Purification and Characterization of a Polygalacturonase Produced by *Penicillium expansum* in Apple Fruit

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ABSTRACT

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A polygalacturonase (PG) was purified from rotted cortical tissue of cv. Golden Delicious apple fruit inoculated with *Penicillium expansum*. The purified protein had a pI of 8.10 and a molecular mass of 34 kDa. The PG was heat labile and most active at pH 5.5. There were no detectable *O*- or *N*-linked glycans associated with the PG polypeptide. The purified enzyme macerated apple tissue in situ and in vitro. PG activity was not affected by purified apple polygalacturonase-inhibiting protein. Based on the amino acid sequences determined, three degenerate oligonucleotides were synthesized and used as primers to amplify the flanking regions on the fungal genome by polymerase chain reaction (PCR). Amino acid sequences predicted from the cloned PCR products matched perfectly with the determined amino acid sequences, indicating the fungal origin of the purified PG. When a cloned 212-bp PCR product was used as a probe, it hybridized with 1.5-kb RNA molecules extracted from *P. expansum* in rotted apple tissue. However, no hybridized signals were readily detected for RNA isolated either from nonrotted apple tissue or fungal mycelia grown in cultures with apple pectin as the sole carbon source. This demonstrated that the fungal PG was mainly, if not specifically, expressed in the invasion and colonization of fruit.

Additional keywords: pectic enzyme, postharvest decay.

The plant cell wall is a major barrier to the establishment of fungal infection on a host. Most plant-pathogenic fungi produce a number of cell wall-degrading enzymes when grown in liquid culture containing pectin (3,6,9,10). One of these enzymes, polygalacturonase (PG, EC 3.2.1.15), has been implicated routinely in facilitating the invasion and colonization of host tissue, particularly in diseases characterized by tissue maceration or soft rot (2, 3). There is abundant correlative evidence supporting the role of PG in pathogenesis. PG is the first pectic enzyme secreted by fungal pathogens grown on isolated host cell walls (11,14,18). Pretreatment of cell walls with PG appears to facilitate the ability of other cell wall-degrading enzymes to attack their substrates (15). Highly purified PGs from many fungal pathogens have the ability to cause cell maceration and kill tissues in a manner similar to that seen in soft-rot diseases (1,2,5,19). However, conclusive experimental proof for a role of PG in the fungal pathogenic process is lacking at present (25,31).

Differences in numbers and characteristics between PG isozymes produced by fungal pathogens in culture compared to host tissue have been demonstrated frequently. For example, a *Botrytis cinerea* strain produced five PG isozymes in liquid culture containing apple pectin as the sole carbon source (28); however, there was only one PG isozyme of fungal origin present in infected apple tissue (29). Similar phenomena have been observed in other plant pathogenic fungi, such as *Rhizoctonia solani* (3) and *Penicillium expansum* Link (27). Therefore, PG produced in culture may not represent the enzyme elaborated by pathogens during host infection and decay.

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P. expansum, the causal agent of blue mold, is the most important postharvest apple pathogen (21). This pathogen produces at least five PG isozymes in culture (C. Yao, W. S. Conway, and C. E. Sams, *unpublished data*); however, only one PG has been isolated from rotted apple extract (8). Based on these observations, a more direct approach was employed in our studies, in which a PG isozyme secreted by *P. expansum* in the infection process was used instead of those produced in culture. We report here the purification and characterization of a PG produced by the pathogen during infection of apple fruit as the first step in our attempts to elucidate its role in fungal pathogenesis.

MATERIALS AND METHODS

Materials. *P. expansum* was isolated from naturally infected apple fruit and maintained on potato dextrose agar. Mature cv. Golden Delicious apple fruit was harvested from a commercial orchard in Pennsylvania. All chemicals used in this work were purchased from Sigma Chemical Co. (St. Louis) unless otherwise specified.

Extraction and purification of PG. Apple fruits were wound-inoculated by dipping in a spore suspension of *P. expansum* (10^5 conidia per ml) as previously described (7). After 8 days of incubation at 20°C, the peel over the lesion was removed, and the decayed cortical tissue was collected for PG extraction. All procedures were carried out at 4°C unless otherwise indicated. Rotted tissue (in 500-g portions) was homogenized in an equal volume of 20 mM 2-[*N*-morpholino]-ethanesulfonic acid (MES), 1 M sodium chloride, and 1.5% polyvinylpolypyrrolidone, pH 6.0. The homogenate was stirred for 30 min and filtered through Miracloth (Calbiochem-Behring, La Jolla, CA). After filtration, the remaining tissue was reextracted, and the filtrates were combined and centrifuged at 17,700 × *g* for 30 min. The supernatant was concentrated to 100 ml by a Minitan ultrafiltration system equipped with 10-kDa cut-off low protein-binding membranes (Millipore Corp., Bedford, MA). After centrifugation at 39,200 × *g* for 30 min, the

