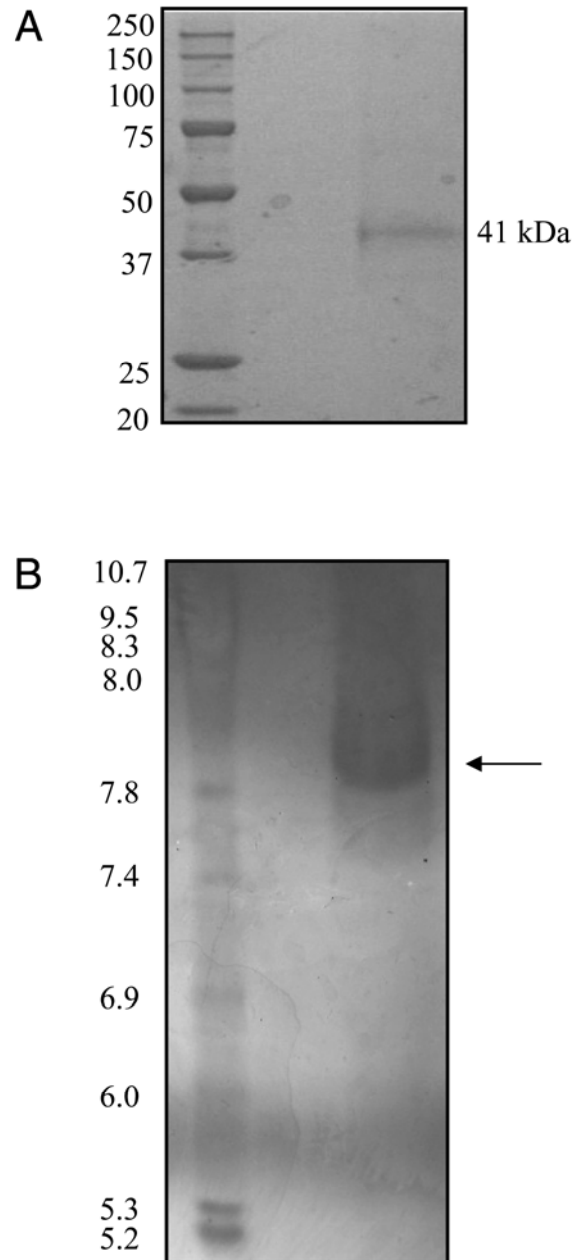
Fig. 3. A, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12%) of purified polygalacturonase produced by *Penicillium expansum*-decayed pear fruit that was stained with Coomassie blue. Sizes of molecular weight markers are indicated in kDa. B, Isoelectric focusing polyacrylamide gel electrophoresis of purified polygalacturonase produced by *P. expansum* in decayed 'Anjou' pear fruit stained with silver. The pIs of standard proteins are indicated on the left.
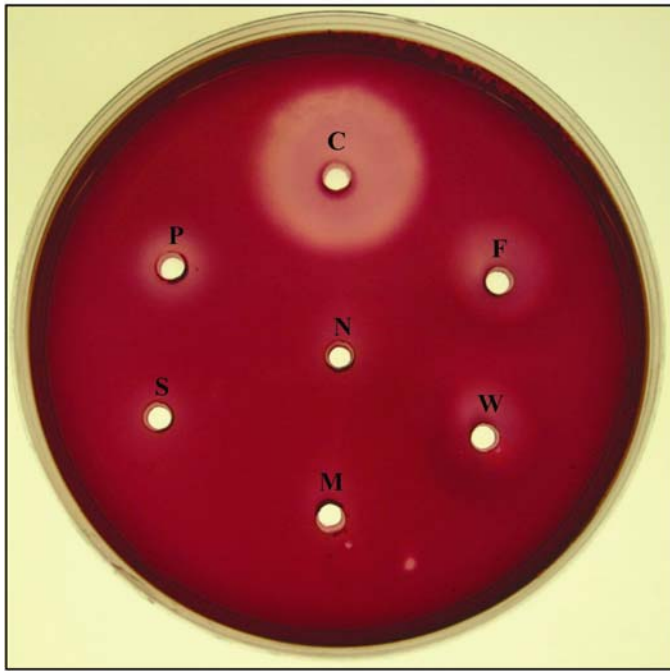
Fig. 4. Polygalacturonase plate activity assay of cation exchange-purified enzyme from *Penicillium expansum*-decayed 'Anjou' pear fruit following fractionation on a glycoprotein-binding column. The samples are represented as C = cation exchange purified polygalacturonase from *P. expansum*-decayed pear fruit; F = column flow through; W = column wash; M = mannose elution step; S = sialic acid elution step; P = pectinase from *Aspergillus niger*; and N = water.

