Identification of Pectinases Produced in Cotton Bolls Infected with *Aspergillus flavus*

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ABSTRACT

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Pectinases from *Aspergillus flavus* liquid cultures or cotton bolls wound-inoculated at 30 days postanthesis (DPA) with the same fungus were analyzed by isoelectric focusing. The same three *A. flavus* pectinases (P1, P2c, and P3) were detected in both liquid cultures containing pectin and the immature lint of inoculated 30 DPA bolls. Pectinase activity was first detected in lint and seed extracts 2 days after inoculation and increased to maximum level in 3–5 days. The major pectinase (P2c) of the three activities examined in both liquid media and infected bolls was identified and partially characterized. Rates of pectolytic-induced viscosity loss and production of reducing sugars, the pattern of release of oligogalacturonide fragments, and the absence of transeliminative products in reactions indicated that P2c was an endopolygalacturonate hydrolase. Activities of P1 and P3 but not P2c were subject to catabolite repression in fungal cultures containing glucose. The early accumulation and continued presence of pectinases within infected tissues suggested that they were involved in establishment of this fungus in locular tissues.

Additional key word: cotton boll rot.

Most of the initial research on plant pathogen-produced pectic enzymes involved bacterial and fungal soft-rotting organisms (4,8,9,16). Pectic enzymes from soft-rotting microbes were shown to macerate plant tissues directly or to render plant cell walls more susceptible to attack by other cell wall-degrading enzymes (5,8).

In addition to causing crop losses due to soft rot and spoilage, some fungal organisms elaborate zootoxigenic and carcinogenic mycotoxins in food crops. *Aspergillus flavus* Link ex. Fries was shown to produce cell wall-degrading enzymes that were associated with fruit spoilage (1) and to produce high levels of aflatoxins during infestation of cottonseed (3). *A. flavus* enters cotton bolls through wounds or natural openings (3,10). The fungus often grows through the lint and into the seed where aflatoxins are produced. Virtually nothing is known about the physiology of the *A. flavus*-cotton interaction. *A. flavus*-produced cell wall-degrading enzymes, such as those catalyzing pectic breakdown, may play a role in this fungal-plant interaction; these enzymes are often associated with wound-parasites. However, the presence of pectolytic activities in cotton bolls infected with *A. flavus* has not been established, and little is known about the characteristics of the individual activities produced by this fungus (1).

The purpose of the current investigation was to identify and characterize pectic enzymes produced in cotton bolls wound-inoculated with *A. flavus* and to compare their electrophoretic and enzymatic properties with activities produced in pure *A. flavus* cultures. This procedure was used to discriminate between *A. flavus* enzymes and pectic enzymes produced by plant tissues or secondary microbial invaders.

MATERIALS AND METHODS

Cultures. *A. flavus* (SRRC 1100) was maintained at 28 C on potato-dextrose agar (PDA) plates or slants. The liquid medium was similar to that employed in a previous investigation (2), but the carbon source was varied for production of pectolytic enzymes. Liquid media (25 ml) containing glucose (5%) (GLM) or grade I (7.7% methoxy content) citrus pectin (1%) (PLM) (Sigma, St. Louis, MO), or a combination of glucose (5%) and pectin (1%) (GPLM) were adjusted to pH 5.0 and sterilized, and each was inoculated with a 5-mm plug of mycelium and spores from a 2-wk-old lawn of *A. flavus*. Cultures were incubated at 28 C without shaking until mycelial mats covered the media surface (4 or 5 days).

Isolation of pectinases from culture filtrates. Mycelial mats were removed with forceps and medium was centrifuged at $2\times10^4\,g$ for 15 min to pellet remaining mycelia and spores. Supernatants were carefully removed and the pellets were combined with mycelial mats for dry weight analyses. Supernatants were dialyzed (12,000 MW cutoff) for 16 hr at 4 C against 9 L of 1% (w/v) glycine. Material retained in dialysis bags was concentrated (about 10-fold) by overlaying dialysis bags with crystalline sucrose at 4 C for 16 hr. The concentrated solutions were used for activity assays, isoelectric focusing (IEF), or native polyacrylamide gel electrophoresis (PAGE). Soluble protein was determined using the Bio-Rad (Richmond, CA) protein assay reagent and method, employing bovine serum albumin for a standard.