supernatant was concentrated further to 15 ml in a stirred-cell ultrafiltration system fitted with a 10-kDa cut-off membrane (Amicon, Beverly, MA). The concentrate was dialyzed against deionized water overnight. After centrifugation at 39,200 × *g* for 30 min, the supernatant was mixed with 1 ml of 40% ampholytes (pH 3 to 10, Bio-Rad, Hercules, CA) and subjected to preparative isoelectric focusing (IEF) in a Rotofor cell, following the procedures recommended by the manufacturer (Bio-Rad). Fractions with PG activity were combined and used for refractionation. The combined active fractions were mixed with equal volumes of 2 M sodium chloride and dialyzed against 20 mM MES (pH 6.0) overnight to remove the ampholytes in the sample. PG was separated from other proteins by fast protein liquid chromatography (Waters Chromatography Division, Milford, MA) with a Mono S HR 5/5 cation exchange column (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). The dialyzed sample was applied to the column, equilibrated with 20 mM MES (pH 6.0), and eluted at 0.5 ml/min with a 30-ml linear gradient from 0 to 1 M sodium chloride in 20 mM MES (pH 6.0). Fractions (1 ml) were collected and used to assay PG activity.

PG activity assay. PG activity was determined by measuring reducing groups released from sodium polypectate, using D-galacturonic acid as the standard (8,13). A 0.1-ml aliquot of the enzyme preparation was mixed with 0.4 ml of the standard assay buffer, consisting of 100 mM sodium acetate and 0.4% polygalacturonic acid (ethanol-washed), pH 5.5. The mixture was incubated at 37°C for 20 min, and 1.2 ml of 100 mM boric acid-borax buffer, pH 9.0 (26), containing 0.1% 2-cyanoacetamide was added to terminate the reaction. After mixing, the sample was immersed in a boiling water bath for 10 min and cooled in ice water. After equilibration to room temperature, absorbance at 276 nm was measured. Stopped enzyme controls, in which boric acid-borax buffer with 2-cyanoacetamide was added prior to the substrate solution, were run simultaneously with each sample. One unit of PG activity was defined as the amount of enzyme required to release 1 μmol of reducing groups per min under the assay conditions employed. The effect of apple polygalacturonase-inhibiting protein (PGIP) on PG activity was evaluated by incubating 0.025 units of purified PG with different amounts of PGIP (33). The pH optimum for PG activity was determined by the standard assay buffer in a pH range of 4 to 7. The heat stability of PG was measured by boiling aliquots of enzyme for different periods of time.

Protein assay and gel electrophoresis. Protein was determined by the method of Bradford (4) with a Bio-Rad protein assay kit with bovine serum albumin as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins was performed in a Bio-Rad Mini-protean II cell according to the method described by Laemmli (17). The molecular mass of purified PG was estimated by comparison to Sigma SDS-7 molecular weight markers. Analytical IEF of purified PG was performed on a SE600 vertical slab gel unit, following the protocol provided by the manufacturer (Hoefer Scientific Instruments, San Francisco). The pI of the PG was determined by calibrating against standard proteins with known pIs (Pharmacia). The gels were stained with a Bio-Rad silver stain kit according to the manufacturer's recommendations.

Deglycosylation of PG. Aliquots of samples containing 1 μg of purified PG were deglycosylated by *N*-glycosidase F and *O*-glycosidase (Boehringer Mannheim Biochemicals, Indianapolis, IN), following procedures described previously (33).

Analysis of hydrolysis products. Purified PG (0.37 units) was used to determine its hydrolysis products in a 0.5-ml reaction mixture under the same conditions as in the PG activity assay. After incubation for 0, 5, 10, 15, 30, and 60 min and 24 h, a 25-μl sample was taken out and heated at 100°C for 30 min. A 4-μl sample from each time was run on silica gel 60 (Merck, Darmstadt, Germany) with the monomer, dimer, and trimer of galacturonic acid as standards. The gel was developed with ethyl acetate/acetic acid/formic acid/water (9:3:1:4) as the mobile phase. Galacturonic acids were detected by spraying 15 ml of 0.2% orcinol in sulfuric acid/methanol (1:9) on the gel plate, followed by heating at 105°C for 3 to 5 min (12).

