

## Characterization of Endopolygalacturonase Produced by *Rhizoctonia solani* in Culture and During Infection of Cotton Seedlings

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### ABSTRACT

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An isolate of anastomosis group 4 of *Rhizoctonia solani* produced endopolygalacturonase (endoPG) in culture in response to cotton seed exudates and in infected cotton seedlings 18 hr after inoculation. No other depolymerizing type of pectic enzyme was detected during fungal growth under these conditions. EndoPG from these sources had pH optima of 5.2 and molecular weights of ~42,000 daltons. Enzyme preparations from culture and infected seedlings readily macerated cotton hypocotyl sections. However, by using isoelectric focusing techniques, it was found that the

endoPG produced by *R. solani* in response to seed exudates had a single isoelectric point (pI) of 7.1 whereas endoPG from infected hypocotyls had a major peak at pI 7.8 and a minor peak at 7.1. Differences in elution patterns were observed when preparations from the two sources were purified by ion exchange chromatography. Results of this study suggest that *R. solani* produces different forms of endoPG and that the ionic properties of the predominant form produced during pathogenesis differ from those of the single-peak form produced in culture.

Cytological and biochemical evidence suggests that pectolytic enzymes contribute to fungus penetration and the initial processes associated with infection of hypocotyls of cotton seedlings by *Rhizoctonia solani* Kühn (4,15). *R. solani* is reported to produce a variety of pectolytic enzymes in culture and during pathogenesis; different investigators report different findings (2,3,11). This study compares the properties of the pectolytic enzymes produced during the early stages of pathogenesis on cotton with those produced in culture on cotton seed exudates by a strain of *R. solani* common in California.

### MATERIALS AND METHODS

**Inoculation of cotton seedlings.** The *R. solani* strain used in this study was isolated from cotton and is a member of one of the anastomosis groups (AG-4) studied by Parmeter et al (8,9). In greenhouse tests it was virulent to cotton, bean, and sugar beet seedlings (15).

To produce inoculum, the pathogen was grown in still culture at 28 C in petri dishes containing 29 ml of a synthetic liquid medium composed of essential macro- and micronutrients plus 2 g/L asparagine and 20 g/L glucose (14).

Fifteen 4-day-old seedlings of cotton (*Gossypium hirsutum* L. 'Acala 4-42') were arranged in horizontal positions on glass plates (16 × 21 cm), and the roots were covered with moistened quartz sand (13). A thin layer of moistened sand was placed between the glass plates and hypocotyls. A 2-mm-diameter disk, cut from the mycelial mat of 4-day-old cultures, was washed and placed on the moist sand adjacent to each hypocotyl. Aluminum foil was wrapped around each glass plate, enclosing seedling roots and hypocotyls. After holes were punched in the aluminum foil, the plates (and plants) were placed in a vertical position in plastic pans containing a layer of water 1 cm deep, and the pans were covered with clear plastic. Inoculated plants were incubated under 3,229 lux of continuous light and a temperature of 28 C.

**Extraction of pectic enzymes from inoculated seedlings.** Cotton hypocotyls were sampled 18 hr after inoculation and segments, 1.8 cm long, were cut at the inoculation site from 15 seedlings on each glass plate. Extracts were prepared by grinding 15 hypocotyl segments from each of three replicate glass plates for 1 min at 4 C in 5 ml of 0.5 M NaCl (pH 6) in a VirTis homogenizer. Each of the three extracts were strained through three layers of cheesecloth and

centrifuged at 6,000 g for 20 min at 4 C. The supernatants were dialyzed overnight against distilled water at 4 C and stored at -18 C. Uninoculated seedlings incubated under these conditions did not contain measurable pectolytic activity (4).

**Collection of cotton seed exudates.** Exudates were obtained by placing 12.6 g of cotton seeds (100 ± 3 seeds) in each of 50 petri dishes containing 15 ml of distilled water. After 3 hr at 22 C, the liquid in the dishes was filtered through a 0.22-μm Millipore filter (Millipore Filter Corp., Bedford, MA 01730) and the volume adjusted to 25 ml per 100 seeds. This solution represented the nonfractionated seed exudates. One hundred milliliters of this exudate solution was dialyzed overnight against 4 L of distilled water at 4 C and is hereafter identified as the dialyzed seed exudates.