Inoculation and sampling of cotton bolls. Cotton plants (Delta Pine 61) were grown in field plots at the Southern Regional Research Center, USDA, in New Orleans, LA. Flowers were labeled on 22 July 1985, and unopened bolls were wound-inoculated 12 or 30 days postanthesis (DPA) by first removing a 2.5-mm plug from the carpel suture area with a hollow tube and inoculating the exposed lint with dry spores (from 2-wk-old cultures growing on PDA slants at 28 C) on the tip of a small paint brush. Bolls inoculated at each of the two different times of development (12 or 30 DPA) were collected for a 1-wk period starting immediately after inoculation. Temperatures averaged 26.1 ±0.9 and 25.6 ±0.6 C during the 1-wk periods following inoculation of the 12 and 30 DPA bolls, respectively. Controls were wounded but received no *A. flavus* spore inoculum. At various times following wounding, several three-boll samples were removed, and certain portions (lint or carpel wall) of single locules were excised for extraction of pectinase activity.

Extraction of pectinases from cotton bolls. Lint containing seed from a single inoculated or uninoculated locule was extracted by placing tissue in 10 ml of cold (4 C) 0.1 M sodium acetate buffer, pH 4.5. Air was squeezed from the lint with a spatula, and tissue was soaked in the buffer with occasional agitation for 15 min. Buffer was squeezed from the lint and seed and retained. Tissue from a single inoculated carpel wall (from about a 1-cm radius

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around the wound site) was ground in 2 ml of 0.1 M sodium acetate, pH 4.5 (4 C), with a mortar and pestle. Extracts from lint or carpel wall tissues were centrifuged for 10 min at 5×10^3 *g* to remove insoluble material. Half of the supernatant liquid from each of the lint extractions was dialyzed and concentrated about fivefold by the same method used for preparation of concentrated pectinase from culture filtrates. Pectinases in the concentrated solution, containing about 0.4 mg of protein per milliliter, were resolved by IEF to assess levels of individual activities. Carpel wall extracts and the remaining half of the lint extractions also were concentrated about fivefold in dialysis bags (with no dialysis) and assayed directly for pectinase activity.

Activity assays. Radial diffusion activity assays used in the present study were a modification of the cup-plate assay (7,22). The assays were conducted using substrate gels containing either 0.5% citrus pectin or polygalacturonic acid (PGA) and 1% agarose (Sigma) in 0.1 M sodium acetate, pH 4.8. Pectin, PGA, and agarose powder mixtures were solubilized in 0.1 M acetate while being stirred at 90 C. The pH was adjusted with 0.1 M NaOH while the agarose was still molten (50 C). Media (10 ml) were poured into 10-cm petri plates, and activity assays were initiated by placing 20 μl (containing about 10 μg of protein) of test solutions into 3-mm-diameter wells cut into the pectin or PGA-agarose layer. Some IEF gels were assayed for pectinase activity by removing 3-mm-diameter plugs along the length of gels and placing them in the 3-mm wells. Plates were incubated for 16 hr at 28 C and then stained for 20 min with 10 ml of 0.05% ruthenium red (Sigma). Plates were rinsed with deionized water, and the radius of clear zones around wells, resulting from pectolytic activity, was measured.

IEF and PAGE gels also were assayed for bands of pectinase activity using a PGA (sodium or nonsodium salt) and/or pectin-agarose overlay method (19). Buffers in some overlays were varied for differential detection of pectolytic enzymes (19). Pectic lyase was assayed in 50 mM Tris-hydrochloride, pH 8.5, containing 1.5 mM $CaCl_2$, and endopolygalacturonases were assayed in 50 mM potassium acetate, pH 4.5, containing 10 mM ethylenediaminetetraacetic acid (EDTA). Overlays remained in contact with gels for 15–30 min at 30 C, after which they were removed, stained with ruthenium red, and air dried overnight. Dried overlays sometimes were placed between two glass plates and scanned for percent transmittance at 548 nm.

The ability of boll extracts to reduce the viscosity of a pectin solution was assayed by addition of about 200 μg of protein in 0.5 ml total volume of dialyzed concentrated extracts to 9.5 ml of 1% pectin in 0.1 M sodium acetate, pH 4.8, containing 20 mM EDTA. Relative viscosity (20) of solutions was measured with a No. 100 Cannon-Fenske viscosimeter after a 2-hr incubation at 28 C. In one experiment, a focused band (after IEF) of pectinase activity was eluted from a gel strip (as described in the next section) and assayed viscosimetrically in 0.5% PGA using the same method for assaying boll extracts. The production of reducing sugars was measured simultaneously in the PGA solution by removing 0.5-ml aliquots from the reaction mixture at various times of incubation and assaying for galacturonic acid (Sigma) equivalents by the method of Somogyi (21) as adapted by Nelson (17).