Tissue maceration assay. Apple fruit was kept at 37°C overnight to equilibrate tissue temperature. To test the tissue maceration ability of PG in situ, 1-cm-deep holes were made in apple fruit with a No. 1 cork borer; after which, 100 μl of 100 mM sodium acetate (pH 5.5), 0.37 units of PG, and the same amount of denatured PG in the sodium acetate buffer were added to each hole made in the apple fruit. PG was denatured by boiling in a water bath for 1 h. Apple fruit was kept at 37°C for 24 h, after which tissue maceration activity was evaluated. For maceration assays in vitro, a plug of cortical tissue was removed from apple fruit with a No. 2 cork borer. After removing the peel, this tissue was cut to a length of 1.5 cm and weighed. Each tissue sample was incubated with 0.37 units of purified PG in 2 ml of 100 mM sodium acetate (pH 5.5). Buffer alone and denatured PG in the buffer were used as controls. Maceration assays from each treatment were repeated four times. After incubation at 37°C for 24 h, tissue samples were removed from the test tubes and weighed.

Protein sequencing. Purified PG (10 μg) was used to run a SDS-PAGE and electroblotted onto a polyvinylidene diflouride membrane (Bio-Rad) according to the method described by Towbin et al. (30) and stained with 0.5% Ponceau S (Sigma) in 0.1% acetic acid (22). Protein digestion and sequencing were performed at the Harvard Microchemistry Facility (Cambridge, MA). After digestion with trypsin, peptides were separated by high performance liquid chromatography. Two tryptic peptides were subjected to protein sequencing with an ABI 494 protein sequencer equipped with an 190 PTH analyzer (Applied Biosystems, Foster City, CA).

Nucleic acids isolation. Apple leaves and *P. expansum* mycelia grown in potato dextrose broth (Difco Laboratories, Detroit) were ground to a fine powder in liquid nitrogen and suspended in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM EDTA, 3% SDS, and 1% 2-mercaptoethanol, followed by incubation at 65°C for 1 h. After successive extractions with phenol/chloroform, nucleic acids were precipitated with ethanol. The pellets were dissolved in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA, and genomic DNA was further purified by equilibrium centrifugation in cesium chloride-ethidium bromide gradients (23).

Fungal mycelia grown in modified Richard's solution (28) containing 0.5% apple pectin were harvested after 2, 4, 6, and 8 days of incubation at 20°C with continuous shaking (100 rpm). Rotted and nonrotted tissue from *P. expansum*-inoculated apple fruit was collected at day 8 after inoculation. Powdered apple tissue and fungal mycelia were suspended in extraction buffer containing 5 M guanidinium thiocyanate, 0.1 M Tris-HCl (pH 7.0), 1% polyvinylpyrrolidone (molecular mass of 40 kDa), 0.5% sodium lauryl sarcosinate, and 1% β-mercaptoethanol. After homogenization in a polytron for 1 min, the homogenates were centrifuged at 12,100 × *g* at 20°C for 10 min. After filtration through Miracloth, the filtrate was layered onto a cushion of 5.7 M cesium chloride and 0.01 M EDTA (pH 7.5) in a clear ultracentrifuge tube and centrifuged overnight to pellet RNA (23).

Polymerase chain reaction (PCR) amplification and cloning. All reagents for PCR, except primers, were purchased from Promega (Madison, WI). A 50-μl mixture, containing 1× reaction buffer, 1.5 mM magnesium chloride, 200 μM of each deoxynucleotide triphosphate, 1 μM of each primer, 50 ng of genomic DNA, and 2.5 units of *Taq* DNA polymerase, was overlaid with 100 μl of mineral oil. Primers were synthesized at Eppendorf (Madison, WI). A negative control containing all reagents, except the template, was run with each primer set. The mixtures were subjected to PCR amplification with a Perkin-Elmer Cetus (Norwalk, CT) thermal cycler for 40 cycles. The temperatures and times used for PCR were as follows: in the first cycle, the mixtures were denatured at 94°C for 4 min, followed by 0.5 min at 40°C for primer

annealing and 1.5 min at 72°C for primer extension; the conditions for the following 39 cycles were the same as the first, except the time for DNA denaturation was reduced to 1 min. The final extension time for PCR amplification was 10 min at 72°C. The resulting PCR products were excised from low-melt agarose gel and purified according to published protocols (23). The purified fragment was cloned into pGEM-T vector following the manufacturer's recommendations (Promega). The plasmid DNA was purified following published protocols (23,35). DNA sequence analysis was performed at the DNA Sequencing Facility, Iowa State University, Ames.