The molecular mass of the protein is consistent with reports for other fungal PGs from postharvest fungal plant pathogens, i.e., *Phomopsis cucurbitae*, *Botrytis cinerea*, and *P. italicum* (3,13,27). The PG produced by *P. expansum* during postharvest decay of pear fruit is larger than the 34 kDa PG that was isolated and characterized from *P. expansum*-decayed apple fruit (26). The pIs of the two enzymes differ (7.8 versus 8.1), although both values are comparable to PGs from other fungi (15). The optimal pH for PG activity is also different (4.5 in pear versus 5.5 in apple). However, both values are within the range of pH optima for various fungal PGs (1,14,18,24).

Biochemical properties such as thermolability, lack of glycosyl moieties, and activity at low temperature are shared among various fungal PGs including those produced by *P. expansum* in pear and apple fruit. Thermolability is a common feature of enzymes as high temperatures disrupt higher order structures that are maintained by hydrophobic interactions, hydrogen bonding and disulphide bridges. The purified PG from *P. expansum*-decayed pear fruit was active at temperatures from 0 to 50°C. Activity of the PG over a broad temperature range gives the fungus the ability to macerate tissue and cause decay during cold storage, in the field in storage bins, or on grocery store shelves. Jurick et al. (11) showed that the purified PG from *P. solitum* was active at temperatures as low as 2°C, which substantiates our findings that PGs from another *Penicillium* species have detectable enzymatic activity at low temperatures. The lack of glycosylation was demonstrated for both *P. expansum* PGs. However, a number of PGs from various fungal species (*Saccharomyces cereviseae*, *Aspergillus kawachii*, and *Colletotrichum acutatum*), have been shown to be glycosylated which seems to be the most common form of posttranslational modification for this class of hydrolytic enzymes (15). Thus, the difference in molecular mass between the two *P. expansum* PGs isolated from decayed pear and apple fruit may result from other forms of posttranslational modifications (e.g., myristolation, stearoylation, and geranyl-geranlation, etc.), alternate splicing of the same gene, or different
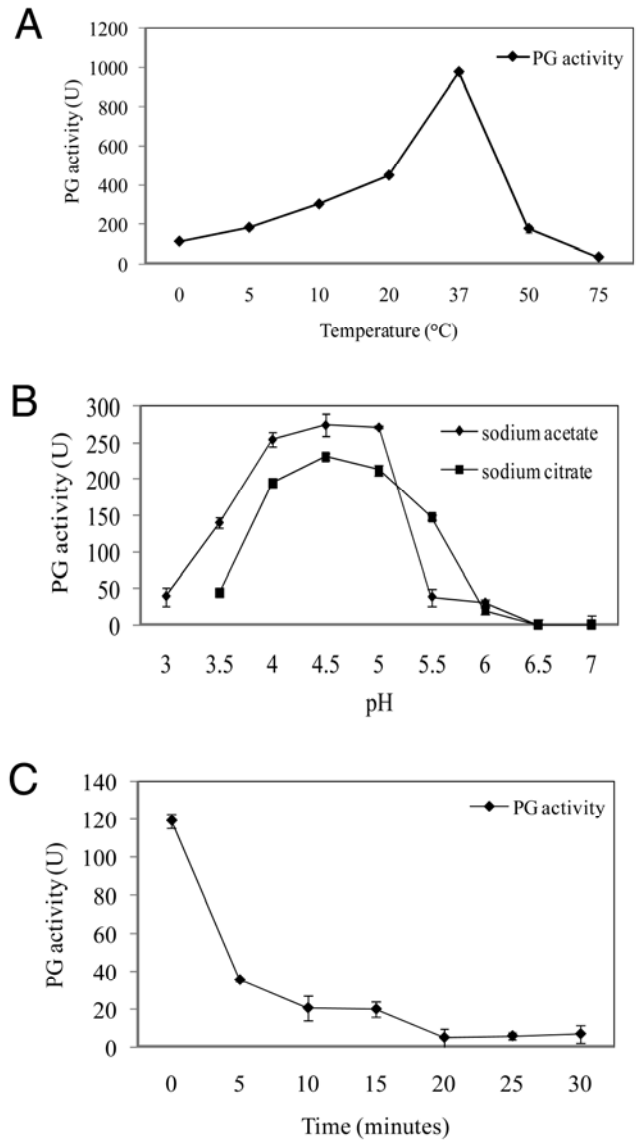