Portions (25 μl) of pectolytic enzyme products released from 0.5% PGA by certain enzyme preparations were analyzed by thin-layer chromatography (TLC) on cellulose plates using butanol:water:acetic acid (5:3:2, v/v/v) as the solvent system (23). Plates were developed for about 4 hr, air dried, and immersed in a solution of bromophenol blue in 95% ethanol (50 mg 100 ml^{-1}) containing 1 mM Tris-hydrochloride (pH 7.5). Oligogalacturonides were visualized as yellow bands against a blue background. Galacturonic acid was used as a TLC standard.

Pectin lyase activity was assayed in extracts of 7-day inoculated bolls and concentrated culture filtrates by placing 50 μl of the enzyme preparation (about 20 μg of protein) into 1 ml of 0.25% pectin or PGA in 0.05 M Tris-HCl, pH 8.5, containing 1 mM $CaCl_2$ (28 C). Absorbance was monitored at 235 nm (16).

Nonequilibrium IEF. The solution for casting vertical polyacrylamide gels for IEF consisted of 5% acrylamide (w/v), 0.5% *N*, *N'*-methylene-bisacrylamide (w/v), 80 μl of 10% (w/v) ammonium persulfate, 50 μl (v/v) of *N*, *N'*-tetramethylenediamine, and 1.5 ml of ampholytes (1.25 ml, pH 3–10; 0.25 ml, pH 2.5–5) (all from Sigma) per 30 ml of total volume. Slab gels (14 × 14 × 0.08 cm) were cast by pouring the solution between two vertical glass plates clamped on a vertical electrophoresis apparatus and allowing polymerization to occur for 1 hr. Concentrated pectinase samples (10 μl) containing about 5 μg of protein and ampholyte buffers (same concentration as in IEF gels) were applied to sample wells (1 cm wide) and overlayed with 50 μl of a solution consisting of 5% sucrose (w/v) and ampholyte buffers (pH 2.5–5.0, 50 μl ml^{-1}) in deionized water. For some experiments, 250 μl of sample (about 100 μg of protein) was applied to extra-wide (4.5-cm) sample wells; the overlay solution was 250 μl volume in this case. Upper and lower reservoir solutions contained 10 mM phosphoric acid and 20 mM NaOH, respectively. IEF was performed at 4 C for 30 min at 200 V and 3.0 hr at 400 V. In all experiments, gels were immediately assayed for bands of activity using pectin-agarose overlays. In some IEF experiments using 4.5-cm-wide sample wells, gels were fractionated along their lengths by slicing at 4-mm intervals beneath the wells. One 4.5 × 0.4-mm gel slice containing a pectinase band was either directly placed into a sample well for PAGE, or pectinase activity was eluted overnight in 0.5 ml of 1% glycine and used for activity assays. Some gel strips were eluted in deionized water overnight for pH determinations.

PAGE. PAGE was conducted using the Laemmli system (12) on slab gels (14 × 14 × 0.15 cm) cast using a solution of 7.5% acrylamide-0.2% bisacrylamide (w/v) for the resolving gel and a solution of 4.5% acrylamide-0.12% bisacrylamide (w/v) for the stacking gel. In some experiments, crude pectinase from concentrated culture filtrates (about 80 μg of protein in 200 μl) was applied to 4.5-cm-wide wells. In other experiments, gel slices taken from IEF gels containing a band of pectinase activity were placed directly into the wells. Electrophoresis was carried out at 4 C for 20 min at 15 mA to achieve stacking and 4 hr at 30 mA to resolve proteins. PAGE gels were soaked for 1 hr in 0.5 L of 0.1 M sodium acetate, pH 4.5, and assayed for bands of pectinase activity using an overlay of sodium PGA-agarose.

RESULTS

Production of pectinases in *A. flavus*-infected bolls. Pectinase activity was detected in extracts of lint taken from single locules wound-inoculated with *A. flavus* at 30 DPA, using viscosimetric or radial diffusion activity assays at pH 4.8. Extracts (0.4 mg of protein ml^{-1}) from lint and seed of infected locules 7 days after inoculation reduced the viscosity of 1% pectin 33% after 2 hr or produced a 4.1-mm radius of activity in pectinase radial diffusion activity assays. Exposure of extracted pectinases to temperatures exceeding 95 C for 2 min destroyed these activities. No detectable pectinase activity was found in extracts of lint and seed from noninfected locules adjacent to infected ones, wounded locules that received no inoculum, and the carpel wall surrounding the site of inoculation. Extracts of inoculated bolls at 12 DPA contained no detectable pectinase activity. However, tissue within punctured locules of both uninoculated and inoculated bolls at 12 DPA showed signs of severe browning and a slimy, watery breakdown (soft rot) resembling pectic maceration. Inoculated lint from locules of bolls at 30 DPA showed a yellow-brown discoloration, which increased in size and severity with time after inoculation, but the tissue demonstrated no sign of soft rot.

