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Host-Pathogen Interactions[1]

XXV. ENDOPOLYGALACTURONIC ACID LYASE FROM *ERWINIA CAROTOVORA* ELICITS PHYTOALEXIN ACCUMULATION BY RELEASING PLANT CELL WALL FRAGMENTS

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KEITH R. DAVIS, GARY D. LYON, ALAN G. DARVILL, AND PETER ALBERSHEIM*
Departments of Chemistry and of Molecular, Cellular, and Developmental Biology, Campus Box 215, University of Colorado, Boulder, Colorado 80309

ABSTRACT

Heat-labile elicitors of phytoalexin accumulation in soybeans (*Glycine max* L. Merr. cv Wayne) were detected in culture filtrates of *Erwinia carotovora* grown on a defined medium containing citrus pectin as the sole carbon source. The heat-labile elicitors were highly purified by cation-exchange chromatography on a CM-Sephadex (C-50) column, followed by agarose-affinity chromatography on a Bio-Gel A-0.5m gel filtration column. The heat-labile elicitor activity co-purified with two α-1,4-endopolygalacturonic acid lyases (EC 4·2·2·2). Endopolygalacturonic acid lyase activity appeared to be necessary for elicitor activity because heat-inactivated enzyme preparations did not elicit phytoalexins. The purified endopolygalacturonic acid lyases elicited pterocarpan phytoalexins at microbial-inhibitory concentrations in the soybean-cotyledon bioassay when applied at a concentration of 55 nanograms per milliliter (1×10^{-9} molar). One of these lyases released heat-stable elicitors from soybean cell walls, citrus pectin, and sodium polypectate. The heat-stable elicitor-active material solubilized from soybean cell walls by the lyase was composed of at least 90% (w/v) uronosyl residues. These results demonstrate that endopolygalacturonic acid lyase elicits phytoalexin accumulation by releasing fragments from pectic polysaccharides in plant cell walls.

Plants can activate any one of several defense mechanisms when invaded by potentially pathogenic microorganisms (4). One well-characterized mechanism of defense involves the accumulation at the site of infection of phytoalexins, which are antimicrobial compounds of low mol wt (1, 2). Evidence has been published showing that phytoalexin accumulation can be important in limiting bacterial growth in several plant species (17 and references therein).

Phytoalexin accumulation can be induced by molecules of microbial origin, called elicitors (1, 2, 34). Elicitors that have been isolated include fungal cell wall glucans and several fungal glycoproteins (27, 34 and references therein) including a fungal endopolygalacturonase (19, 20). Elicitors produced by bacteria have not been as well characterized as those of fungi. There have been reports of elicitor activity being associated with bacterial cell envelopes (8) and extracellular polysaccharides (26); however, these observations could not be confirmed in this laboratory (13).

Elicitors of phytoalexin accumulation have also been obtained from plant tissues. Heat-stable constitutive elicitors have been obtained from *Phaseolus vulgaris* hypocotyls by freeze-thawing or hot water extraction procedures (15, 16). The chemical nature of these elicitors was not determined. Recent studies have shown that the heat-stable elicitors released from soybean cell walls by acid hydrolysis were pectic oligogalacturonides (14, 25). Similar oligogalacturonide elicitors solubilized from cell walls, called endogenous elicitors, have also been obtained by partial acid hydrolysis of the walls of suspension-cultured tobacco, sycamore, and wheat cells (14) and from citrus pectin (25).

The Gram-negative bacterium *E. carotovora* has been shown to elicit the accumulation of pterocarpan phytoalexins in wounded soybean cotyledons (33). Ninety-five per cent of this elicitor activity was abolished by killing the bacteria with heat treatments or antibiotics (13). These results suggested that viable *E. carotovora* cells produce an elicitor when in contact with plant tissue. We report in this paper the purification and characterization of heat-labile elicitors produced by *E. carotovora* grown on a defined medium containing citrus pectin. These elicitors were found to be PGA[2] lyases, pectin-degrading enzymes that have been shown to be secreted by many plant pathogens (3). We also present evidence that the release, by PGA lyase, of oligosaccharides from the pectic polymers of plant cell walls triggered the elicitation of phytoalexin accumulation. This evidence suggests that the release of endogenous elicitors (14, 25) from plant cell walls by pectin-degrading enzymes plays a role in general plant disease resistance to microorganisms. A preliminary report of these results has been published (10).