Northern analysis. Gel electrophoresis of 5 μg of total RNA and blotting followed procedures described previously (34). RNA was UV–cross-linked to Hybond N+ membranes (Amersham Corp., Arlington Heights, IL) with a total energy of 125 mJ with a Bio-Rad GS gene linker. A digoxigenin (DIG)-labeled antisense RNA probe was generated with T7 RNA polymerase by a DIG-RNA labeling kit (Boehringer Mannheim) with 1 μg of *Pst*I-linearized pPGPCR. Labeling efficiency was determined by dot blot analysis according to the manufacturer's recommendations. The membranes were prehybridized at 68°C for 1 h in a solution consisting of 5× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate, pH 7.0), 2% blocking reagent (Boehringer Mannheim), 0.1% sodium lauroylsarcosine, 0.2% SDS, and 50% formamide. The hybridization solution was prepared by adding RNA probe to a final concentration of 50 ng/ml in fresh prehybridization solution. Hybridizations were performed at 68°C overnight. Membranes were washed at room temperature for 15 min in 2× SSC and 0.1% SDS twice, then at 68°C for 15 min in 0.1× SSC and 0.1% SDS twice. Hybridization signals were detected with a Genius 7 luminescent detection kit according to the procedures recommended by the manufacturer (Boehringer Mannheim). After addition of chemiluminescent substrate (Lumigen PPD [Boehringer Mannheim]) and incubation at 37°C for 15 min, membranes were exposed to Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, NY) at room temperature to detect hybridization signals.

TABLE 1. Extraction and purification of polygalacturonase (PG) from cv. Golden Delicious apple cortical tissue (500 g) decayed by *Penicillium expansum*

Method	Total volume (ml)	Total protein (mg)	Total activity (unit)	Specific activity (units/mg protein)
Crude extract	1,200	51.6	663	12.8
Minitan	100	9.75	205	21.0
Stir cell	15.0	4.10	152	37.1
Rotofor 1	4.30	0.345	46.1	133.6
Rotofor 2	4.10	0.229	35.5	155.0
Mono S	3.00	0.094[a]	27.4	291.5

[a] Amino acid analysis showed that 5 μg of PG was obtained from 500 g of rotted tissue.

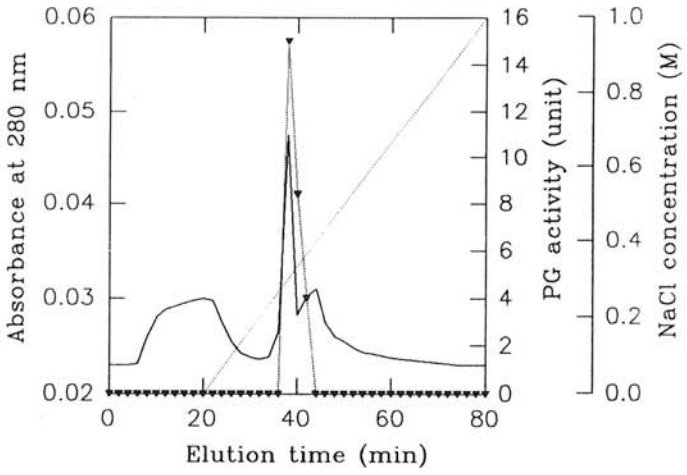

Fig. 1. Elution profile of a polygalacturonase (PG) sample on a Mono S column. Proteins were eluted with a 30-ml linear 0 to 1 M sodium chloride gradient in 20 mM 2-[*N*-morpholino]-ethanesulfonic acid (pH 6.0). Absorbance of fractions at 280 nm (solid line); PG activity (▼); salt gradient (dotted line).

RESULTS

PG purification. PG produced by *P. expansum* during infection and colonization of apple fruit was purified in five steps, involving ultrafiltration, preparative IEF, and cation exchange chromatography (Table 1). The high reading in terms of total activity (663 units) in the crude extract was partially caused by the presence of hydrolysis products produced by PG in situ and other reducing sugars in apple fruit. After removal of the contaminants from the crude extract by dialysis, 51% of the total activity (338 units) remained. Therefore, the low recovery rate by Minitan ultrafiltration was due mainly to the elimination of contaminants instead of the loss of PG protein. After concentration of the extract by ultrafiltration and extensive dialysis against deionized water, the concentrate was used for preparative IEF in a pH range of 3 to 10. The PG protein was effectively focused in the alkaline range, with pH 8.17 in the most active fraction. Preparative IEF removed most nonprotein materials and other proteins in the extract. After refractionation, the combined active fractions were subjected to SDS-PAGE analysis. The results showed abundant protein in the sample, with a few others present in minor amounts (data not shown). Contaminating proteins were removed further by cation exchange chromatography. The PG present in the sample was eluted in 0.3 M sodium chloride when applied to a Mono S column equilibrated with 20 mM MES (pH 6.0) (Fig. 1).