Comparison of pectinases, after IEF produced in infected bolls and in culture media. Figure 1 shows the resolution by IEF of pectinase enzymes produced 4 days after infection of lint and seed by *A. flavus* within unopened, 30-day-old cotton bolls. Three major pectinase activity bands were detected in IEF gels using the pectin-agarose overlay method (Fig. 1A) and radial diffusion assays (Fig. 1B). The pectinase activity band demonstrating the most apparent activity by radial diffusion and pectin-agarose overlay detection methods was designated P2c. At least two other activities (P1 and P3) also were detected (Fig. 1A and B). Activity band P2c actually consisted of at least two incompletely resolved

pectinases. As will be shown later, activity eluted from IEF gel slices containing P2c separated into two pectinase activities during PAGE. However, because P2c ran as one major band during IEF (Fig. 1), activity(ies) P2c will be referred to singularly throughout the text. Figure 2 shows a comparison of pectinase activities after IEF of enzymes obtained from *A. flavus*-infected cotton bolls and from pure liquid cultures of the fungus growing on pectin as the sole carbon source. Stained pectin-agarose overlays demonstrated essentially identical activity profiles for pectinases obtained from infected bolls and from pure fungal cultures.

Production of pectinases in bolls at various times after inoculation. The first detectable pectinase activity in single *A. flavus*-infected locules occurred 2 days after inoculation, as determined by radial diffusion and viscosimetric assays (Fig. 3A). Enzyme activities continued to increase until 3–5 days after inoculation; accumulation of activity then nearly ceased and remained approximately the same at least 7 days after inoculation. An experiment was conducted to assess the individual pectinase activities (P1, P2c, and P3) at various times after inoculation (Fig. 3B). Pectinase activity extracted from three inoculated bolls at various times after inoculation was pooled and subjected to IEF, and gels were assayed for activity using pectin overlays. Stained overlays representing each time period were scanned for zones of pectolytic degradation. The same pattern of results was obtained from gel scans (Fig. 3B) as in radial diffusion and viscosimetric assays of the same extracts in the previous experiment (Fig. 3A). *A. flavus* pectinase activities, P1, P2c, and P3, were all detected about 3 days after inoculation. However, only activities associated with P1 and P2c continued to increase after 3 days, suggesting an accumulation of these two activities in infected bolls. The increase in P2c continued until at least 4 days after inoculation and then ceased; some P1 accumulation continued throughout the 7-day time course, but never reached the activity level attained by P2c. When the time course experiments shown in Figure 3A and B were

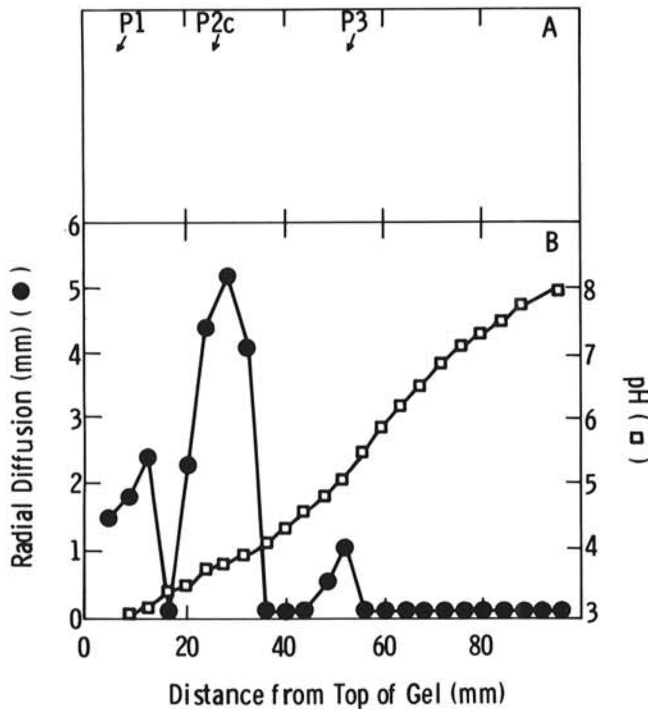

Fig. 1. Isoelectric focusing (IEF) of pectinases extracted from cotton bolls 4 days after inoculation with *Aspergillus flavus*. **A,** Ruthenium red was used to stain pectin-agarose overlays for detection of bands of pectinase activity (unstained regions). **B,** Pectinase activity detected by the radial diffusion method on pectin-agarose. Plugs (3 mm diameter) were removed along the length of IEF gels, placed in 3-mm-diameter wells cut in a layer of pectin-agarose, incubated 16 hr, and stained with ruthenium red. The radius of diffusion of pectolytic activity indicated relative levels of pectinase along the length of the IEF gel.