MATERIALS AND METHODS

Chemicals. Electrophoresis-grade acrylamide and *N,N'*-methylenebisacrylamide, low-mol-wt protein standards, silver-stain reagents, dye reagent concentrate for protein assay, and Bio-Gel A-0.5m agarose beads were from Bio-Rad. Sephadex G-15, CM-Sephadex (C-50), BSA (fraction V), streptomycin sulfate, and Tris were from Sigma. Ion-exchange resin PB 118 and Pharmalyte pH 8–10.5 ampholytes were from Pharmacia. Electrophoresis-grade SDS was from BDH Chemicals Ltd., D-galacturonic acid was from Aldrich, and D-glucose was from Fisher. All other chemicals and solvents were of reagent grade or better.

Polysaccharides. Sodium polypectate (grade II), polygalacturonic acid (grade III), and citrus pectin were from Sigma. Cell walls from soybean stems were prepared by M. Woodward and M. Hahn, as described (14). Before use, the cell walls (1 g) were washed with 1 L 5 mM Tris-HCl, 1 mM $CaCl_2$ at pH 8.5, and 1 L deionized H_2O. The walls were then dried in a vacuum oven. A crude glucan elicitor from *Phytophthora megasperma* var *glycinea* was a gift from J. K. Sharp of this laboratory. α-1,4-

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[2] Abbreviation: PGA, α-1,4-endopolygalacturonic acid.

Digalacturonic acid and α-1,4-trigalacturonic acid were prepared as described (12).

Plant Material. Foundation-quality soybean seed (*Glycine max* L. Merr. cv Wayne) was obtained from the Committee for Agriculture, Ames, IO. Seeds were planted, and seedlings were grown as described (33), except that the germinating seeds were not watered on the 1st day after planting.

Bacterial Cultural Methods. *Erwinia carotovora* (Jones) Holl. was obtained from the American Type Culture Collection (ATCC No. 495) and maintained on trypticase soy broth (Baltimore Biological Labs) agar plates. Bacterial cultures were maintained on stock plates that were prepared every 3 to 4 weeks and stored at 4°C. Cultures producing pectin-degrading enzymes were obtained by inoculating a single colony of *E. carotovora* into 100 ml of EDTA-free medium containing citrus pectin, as described by Moran *et al.* (23). Cultures were incubated in the dark at 24°C on a gyratory shaker (100 rpm) for 72 h. Ten ml of the 72-h culture were added to 100 ml of fresh medium, and incubated as above for 24 h. Each 24-h culture was poured into 500 ml of fresh medium, and incubated as above for 30 h. This procedure resulted in the production of cultures that had an *A* at 580 nm of approximately 1.5. These cultures were used as the source of heat-labile elicitors.

Analytical Methods. Protein concentrations were determined by the Bradford method (6), with BSA as the standard. Hexose concentrations were obtained by the anthrone method (11), with glucose as the standard. Uronic acid concentrations were determined by the *m*-hydroxydiphenyl method (5), with galacturonic acid as the standard.

SDS-PAGE was performed with polyacrylamide slab gels and the buffer system of Laemmli (18). Protein bands were detected by silver staining (22).

Isoelectric points of proteins were determined by column chromatofocusing (28, 29) at 4°C with ion-exchange resin PB 118. The column (1.0 × 29.5 cm) was equilibrated with 25 mM triethylamine at pH 11.0. The bound enzymes were eluted with a 1:45 dilution of pH 8–10.5 carrier ampholytes adjusted to pH 8.0 at a flow rate of 23 ml/h. Fractions containing 5.0 ml were collected. The pH of each eluted fraction was determined at 4°C.

Enzyme Assays. PGA lyase activity was determined by the change in *A* at 235 nm as described (24), with sodium polypectate (grade II) or polygalacturonic acid (grade III) as the substrate. Reaction mixtures contained 0.25% (w/v) substrate, 50 mM Tris-HCl, and 1 mM $CaCl_2$ at pH 8.5 in a total volume of 2 ml. One unit of PGA lyase activity was the amount required to release 1 μmol unsaturated products/min at 30°C. This is equivalent to a change in the *A* at 235 nm of 2.6 (23).