**Production of pectolytic enzymes by *R. solani* in culture.** Seed exudates were used most frequently to induce *R. solani* to produce pectolytic enzymes in culture. In some comparisons, sodium polypectate (1 mg/ml) or cell walls isolated from cotton hypocotyls by the method of English et al (5) were tested as polygalacturonase inducers in culture.

Twenty mycelial disks, 2 mm in diameter, were cut from 3-day-old cultures of *R. solani* grown on the liquid medium and placed in 25-ml Erlenmeyer flasks containing 10 ml of sterile distilled water. The flasks were placed on a water bath shaker and incubated at 28 C at 40 oscillations per minute. After 18 hr the water was decanted and 10 ml of seed exudates were added to each flask. Samples were removed from flasks at various time intervals, dialyzed overnight against distilled water, and assayed for pectolytic enzyme activity.

**Pectolytic enzyme assay.** Results of previous work showed that the isolate of *R. solani* used in this study produced only endopolygalacturonase (endoPG; EC3.2.1.15) during infection of cotton seedlings and in response to cotton seed exudates (4). Direct spectrophotometric and periodic thiobarbituric acid assay procedures (2) failed to demonstrate pectate lyase and pectin lyase in seed exudate-culture filtrates or in extracts of infected cotton seedlings up to 72 hr after inoculation (4). Similarly, pectinesterase did not increase in activity during pathogenesis when assayed in the presence of 0.2 N NaCl; it was not detected in lesion extracts when NaCl was omitted from reaction mixtures. To measure the activity of endoPG, 5 ml of 0.96% sodium polypectate (Sunkist Growers, Inc., Ontario, CA 91764) in 0.1 M sodium acetate buffer (pH 5.2) and 1 ml of enzyme preparation was incubated in a size-300 Cannon-Fenske viscometer (Kimble, Toledo, OH 43666) in a water bath at 28 C. Relative changes in viscosities of the incubation mixture were followed. Units of enzyme activity were determined by dividing

1,000 by the minutes required for 1 ml of enzyme preparation to reduce the viscosity by 50%.

**Paper chromatography.** One-dimensional descending paper chromatography was used to determine the reaction products released from polygalacturonic acid by the pectic enzymes produced by *R. solani* in cotton hypocotyls and in seed exudate-culture. The reaction mixtures consisted of 1 ml of 0.5% polygalacturonic acid in 0.1 M sodium acetate buffer (pH 5.2) plus 1 ml of enzyme preparation and one drop of toluene. After 0, 2, 8, 24, and 96 hr at 28 C, 0.2 ml of the reaction mixture was heated in a boiling water bath for 10 min, cooled, and then 50  $\mu$ l was applied to Whatman No. 1 filter paper. Fifty micrograms of D-galacturonic acid in a volume of 5  $\mu$ l were used as a standard. The solvent system consisted of 85 ml of 77% ethanol and 15 ml of 88% formic acid (7). After development for 10 hr, the chromatograms were dried and sprayed with an indicator solution. The indicator solution was prepared by adding 59 mg of bromophenol blue to 100 ml of 95% ethanol and then adjusting the pH to 6.5 with 0.1 M NaOH (7). The sprayed chromatograms were developed by autoclaving for 5 min at 121 C.