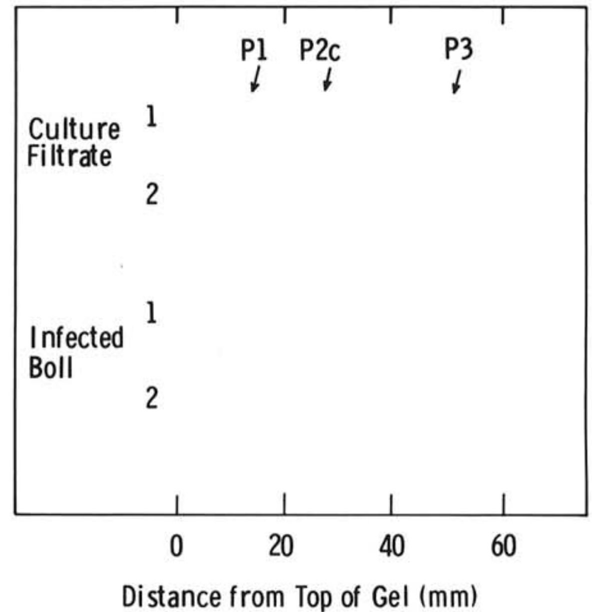

Fig. 2. Comparison of pectinases from *Aspergillus flavus* liquid cultures containing pectin as the carbon source and cotton bolls infected with this fungus (5 days after inoculation). Pectinase activities were resolved by isoelectric focusing and visualized by the pectin-agarose overlay method. Two replicate overlays are shown for each experiment.

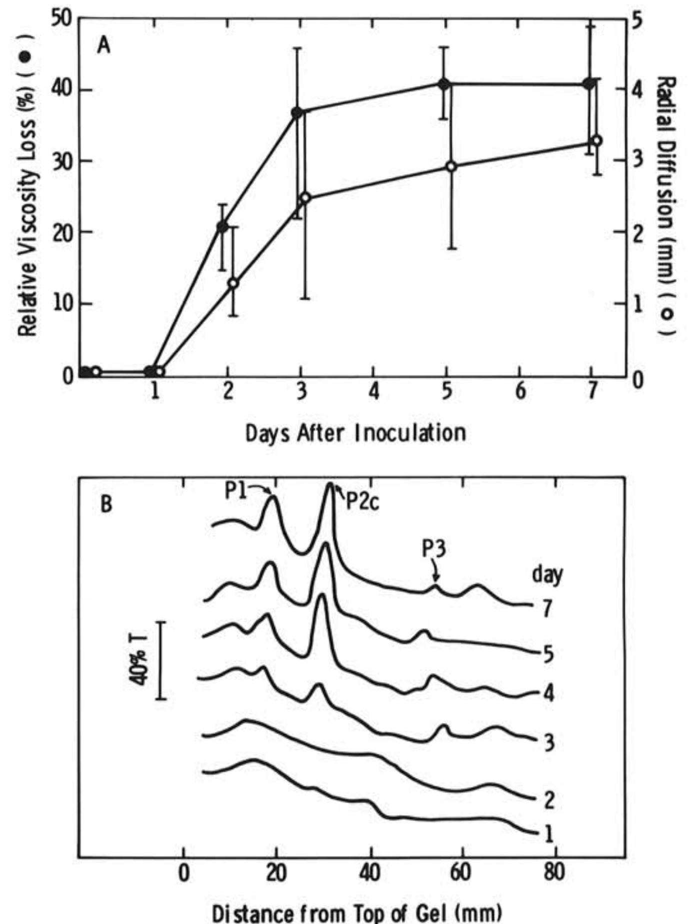

Fig. 3. Levels of pectinase activity in cotton bolls at various times following inoculation with *Aspergillus flavus*. **A,** Radius of diffusion of activity (o) and relative viscosity (%) loss (•) resulting from activity in lint extracts (Materials and Methods). Vertical bars show the range about the mean obtained from three replicate boll samples. **B,** Scans (% T) at 548 nm of stained pectin-agarose overlays obtained after isoelectric focusing of activities present in lint at various times after inoculation.

repeated, essentially the same pattern of results was obtained.