Polygalacturonase activity was determined at 30°C by the increase of reducing sugars in a 1-ml reaction mixture containing 0.1% sodium polypectate (grade II), 50 mM sodium acetate, and 100 mM $NaCl_2$ at pH 5.2. Reactions were terminated with 1 ml alkaline copper reagent. Reducing groups were measured by the Somogyi method as adapted by Nelson (24, 30), with galacturonic acid as a standard. One unit of polygalacturonase activity was the amount necessary to release 1 μmol reducing groups/min at 30°C.

Assay for Elicitor Activity. The soybean-cotyledon assay was used as previously described (21), except that the cotyledons were incubated for 24 h rather than 20 h before determining the *A* at 286 nm of the wound-droplet solutions. Samples to be assayed for elicitor activity were diluted with filter-sterilized H_2O to a final volume of 2.0 ml and contained 200 μg/ml streptomycin sulfate. A 90-μl aliquot of the solution was applied to the wounded surface of each cotyledon. The elicitor activity is presented as the *A* at 286 nm/cotyledon or has been converted to μg QAE-purified endogenous elicitor eq/cotyledon (21). The *A* at 286 nm has previously been shown to be proportional to the amount of the phytoalexins glycinol and glyceollin in the wound droplets (1, 14).

The following experiment was conducted to compare the elicitor activities of the heat-labile elicitors at each purification step. The heat-labile elicitor activity after each purification step was determined at three to five different concentrations and converted to μg endogenous elicitor eq/cotyledon. The data were plotted as protein concentration *versus* μg endogenous elicitor eq/cotyledon, and analyzed by linear regression. The amount of protein necessary to give a response equivalent to 10 μg endogenous elicitor eq/cotyledon (1 unit of elicitor activity) was determined from these regression analyses, and this quantity was used to compare the specific elicitor activity at each purification step. Each regression analysis contained at least one protein concentration lower and at least one concentration higher than that necessary to cause a response equivalent to 1 unit of elicitor activity. Assay of the glucan elicitor at 0.18 μg/cotyledon was included as a positive control in all elicitor assays to estimate the day-to-day variability of the assay.

RESULTS

Purification of PGA Lyase Elicitors. All purification procedures were performed at 4°C unless otherwise stated. Late log phase cultures of *E. carotovora* were subjected to three cycles of freeze-thawing (−60°C to 20°C). The bacteria were removed by centrifugation and the supernatant was recovered. This freeze-thawed culture filtrate was brought to 55% acetone (v/v) as described (23) and the precipitate recovered by centrifugation. The acetone-precipitated proteins were dissolved in 50 mM sodium acetate at pH 5.2 and dialyzed extensively against the same buffer.

This dissolved protein dialysate was applied to a CM-Sephadex C-50 column. The bound proteins were eluted with a linear gradient of increasing sodium-acetate concentration. Two peaks of PGA lyase activity were resolved, and these were followed by a peak of polygalacturonase activity (Fig. 1). Elicitor assays of aliquots removed from selected fractions from this column demonstrated that elicitor activity was associated with each of the PGA lyase peaks. This elicitor activity was shown to be heat labile (data not shown).

The fractions containing the two PGA lyase peaks were pooled separately, as indicated by the bars in Figure 1. The pooled fractions—CM I and CM II—were dialyzed extensively against 50 mM sodium acetate at pH 5.2. The dialysates were concentrated on a small (0.75 × 2 cm) column of CM Sephadex C-50. The bound proteins were eluted with 5 ml of 1 M sodium acetate at pH 5.2. These eluants were dialyzed against 50 mM Tris-HCl containing 1 mM $CaCl_2$ at pH 8.5.

The dialysates were applied separately to a Bio-Gel A-0.5m agarose column. The elution profile of CM I on the agarose column is shown in Figure 2A. A single peak of PGA lyase activity, PGA lyase I, eluted after the included volume of the column. The elution profile of CM II on the agarose column is shown in Figure 2B. Two peaks of PGA lyase activity eluted after the included volume of the column. The major peak of PGA lyase activity, PGA lyase II, eluted first, followed by a PGA lyase that eluted in the same volume as PGA lyase I. Elicitor assays on selected fractions from the CM I and CM II Bio-Gel A-0.5m column runs demonstrated that elicitor activity was associated with those fractions containing PGA lyase activity and that this elicitor activity was heat labile (data not shown). The fact that the PGA lyases eluted after the included volume of the agarose column indicated that these enzymes have an affinity for the agarose matrix. Similar observations were made during the purification of endopolygalacturonases secreted by *Colletotrichum lindemuthianum* (12) and *Rhizopus stolonifer* (19).