PG characterization. The purity of three pooled active PG fractions from the Mono S column was evaluated by SDS-PAGE and IEF. In both analyses, a single protein component was identified on the gel, indicating the PG had been purified to homogeneity (Figs. 2 and 3). The purified PG had a molecular mass of 34 kDa (Fig. 2), and a pI of 8.10 (Fig. 3). When the enzyme was treated with *O*- and *N*-glycosidases, there was no apparent change in mobility on the gel (Fig. 2). This result indicated that either there were no oligosaccharides associated with the protein, or the glycans on the PG polypeptide were not accessible to the glycosidases tested.

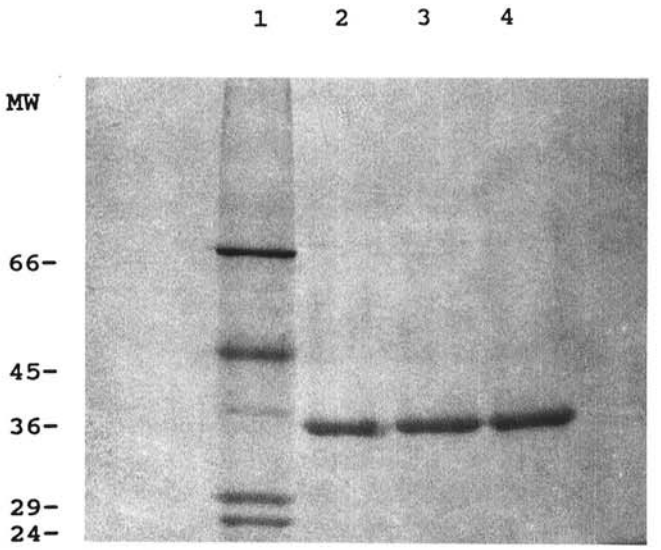

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of purified polygalacturonase produced by *Penicillium expansum* in apple fruit. Lane 1, molecular weight markers; lane 2, nontreated; lane 3, *O*-glycosidase treated; and lane 4, *N*-glycosidase treated.

The pH optimum of purified PG was determined in the pH range of 4 to 7 with 0.32% sodium polypectate in 80 mM sodium acetate buffer as the substrate. The PG was most active at pH 5.5. Only residual activities were detected when the pH was lower than 4.5 or higher than 6.5 (Fig. 4).

The heat stability of PG protein was tested by boiling aliquots of PG preparation for 5, 10, 15, 20, 25, 30, and 60 min. The purified PG was heat sensitive, because 81% of the activity was destroyed when heated at 100°C for 10 min, and no activity remained after heating for 30 min (Fig. 5). When PG was incubated with 1 to 5 units of purified apple PGIP under standard assay conditions, no inhibitory effect was detected.

Purified PG produced by *P. expansum* in apple fruit hydrolyzed polygalacturonic acid in a mixed manner. After incubation with the substrate for 5 min, the hydrolysis products were a mixture of monomers, dimers, and trimers of galacturonic acid, as well as unresolved oligomers. After incubation for 1 h, the principal products were composed of monomers, dimers, and trimers of galacturonic acid (Fig. 6). When apple pectin was used as the substrate, similar results were observed, although the hydrolysis was much less efficient (data not shown). When the substrate was trigalacturonic acid, the enzyme also produced hydrolysis products, although at a very low rate (Fig. 6). The hydrolysis of digalacturonic acid was very minimal (Fig. 6). The standard reducing groups assay was not sensitive enough to detect any PG activity when using di- and trigalacturonic acids as the substrates. The high background in the standard reducing groups assay with pectin as the substrate excluded the application of this method to measure PG activity. The results demonstrated that the purified PG behaved primarily as an endoenzyme, with limited exoactivity.

Tissue maceration. Purified PG was added to holes made in apple fruit to test its tissue macerating ability. After incubation at 37°C for 24 h, both the diameter and the depth of the hole increased with added PG. However, there were no apparent changes in holes incubated with sodium acetate buffer and denatured PG. To measure this activity quantitatively, apple tissue plugs were incubated with the buffer, denatured PG and PG, respectively, and the weight change of each treatment was measured. The results showed that the purified PG caused complete maceration and significant weight loss of apple tissue plugs compared to the other two treatments (Fig. 7).