Radial diffusion (modified cup-plate) and pectin-agarose overlay methods corresponded well, on a relative basis, with results from the better-known viscosimetric assay method (Fig. 3A and B). The radial diffusion and overlay methods were less cumbersome than viscosimetric assays. Reducing sugar assays for pectinase activity proved unacceptable when assaying boll extracts, because the bolls possessed high background levels of reducing sugars.

Characteristics of *A. flavus* pectinases. Several experiments were conducted to determine the characteristics of *A. flavus* pectinases. Individual experiments were repeated at least two times with the same pattern of results. Figure 4A shows results from one experiment performed to determine the effects of different carbon sources in *A. flavus* cultures on induction/repression of individual pectinases resolved by IEF. Activities P1, P2c, and P3 all were produced in cultures containing PLM, which produced an average of 0.10 gm mycelial dry weight per culture. The presence of glucose in the pectin medium (GPLM) did not inhibit activity of P2c but abolished activities of P1 and P3. Mycelial growth was enhanced (0.35 gm dry weight per culture) in GPLM relative to PLM and GLM cultures. Only P2c activity was detected in the medium with glucose as the sole carbon source (GLM); 0.23 gm mycelial dry weight per culture was produced in the glucose medium.

The effects of varying pectinaceous substrates and pH in agarose overlays (Fig. 4B) on pectinase activities from *A. flavus* (grown on PLM) culture filtrates were assessed. Addition of EDTA to pectin-agarose (pH 4.8) did not abolish any of the three activities. However, none of the activities were detected using pectin-agarose at pH 8.5 containing $CaCl_2$. Only activity P2c was detected using PGA-agarose overlays. However, the peak height of P2c was slightly reduced (Fig. 4B), and the band on stained PGA-agarose overlays was visibly more faint.

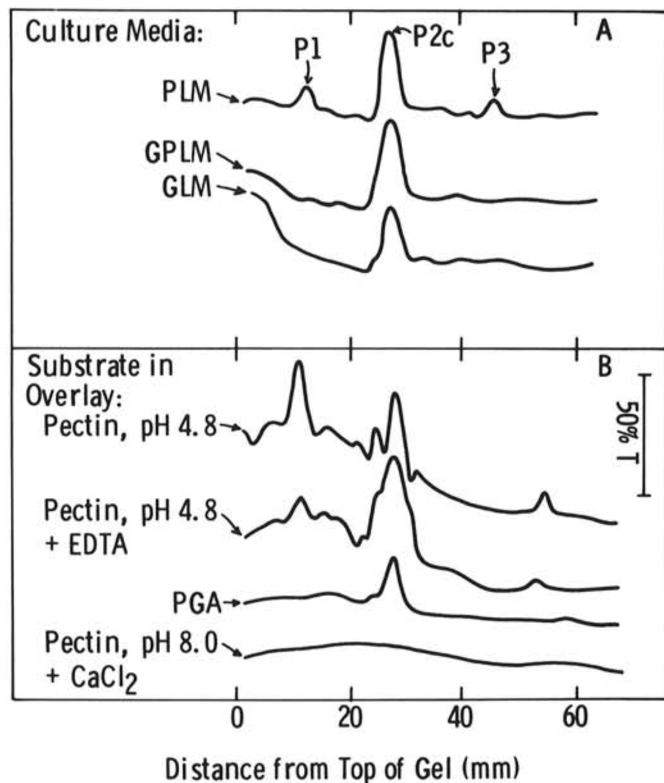

Fig. 4. Characteristics of *Aspergillus flavus* pectinases. **A,** Activities (% T) produced by *A. flavus* cultures with varying carbon sources. PLM, GLM, and GPLM represent liquid media containing pectin, glucose, or pectin plus glucose, respectively, as the carbon sources. **B,** Effects of varying pH or substrate on activities produced by *A. flavus* growing on pectin (PLM) as a carbon source (see A). Pectin at pH 4.8 (plus or minus ethylenediaminetetraacetic acid) and pH 8.0 (plus $CaCl_2$) and PGA at pH 4.8 were used in agarose overlays.

Pectinase activities in 7-day inoculated bolls and in concentrated culture filtrates were tested for the presence of pectin lyase using pectin or PGA as substrates. An increase in absorbance at 235 nm, indicating pectin lyase activity, was not observed in any of the preparations after 4 hr of incubation at 28 C.

Activity P2c was partially purified from the *A. flavus* GLM culture filtrate by IEF and elution of gel slices containing P2c. Addition of the partially purified P2c to a solution of PGA resulted in 50% viscosity loss in 7 min with only about 0.5% hydrolysis of the polymer (Fig. 5). Analysis by TLC of oligogalacturonide fragments released by P2c in the above PGA solution revealed the accumulation of several multimeric fragments after 1 hr of incubation. The R_{gal} (relative to the galacturonic acid monomer) values of four fragments (0.74, 0.51, 0.34, and 0.25) closely resembled the values obtained previously (23) for dimeric through multimeric fragments, respectively.