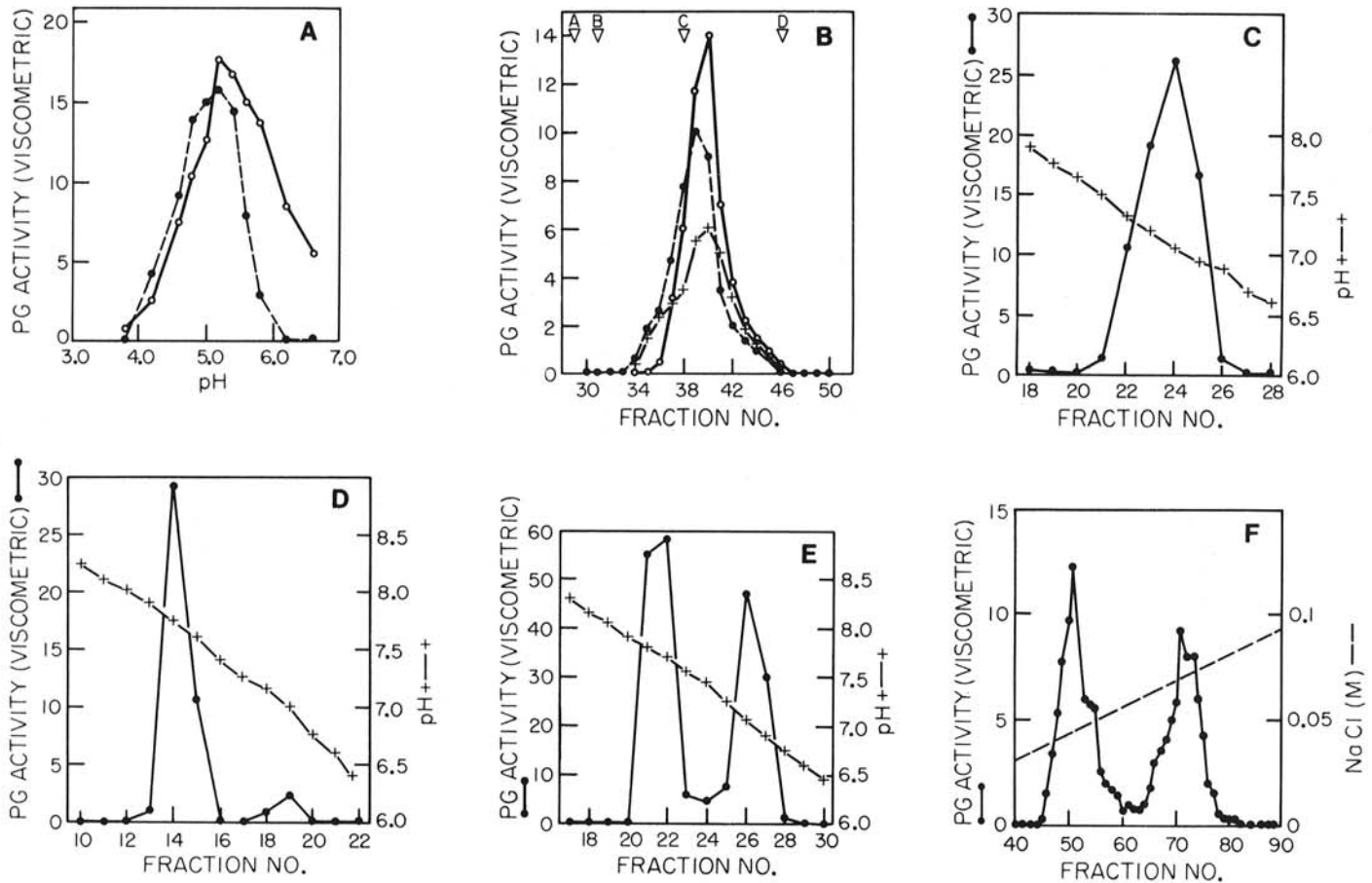
**Gel filtration, isoelectric focusing, and ion exchange chromatography.** Gel filtration chromatography was used to determine the molecular weights of endoPG produced by *R. solani* on cotton seedlings and in culture (1). A column (2 cm  $\times$  40 cm) of Sephadex G-75 (Pharmacia Fine Chemicals, Uppsala, Sweden)

was equilibrated with 0.1 M KCl in 0.01 M sodium acetate buffer (pH 5.2) at 4 C. Four milliliters of the enzyme preparation from seedlings or cultures were applied to the column. In another experiment, the two enzyme preparations were mixed and then applied to the column. The endoPG was eluted from the column by passing the above salt-buffer mixture through the column at a flow rate of 7 ml/hr. Fractions (2-ml) were collected. Blue dextran (mol wt 2,000,000), bovine serum albumin (mol wt 67,000), ovalbumin (mol wt 45,000) (all obtained from Sigma Chemical Co., St. Louis, MO 63178) and  $\alpha$ -chymotrypsinogen (mol wt 12,500) (Calbiochem, La Jolla, CA 94037) were used as standards.