Fig. 5. A, Activity profile of purified polygalacturonase produced by *Penicillium expansum* in 'Anjou' pear fruit at various temperatures. **B,** Activity profile of purified polygalacturonase produced by *P. expansum* in decayed 'Anjou' pear fruit at different pHs. **C,** Activity profile of purified polygalacturonase produced by *P. expansum* in decayed 'Anjou' pear fruit following incubation at 100°C for different periods of time. Each data point represents the mean of three replicates with bars representing standard deviation of the means. All experiments were repeated and data from a representative experiment is shown.

PG genes encoding two distinct polypeptides. The latter possibility is in accord with the earlier observation that *P. expansum* produces at least five PG isozymes in culture (C. L. Yao, *unpublished data*).

The purified PG hydrolyzed sodium polypectate in a mixed manner exhibiting both endo and exo-activity. PG was unable to hydrolyze digalacturonic acid but cleaved the trimer into a monomer and dimer. This result shows that the enzyme requires a minimum length of three galacturonic acid residues for enzymatic cleavage. Mixed PG activity has also been reported for other purified PGs from the various fungal plant pathogens *P. cucurbitae* and *P. expansum* (26,27). However, the PG produced by *P. expansum* during apple fruit decay produced monomers, dimers, and trimers of galacturonic acid after 24 h, whereas the PG produced by the same fungus during pear decay produced monomeric, dimeric, trimeric, and oligomeric forms of galacturonic acid.
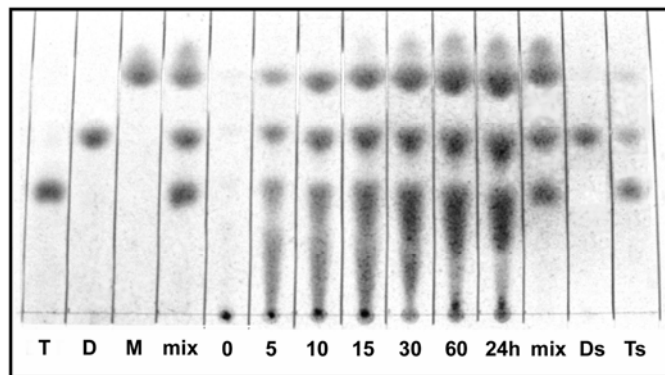
Fig. 6. Thin-layer chromatography of hydrolysis products from purified polygalacturonase produced by *Penicillium expansum* in decayed 'Anjou' pear fruit with different substrates. Lanes 1 through 4, trimer, dimer, monomer and a mix of standard galacturonic acid, respectively; lanes 5 through 11, hydrolysis products of sodium polypectate after incubation with 6 U of purified polygalacturonase enzyme for 0, 5, 10, 15, 30, and 60 min and 24 h, respectively; lane 12, a mixture of monomer, dimer and trimer galacturonic acid standards; lane 13, hydrolysis products of digalacturonic acid for 24 h; and lane 14, hydrolysis products of trigalacturonic acid for 24 h.

An approximately 30% reduction in mass resulted when pear tissue plugs were incubated with native *P. expansum* PG obtained from decayed pear fruit. The reduction in mass indicates the ability of the purified PG to degrade pear fruit tissue in vitro and substantiates the role of this enzyme in postharvest decay of pear by *P. expansum*. Interestingly, a 30% reduction in mass of muskmelon fruit plugs resulted from incubation with a purified PG from *Phomopsis cucurbitae* in vitro which is consistent with data presented in this work (27). The authors concluded that PG may be involved in Phomopsis fruit rot of muskmelon based on the ability of the purified enzyme to macerate muskmelon fruit tissue in vitro. In contrast, only an ≈17% reduction in mass of fruit tissue occurred when the native PG from decayed pear fruit was incubated with apple fruit tissue. The different PG enzymes secreted by the same pathogen on different hosts may offer a "fitness" advantage, by expression of host specific enzymes that maximize degradation of the tissue and colonization. This assertion is further supported by the results from tests where the purified PG produced higher levels (≈5.1-fold increase) of soluble polyuronides in vitro when incubated with pear fruit tissue compared to increase (≈1.8-fold) in buffer with apple fruit tissue. Residual levels of soluble polyuronides detected in the buffer only treatments may have been due to host-derived PGs that were active during the incubation period or may have resulted from nonenzymatic activity as they may have leached out into the buffer from damaged and or dead cells when plugs were cut from intact fruit.