PG origin. Amino acid sequences of two tryptic peptides prepared from purified PG were determined. They were designated

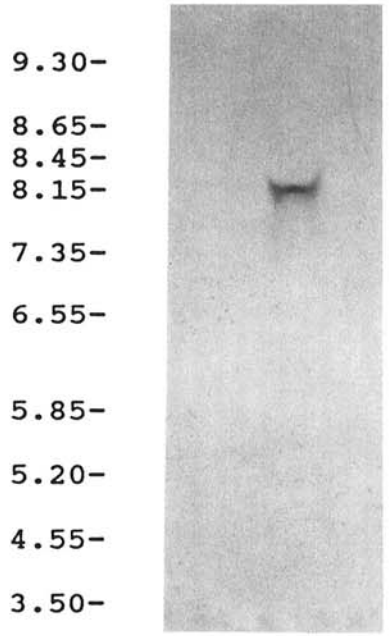

Fig. 3. Analytical isoelectric focusing of purified polygalacturonase produced by *Penicillium expansum* in apple fruit. The pIs of standard proteins are indicated on the left.

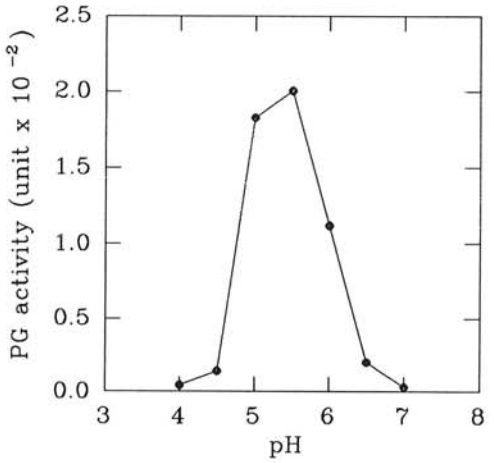

Fig. 4. Activity profile of purified polygalacturonase (PG) produced by *Penicillium expansum* in apple fruit at different pHs.

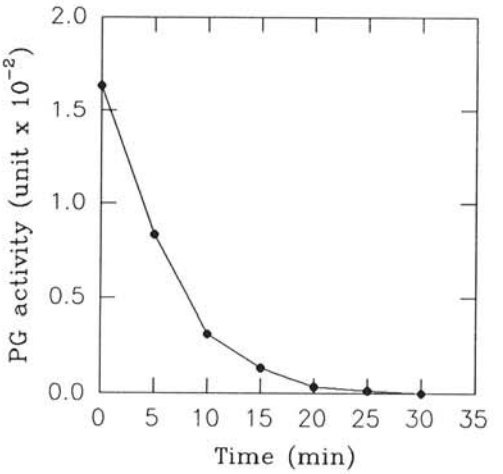

Fig. 5. Activity profile of purified polygalacturonase (PG) produced by *Penicillium expansum* in apple fruit after boiling for different periods of time.

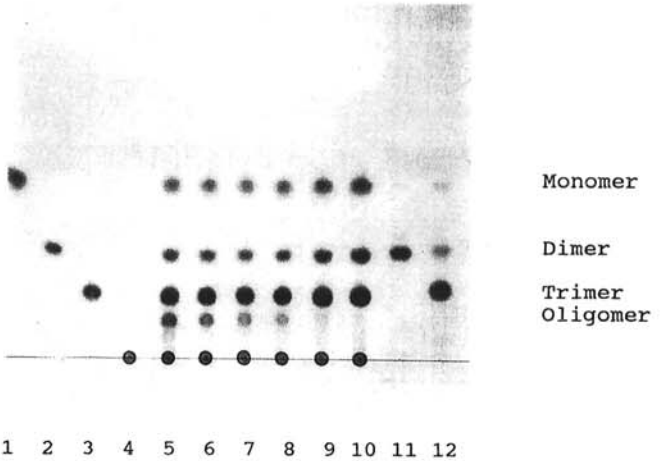

Fig. 6. Thin-layer chromatographic analysis of purified polygalacturonase hydrolysis products from *Penicillium expansum* in apple fruit with different substrates. Lanes 1 through 3, monomer, dimer, and trimer of standard galacturonic acid, respectively; lanes 4 through 10, hydrolysis products of sodium polypectate after incubation with enzyme for 0, 5, 10, 15, 30, and 60 min and 24 h, respectively; lane 11, hydrolysis products of digalacturonic acid; and lane 12, hydrolysis products of trigalacturonic acid.