An LKB 8100 ampholine electrofocusing apparatus (LKB-Produkt AB, Bromma, Sweden), equipped with a 110-ml column containing pH 3-10 ampholine carriers in a sucrose gradient, was used to determine the isoelectric points of the endoPG produced on cotton seedlings and in cultures.

Six milliliters of one of the enzyme preparations was applied to the column. For each determination, the voltage was initially set at 100 V and raised 100 V/day to 300 V. After 2 days at 300 V, 2-ml fractions were collected at a flow rate of 1 ml per min. The pH and endoPG activity of each fraction were determined.

A column (1.4 cm  $\times$  20 cm) of diethylaminoethylcellulose in the hydrogen form and equilibrated with 0.005 M Tris-HCl buffer (pH 8.4) at 4 C was used for ion exchange chromatography. For each determination, 4 ml of enzyme sample containing 0.1 ml of 0.1 M



**Fig. 1.** Physical separation and pH optima for activity of forms of endopolygalacturonase (endoPG) produced in culture and in cotton stems infected by *Rhizoctonia solani*. In A-F activity in each of the 2-ml fractions was determined viscometrically by using 0.96% sodium polypectate in 0.1 M acetate buffer (pH 5.2) as the substrate. **A**, Polygalacturonase (PG) activity as a function of pH. Enzyme preparations were dialyzed extracts obtained from cotton seedlings 18 hr after inoculation (●—●), and filtrates obtained from cultures 24 hr after cotton seed exudates were added to mycelial disks of *R. solani* (○—○); **B**, Mobility of endoPG in Sephadex G-75. The enzyme preparations applied to the column were extracts prepared from cotton hypocotyls 18 hr after inoculation with *R. solani* (+—+); filtrates obtained from cultures 24 hr after cotton seed exudates were added to mycelial disks of the fungus (○—○); and a mixture of the above two preparations (●—●). Two-milliliter fractions were collected at a flow rate of 7 ml/hr. The markers A, B, C, and D represent the elution volumes of blue dextran, bovine serum albumin, ovalbumin, and  $\alpha$ -chymotrypsinogen, respectively; **C**, Isoelectric focusing of endoPG produced in culture by *R. solani* in response to cotton seed exudates; **D**, Isoelectric focusing of endoPG extracted from cotton hypocotyls 18 hr after inoculation with *R. solani*; **E**, Isoelectric focusing of a mixture of polygalacturonase produced by *R. solani* in response to seed exudates in culture and polygalacturonase extracted from cotton hypocotyls 18 hr after inoculation with the pathogen; **F**, DEAE cellulose chromatography of a mixture of polygalacturonase produced by *R. solani* in response to seed exudates in culture and polygalacturonase extracted from cotton hypocotyls 18 hr after inoculation with the pathogen.

Tris-HCl buffer (pH 8.4) was passed through the column and the sample was then eluted with a linear gradient of NaCl in this buffer at 4 C. The flow rate was 30 ml/hr. Fractions (2-ml) were collected and assayed for endoPG.

## RESULTS

**Optimum pH for activity.** The optimum pH for activity was determined for endoPG produced in seed exudate-culture or extracted from cotton hypocotyls. Both enzyme preparations were assayed viscometrically at a range of pH values between 3.8 and 6.6. Both the polygalacturonase extracted from cotton seedlings 18 hr after inoculation and the one from filtrates of cultures grown on seed exudates had pH optima of 5.2 (Fig. 1A).