Separation of pectinase activities in peak P2c by PAGE. The dialyzed, concentrated filtrates from *A. flavus* GLM cultures, in which only activity peak P2c was detected using pectin (Fig. 4A), PGA, or sodium PGA-agarose overlays (not shown), and fractions from IEF gels containing only P2c were subjected to native PAGE. PAGE separated activities in both crude and partially purified preparations containing P2c activity into two predominant activities; the position of the activity bands from crude or partially purified (by IEF) sources was similar, as determined by sodium PGA-agarose overlays (Fig. 6).

DISCUSSION

At least three acidic pectinases, P1, P2c, and P3, were produced in *A. flavus*-infected cotton bolls at 30 DPA and in pure *A. flavus* culture containing citrus pectin as the sole carbon source. This finding indicated that only *A. flavus*-produced pectinases accumulated in boll tissues after wound-inoculation and demonstrated the absence of other microbial pectinases or endogenous plant pectinases. The accumulation of *A. flavus* pectinases during invasion of boll locules indicates that these enzymes may be important during the establishment of the fungus in host tissues. In support of this hypothesis, a prior investigation demonstrated that *A. flavus* produced pectinases during invasion of tomato fruit (1). Infection of tomato fruit by *A. flavus* resulted in a dry rot, whereas *A. fumigatus* infection produced a softening of tomato fruits, typical of pectic maceration. *A. flavus* can invade wounded cotton bolls, causing a boll rot (18) and damage to the lint (6,14), suggesting the involvement of cell wall-degrading activities in these previous investigations.

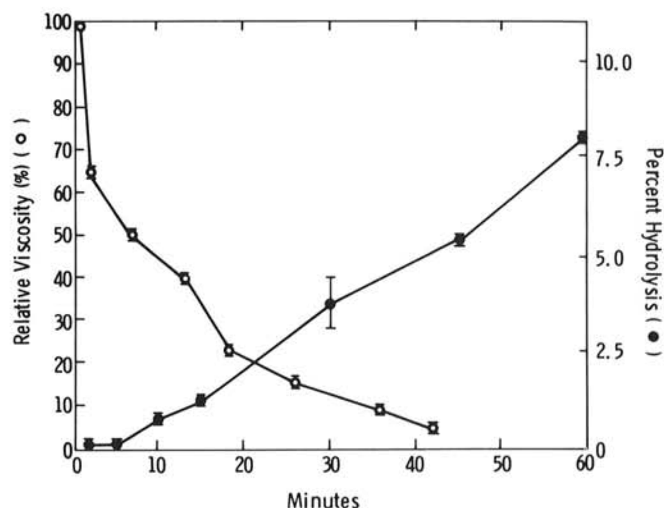

Fig. 5. Reduction in relative viscosity of (o) and percent of bonds hydrolyzed in (•) PGA by P2c. P2c was partially purified by isoelectric focusing before addition to the substrate.

A. flavus pectinases were detected only in extracts of immature lint and seed, suggesting that the fungus was colonizing only these tissues; no pectolytic activity was detected in carpel walls at the site of inoculation. The fungus probably had penetrated only into outer lint layers 2 days after inoculation when pectinase activity was first detected. Other workers demonstrated that at least 4 or 5 days were required for *A. flavus* to grow deeper into locular tissues and reach the seed (13).

A prior investigation showed that polygalacturonic acid, a substrate for pectinases, is present in cotton fibers of certain developmental stages of the cotton boll (15). Chemically extractable uronic acids increased from 4 to 10×10^{-10} g/mm fiber length between 4 and 16 DPA, respectively. Thereafter, a sharp drop in uronic acids occurred, yielding 5×10^{-10} g/mm fiber after 18 DPA; uronic acid content appeared to stabilize at this stage in development and remained approximately the same after 22 DPA. Digestion of fibers with *Colletotrichum* endopolygalacturonase resulted in solubilization of 60–70% of the total uronic acids (15). Thus, pectinaceous materials are present in cotton fibers at least through 22 DPA and might remain available for digestion and solubilization by *A. flavus* pectinases produced in the lint during the present study. It was not surprising that *A. flavus* pectinases were not detected in inoculated bolls at 12 DPA. The optimal stage of boll development for *A. flavus* infection after wound-inoculation has been shown to occur at about 33 DPA (13).