PEPG56 (-SNNVVETVHISSTQVVNSQNGVR-) and PEPG99 (-GVTFQDITLSGITSQGITIR-). Both peptide sequences were compared with the entire set of sequences available in GenBank (release 84) and EMBL (release 39) by the TFASTA comparison algorithm under GCG protocols. No identical sequences were found. There were no significant similarities between the two peptide sequences and PG sequences from plants and bacteria. However, the lists of best matches consisted of fungal PG sequences, suggesting the purified PG was of fungal origin. Three degenerate oligonucleotides were synthesized based on the determined amino acid sequences (Fig. 8). When oligonucleotides 1 and 3 and the genomic DNA from *P. expansum* were used to perform PCR amplification of the flanking region on the fungal genome, a 212-bp fragment was generated. The specificity of the PCR product was tested with oligonucleotides 1 and 2. As expected, a 176-bp fragment was produced. No PCR products were produced when apple genomic DNA was used as the template. The PCR products were cloned and subjected to sequencing analysis. The amino acid sequence derived from the cloned DNA fragment matched perfectly with the determined amino acid sequences, indicating that the cloned DNA fragment is derived from the authentic copy of the fungal PG gene. The 212-bp cloned DNA fragment contained a 60-bp intron ranging from nucleotides 50 to 109 (Fig. 8). The intron nature of this 60-bp region was supported by the presence of an in-frame stop codon (TAA) and typical intron GT-AG splice junctions. When compared to the published sequences in genetic databases, the fragment showed significant similarities in both nucleotide and amino acid sequences to previously characterized fungal PGs, such as *Aspergillus flavus* 70, *A. oryzae*, *Sclerotinia sclerotiorum*, *Cochliobolus carbonum*, and *A. niger*.

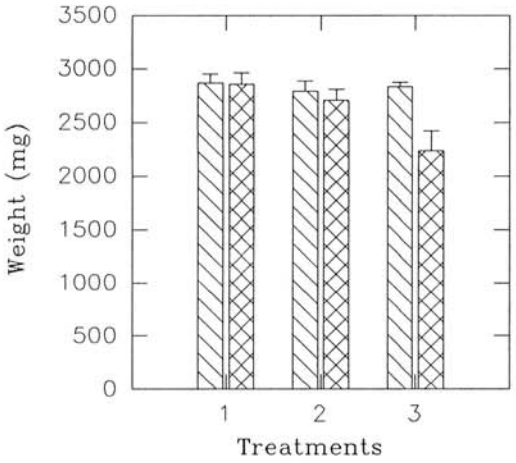

Fig. 7. Fresh weight changes of apple tissue plugs after different treatments. Treatment 1, 100 mM sodium acetate buffer (pH 5.5); treatment 2, denatured polygalacturonase produced by *Penicillium expansum* in buffer; and treatment 3, native polygalacturonase in buffer. Weight before treatment: columns with slash pattern; weight after treatment: columns with x pattern. Vertical bars represent the standard deviations.

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GTGGAGACTGTGCACATCAGCAGCACCCAGGTCGTCAACT
V  E  T  V  H  I  S  S  T  Q  V  V  N  S
    Oligo 1
CTCAGAATGGTAAGCCCGAGCCGCATAATGACTAAGTTTT
  Q  N  G
CCCGTTTCTTGAACTGATATTTCTGTCAGGTGTCCGTGTC
                          V  R  V
AAGGCTGTCTCTGGTGCCACCGGTACTATCAAAGGCGTGA
K  A  V  S  G  A  T  G  T  I  K  G  V  T
CTTTCCAGGATATTACCCTCTCCGGCATTACGAGCCAGGG
  F  Q  D  I  T  L  S  G  I  T  S  Q  G
    Oligo 2
TATCACGATCCG
 I  T  I  R
  Oligo 3
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Fig. 8. DNA and deduced amino acid sequences of the cloned polymerase chain reaction product of polygalacturonase produced by *Penicillium expansum* in apple fruit. Amino acid sequences used to design oligonucleotides 1 through 3 are underlined. The determined amino acid sequences are in bold. The intron is marked by the blank amino acid sequence.

PG gene expression in culture and fruit. Total RNA extracted from rotted and nonrotted apple tissue, as well as RNA isolated from fungal mycelia grown in cultures containing apple pectin, was used to determine the presence of PG gene transcripts. When antisense RNA generated from a cloned PCR product (212 bp) was used as a probe, it hybridized with 1.5-kb RNA molecules extracted from rotted apple tissue, but no hybridization was observed for RNA isolated from nonrotted apple tissue (Fig. 9). There was no detectable hybridization signals between the probe and RNA isolated from fungal mycelia grown on apple pectin for 4 and 6 days. However, weak signals were visible with RNA from 2-day cultures after prolonged exposure (data not shown). No sufficient amount of RNA was isolated from 8-day cultures.