**Reaction products.** The sequence of reaction products released from polygalacturonic acid by the polygalacturonase produced in culture was determined with paper chromatography. Two culture enzyme preparations were used. One consisted of the dialyzed filtrate obtained 24 hr after dialyzed seed exudates were added to mycelial disks and the other consisted of the dialyzed filtrate obtained 24 hr after 0.1% sodium polypectate was added to mycelial disks. Pectolytic activity in both culture preparations as found with diseased tissues (4), released polymers rather than monomers from polygalacturonic acid as the initial reaction products. As reported previously, approximately 0.5% hydrolysis was sufficient to reduce viscosity by 50% (4). Together, these data indicate that the polygalacturonase produced by *R. solani* on cotton hypocotyls and in the liquid cultures both attacked polygalacturonic acid in an endo manner.

**Gel filtration chromatography.** Gel filtration chromatography with a column of Sephadex G-75 revealed that the endoPG produced by the fungus in seed exudate-culture and the endoPG extracted from infected cotton hypocotyls had similar molecular weights. A single peak of activity was detected in approximately the same fraction when the polygalacturonase from these two sources was applied separately or mixed (Fig. 1B). From the elution volumes of blue dextran, bovine serum albumin, ovalbumin, chymotrypsinogen, and the endoPG's, it was estimated that the molecular weight of the endoPG produced in the plant and in culture was about 42,000 daltons. The partition coefficient ( $K_{av} = (V_e - V_0)/(V_t - V_0)$  in which  $V_e$  = elution volume,  $V_0$  = void volume, and  $V_t$  = total volume of the gel bed) was 0.155 for the polygalacturonase from these two sources.

**Isoelectric focusing.** Isoelectric focusing was used to compare the endoPG produced by *R. solani* in flasks containing cotton seed exudates as the inducer with the endoPG extracted from inoculated cotton seedlings. The endoPG produced by the fungus in response to cotton seed exudates had a single isoelectric point of about 7.1 (Fig. 1C). Hypocotyl extracts obtained from cotton seedlings contained two forms of endoPG (Fig. 1D). The predominant polygalacturonase in hypocotyl extracts had an isoelectric point of about 7.8, whereas a minor endoPG activity peak had an isoelectric point similar to that observed for the endoPG produced by the fungus in seed exudate-culture. When enzyme preparations obtained from flasks containing seed exudates were combined with extracts from inoculated cotton seedlings and then added to the same column, two peaks of endoPG activity were observed, representing isoelectric points of 7.8 and 7.1 (Fig. 1E).

Enzyme preparations from flasks containing mycelial disks of the fungus and either cotton hypocotyl cell walls or sodium polypectate were subjected to isoelectric focusing. The polygalacturonase produced by *R. solani* in response to these inducers behaved as a single isozyme with an isoelectric point of 7.1.

**Ion exchange chromatography.** With ion exchange chromatography, the predominant endoPG in extracts from cotton seedlings eluted well before the endoPG in filtrates from seed exudate cultures. When the enzyme preparations from these two sources were mixed, applied to a column, and eluted with the same NaCl gradient, two distinct peaks of endoPG activity were detected (Fig. 1F). Thus, both ion exchange chromatography and isoelectric focusing demonstrated that the predominant endoPG obtained from inoculated seedlings and the endoPG obtained from seed exudate-culture apparently possess different ionic properties.

## DISCUSSION

The endoPG produced by *R. solani* in response to cotton seed exudates in culture was similar to the endoPG extracted from infected cotton hypocotyls on the basis of catalytic properties and molecular weight. However, ion exchange chromatography and isoelectric focusing techniques revealed that the predominant endoPG produced in the plant had different ionic properties than the polygalacturonase produced in culture. These results suggested that *R. solani* produced two molecular forms of endoPG and that the nature of the form produced was dependent upon the conditions of growth. It is particularly interesting that the same form of endoPG was produced in liquid culture regardless of whether the inducing materials were seed exudates, isolated cotton hypocotyl cell walls, or pectate.

Dichotomies between the forms of individual types of pectic enzymes produced in culture and during pathogenesis by plant pathogens were observed in several investigations. For example, Pupillo et al (10) discovered that several strains of *E. chrysanthemi* produced a molecular form of an acid endopectate lyase in infected tissue not produced in culture. Studies with *Fusarium solani* f. sp. *cucurbitae* also revealed differences in the isoelectric points and certain other properties between the form of endopectate lyase produced in culture and in infected tissue (6).