Activity P2c clearly was predominant over activities P1 and P3; therefore, it was further characterized. Pectinase P2c is probably an endopolymethylgalacturonate hydrolase. The rapid reduction in viscosity of solutions of PGA with little associated increase in reducing equivalents (Fig. 5) and the release of various sizes of galacturonide fragments in the presence of activity P2c (partially purified by IEF) indicated an "endo" specificity (23). Pectin and PGA-agarose overlays (Fig. 4B) and radial diffusion assays using P2c (data not shown) on pectin and PGA-agarose plates indicated a slight preference for partially methoxylated pectin over PGA as the substrate; zones of pectolytic activity from P2c were larger, using pectin as the substrate. However, the substrate preference for P2c remains unclear, since the activity of this enzyme was substantial even on PGA. P2c was active in the presence of EDTA at an acidic pH, conditions that are usually not optimal for pectin lyases (19). P2c was not active at a pH optimal for pectin lyases. Thus, a hydrolytic function for P2c is indicated. None of the pectinase preparations in the present investigation contained any detectable pectin lyase activity as judged from the lack of any UV-absorbing transeliminative products released by the activities from pectin or PGA.

Isozymes and/or subunit multimers of pectinase P2c probably are produced both in culture and in *A. flavus*-infected bolls. Occasionally, the nonequilibrium IEF method used during the current study partially resolved activities within P2c, resulting in one major peak with one or two "shoulders" of activity (Fig. 4B). Pectinase activity from GLM cultures, a medium in which only P2c activity was detected, and P2c activity partially purified by IEF resolved into at least two activities when subjected to native PAGE (Fig. 6). The activities resolved by the PAGE method probably compose part of the same incompletely resolved P2c complex occasionally observed on IEF gels. Verification of a complex of pectinase isozymes or subunit multimers in activity peak P2c will require further purification and characterization of the protein(s) involved.

Production of P2c in culture exhibited an apparent lack of catabolite repression in the presence of glucose. Production of the other two major activities (P1 and P3) was repressed in both of the glucose-containing media, a phenomenon commonly occurring with other microbial pectinases (11).

Pectic enzymes P1 and P3 demonstrated an absolute preference for citrus pectin over PGA as a substrate (Fig. 4B) and, therefore, are not part of the pectinase complex associated with P2c. P1 and P3 were only active at pH 4.8 (EDTA present) indicating a hydrolytic mechanism similar to P2c. However, nothing is known at present about the sites of cleavage for P1 and P3 on the pectin polymer.

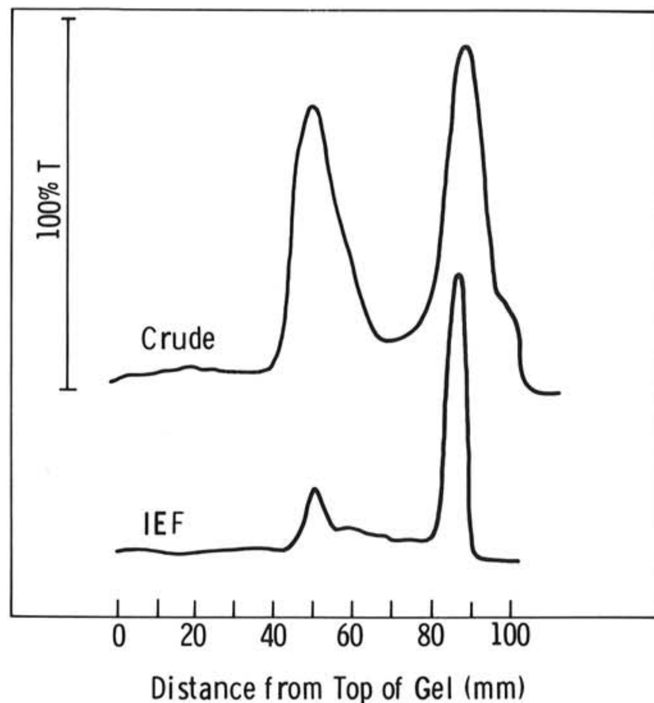

Fig. 6. Separation of pectinase activities in P2c by PAGE. Top curve represents PAGE of dialyzed, concentrated filtrates from liquid medium glucose cultures (demonstrating only peak P2c after isoelectric focusing, see Fig. 4A). Bottom curve represents PAGE of P2c after its partial purification by IEF.

Complete characterization of *A. flavus* pectinases will have to await their purification. However, the present study identified, partially characterized, and established the presence and time course of the activities during invasion of cotton bolls by *A. flavus*. The present investigation sets the stage for further investigations into the physiology of the *A. flavus*-cotton interaction.

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