Results from the activity assays indicate that the PG purified from *P. expansum*-decayed pear fruit is of fungal origin. This conclusion is supported by the lack of detectable PG activity from either wounded or intact pear fruit tissue in the plate assay with the 90% ammonium sulfate pellets, and a clear zone of activity on plates with the 90% ammonium sulfate pellet from the *P. expansum*-decayed pear fruit. Also, no PG activity in healthy intact or wounded pear fruit was detectable in crude extracts or ammonium sulfate fractions (40 and 90% supernatant and pellets) using the PG plate activity assay or the quantitative reducing sugar assay (data not shown). Unfortunately, attempts to obtain N-terminal sequence data (Edman degradation method) from the purified PG produced by *P. expansum* during pear fruit decay were unsuccessful as it was determined that the N terminus was blocked.

This is the first report detailing the isolation, purification and biochemical characterization of PG from *P. expansum*-decayed pear tissue, also showing for the first time that the same pathogen
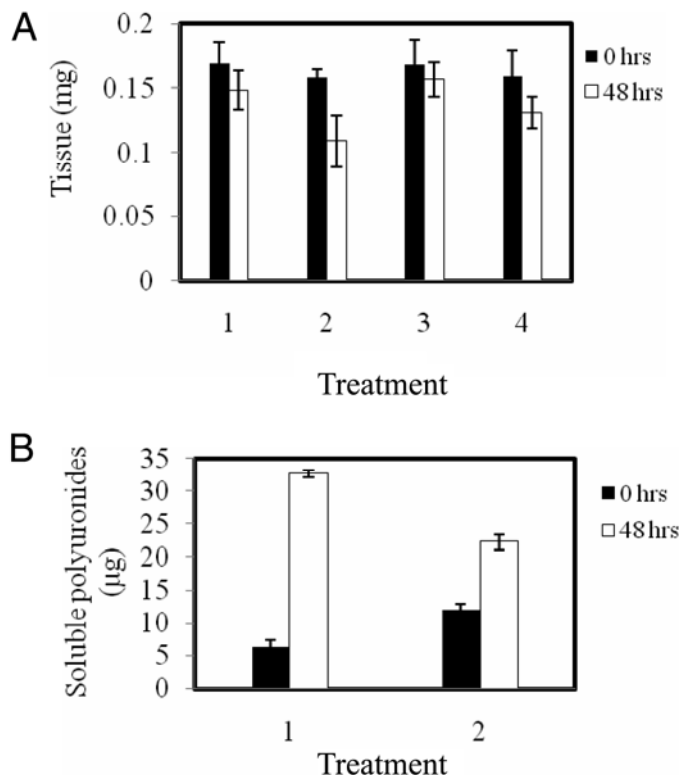

Fig. 7. A, Fresh weight changes of pear and apple tissue plugs after different treatments. Treatment 1, buffer alone with pear tissue; treatment 2, polygalacturonase purified from decayed pear in buffer with pear tissue; treatment 3, buffer alone with apple tissue; and treatment 4, native polygalacturonase in buffer with apple tissue. **B,** Total soluble polyuronides liberated in 100 mM sodium acetate buffer (pH 4.5) from pear and apple tissue in vitro. Treatment 1, pear tissue with native polygalacturonase; and treatment 2, native polygalacturonase in buffer with apple tissue. All experiments were repeated and the results from a representative test are shown.

on a different host secretes a different PG enzyme. Moreover, this work also provides a foundation for molecular biological studies of PG in *P. expansum*–pear fruit interactions. Elucidation of the role of PG by molecular cloning and functional analysis will provide further insight into the mechanisms of invasion and colonization by this pathogen, and will lead to development of more specific and effective control strategies against postharvest pathogens.

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