While the present study indicates that the predominant molecular form of endoPG produced by *R. solani* in infected tissue possesses a significantly different isoelectric point from the molecular form of endoPG produced in culture, it is possible that post-synthetic structural modification accounts for these differences. However, since identical purification techniques were applied to enzymes from culture and infected tissue, it is doubtful that these procedures would alter their properties in the sense discussed by Swinburne and Corden (12). Definitive answers to these questions will require genetic studies and further detailed work on the molecular nature of the two endoPG variants.

## LITERATURE CITED

1. ANDREWS, P. 1964. Estimation of molecular weights of proteins by Sephadex gel-filtration. *Biochem. J.* 91:222-233.
2. AYERS, W. A., G. C. PAPAIVIZAS, and A. F. DIEM. 1966. Polygalacturonate transeliminase and polygalacturonase production by *Rhizoctonia solani*. *Phytopathology* 56:1006-1011.
3. BATEMAN, D. F. 1963. Pectolytic activities of culture filtrates of *Rhizoctonia solani* and extracts of *Rhizoctonia*-infected tissues of bean. *Phytopathology* 53:197-204.
4. BROOKHOUSER, L. W., and A. R. WEINHOLD. 1979. Induction of polygalacturonase from *Rhizoctonia solani* by cotton seed and hypocotyl exudates. *Phytopathology* 69:599-602.
5. ENGLISH, P. D., J. B. JURALE, and P. ALBERSHEIM. 1971. Host-pathogen interactions. II. Parameters affecting polysaccharide-degrading enzyme secretion by *Colletotrichum lindemuthianum* grown in culture. *Plant Physiol.* 47:1-6.
6. HANCOCK, J. G. 1976. Multiple forms of endo-pectate lyase formed in culture and in infected squash hypocotyls by *Hypomyces solani* f. sp. *cucurbitae*. *Phytopathology* 66:40-45.
7. PAGE, O. T. 1961. Quantitative paper chromatographic techniques for the assay of products of polygalacturonase activity of fungus cultures. *Phytopathology* 51:337-338.
8. PARMETER, J. R., Jr., R. T. SHERWOOD, and W. D. PLATT. 1969. Anastomosis grouping among isolates of *Thanateophorus cucumeris*. *Phytopathology* 59:1270-1278.
9. PARMETER, J. R., Jr., and H. S. WHITNEY. 1970. Taxonomy and nomenclature of the imperfect state. Pages 7-19 in: J. R. Parmeter, Jr., ed. *Rhizoctonia solani: Biology and Pathology*. University of California Press, Berkeley, Los Angeles, and London.
10. PUPILLO, P., U. MAZZUCCHI, and G. PIERINI. 1976. Pectic lyase isozymes produced by *Erwinia chrysanthemi* Burkh. et al in polypectate broth or in *Dieffenbachia* leaves. *Physiol. Plant Pathol.* 9:113-120.
11. SHERWOOD, R. T. 1966. Pectin lyase and polygalacturonase production by *Rhizoctonia solani* and other fungi. *Phytopathology* 56:279-286.
12. SWINBURNE, T. R., and M. E. CORDEN. 1969. A comparison of the

- polygalacturonases produced *in vivo* and *in vitro* by *Penicillium expansum* Thom. J. Gen. Microbiol. 55:75-87.
13. WEINHOLD, A. R., T. BOWMAN, and R. L. DODMAN. 1969. Virulence of *Rhizoctonia solani* as affected by nutrition of the pathogen. Phytopathology 59:1601-1605.
  14. WEINHOLD, A. R., R. L. DODMAN, and T. BOWMAN. 1972. Influence of exogenous nutrition on virulence of *Rhizoctonia solani*. Phytopathology 62:278-281.
  15. WEINHOLD, A. R., and J. MOTTA. 1973. Initial host responses in cotton to infection by *Rhizoctonia solani*. Phytopathology 63:157-162.