DISCUSSION

A PG produced by *P. expansum* was purified to apparent homogeneity, supported by data from SDS-PAGE, IEF, and amino acid sequence analyses. For the first time, the fungal origin of PG isolated from diseased tissue has been demonstrated conclusively by

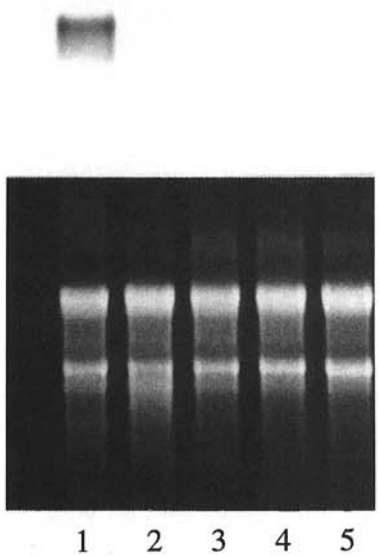

Fig. 9. Analysis of the presence of polygalacturonase gene transcripts in RNA isolated from different sources. Top panel: Northern blot probed with the 212-bp polymerase chain reaction product. Bottom panel: Ethidium bromide-stained gel showing the amount of total RNA present in each lane. Lane 1, rotted apple tissue; lane 2, nonrotted apple tissue; and lanes 3 through 5, *Penicillium expansum* mycelia harvested from cultures grown in apple pectin for 2, 4, and 6 days, respectively.

cloning a segment of DNA from the fungal genome. This provides a necessary prerequisite for further studies on the role of PG in fungal invasion and colonization of host apple tissue.

The purified PG hydrolyzed sodium polypectate and apple pectin in a mixed manner, as opposed to the endoactivity reported previously (27). In the current study, the initial hydrolysis products of PG were composed of monomers, dimers, and trimers of galacturonic acid, as well as unresolved oligomers. After extended incubation, the principal products were still monomers, dimers, and trimers of galacturonic acid. When using dimers and trimers of galacturonic acids as substrates, the enzyme also showed the capability of hydrolyzing the acids, although much less efficiently. This property of the enzyme provides some advantages to the pathogen in utilizing the hydrolysis products as nutrients and more efficiently degrading host cell walls.

The majority of the published PG studies have used enzymes isolated from culture filtrates due to the difficulties in isolating sufficient amounts of enzyme from diseased tissue. It is becoming clear that plant pathogens may produce different sets of enzymes during their saprophytic growth compared to their pathogenic stages. In the case of PG production, both *P. expansum* and *B. cinerea* produced five PG isozymes when grown in culture medium containing apple pectin (28; C. Yao, W. S. Conway, and C. E. Sams, *unpublished data*), but only one PG from each pathogen was isolated from diseased apple tissue (8,29). The bacterial pathogen *Erwinia chrysanthemi* EC16 secreted multiple pectate lyase isozymes when grown in culture. Mutations of all four pectate lyases encoding genes did not eliminate its ability to macerate the host tissue, although the virulence of the mutant was reduced. A second set of plant-inducible pectate lyase isozymes were identified in diseased chrysanthemum tissues (16). The diversity of cutinase isozymes produced at the saprophytic and pathogenic stages also was reported in the plant-pathogenic fungus *Alternaria brassicicola* (34). Because many plant pathogens must live on dead plant materials at some time during their life cycle, it is not surprising to find they possess two sets of enzymes. One set supports saprophytic growth of the organism, whereas the other set facilitates infection and colonization of host tissue. The finding that the gene encoding the purified PG was mainly expressed by the fungal pathogen during infection of its host further supports this hypothesis. However, this does not suggest that cell wall-degrading enzymes produced in culture may not be present in host tissue, as was found in *E. carotovora* subsp. *carotovora* (32).

The potential role of purified PG in fungal infection of apple fruit was supported by the evidence that it can cause complete tissue maceration in situ and in vitro, a major characteristic of soft-rot diseases. Apple PGIP, a proposed general defense mechanism in apple fruit, showed no inhibitory activity toward the purified PG. This further indicated a role for PG in overcoming host defense mechanisms. PG is a virulence factor in the plant-pathogenic bacteria *Pseudomonas solanacearum* and *Agrobacterium tumefaciens* biovar 3, because the mutants with inactivated PG genes were less virulent on their hosts (20,24). A targeted gene disruption of the fungal PG gene was performed only in a maize pathogen, *C. carbonum*, and had no effect on pathogenicity (25). However, there is no data suggesting this PG is produced by the fungus during infection of maize plants. The PG purified in this study was apparently the enzyme produced by *P. expansum* in its pathogenic stage. The elucidation of its role by gene cloning and disruption will provide a better understanding of the mechanisms of fungal invasion and colonization of host tissues, leading to the employment of more specific and effective strategies to control plant diseases.

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