The purifications of the PGA lyases and the heat-labile activ-

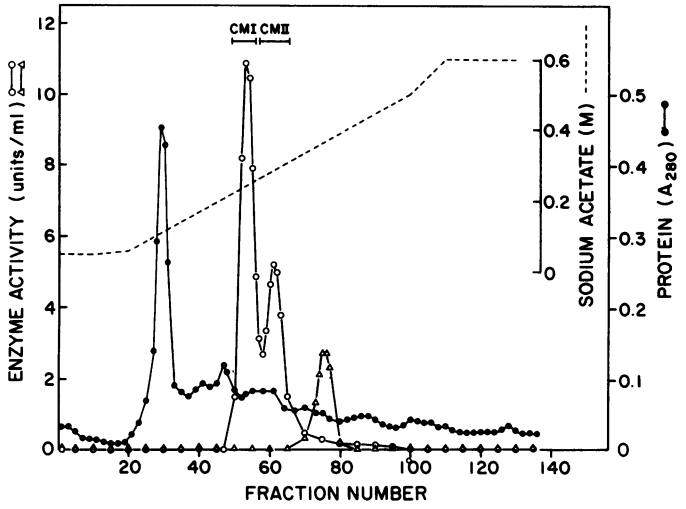

FIG. 1. Cation-exchange chromatography of dialyzed, acetone-precipitated proteins on a CM-Sephadex column. The proteins obtained from freeze-thawed *E. carotovora* cultures were loaded onto a CM-Sephadex (C-50) column (3.0 × 16.5 cm) equilibrated with 50 mM sodium acetate at pH 5.2. The column was washed with 200 ml of buffer, and the bound proteins eluted with a linear gradient of increasing sodium-acetate concentration at a flow rate of 10 ml/h. Fractions of 8.5 ml were collected. Aliquots were assayed for PGA lyase activity (5 μl, O), polygalacturonase activity (25 μl, Δ), *A* at 280 nm (●), and elicitor activity (25 μl) as described in "Materials and Methods." Fractions 49 thru 56 (CM I) and 57 thru 65 (CM II) contained both PGA lyase and heat-labile-elicitor activity, and were pooled separately for further purification. Fractions 71 thru 80 contained PGA lyase, polygalacturonase, and heat-labile-elicitor activity. The polygalacturonase activity was lost upon extensive dialysis against 50 mM Tris-HCl and 1 mM $CaCl_2$ at pH 8.5. These fractions were not studied further.

ities are compared in Table I. PGA lyase I was purified 81-fold and accounted for 17% of the original PGA lyase activity. PGA lyase II was purified 21-fold and accounted for 7% of the original PGA lyase activity. Heat-labile elicitor activity co-purified with each PGA lyase. The heat-labile elicitor activity associated with PGA lyase I was purified 97-fold and accounted for 20% of the original heat-labile elicitor activity. The heat-labile elicitor activity associated with PGA lyase II was purified 47-fold and accounted for 15% of the original heat-labile elicitor activity.

Comigration of the PGA Lyases and the Heat-Labile Elicitors during Column Chromatofocusing. The agarose-purified PGA lyase I and PGA lyase II preparations were subjected individually to column chromatofocusing (Fig. 3). Both of the agarose-purified PGA lyases exhibited a single peak of lyase activity and were recovered quantitatively. Assays of the elicitor activity of fractions from both chromatofocusing runs demonstrated that, in each case, a single peak of elicitor activity eluted from the column coincident with a single PGA lyase (Fig. 3). The elicitor activity was heat labile (data not shown). These results further established that the PGA lyases were the elicitor-active components of the agarose-purified fractions.

Response of Soybean Cotyledons Treated with PGA Lyases I and II. The relative amounts of phytoalexins accumulated by soybean cotyledons treated with increasing amounts of agarose-purified PGA lyase I and II are illustrated in Figure 4. PGA lyase I elicited a maximum response when 3 ng/cotyledon was applied, while approximately 6 ng/cotyledon of PGA lyase II was needed to give a similar response. These concentrations of lyase corresponded to concentrations of 7.8×10^{-10} M and 1.6×10^{-9} M for PGA lyase I and II, respectively. These concentrations were calculated using the apparent mol wt determined as described in

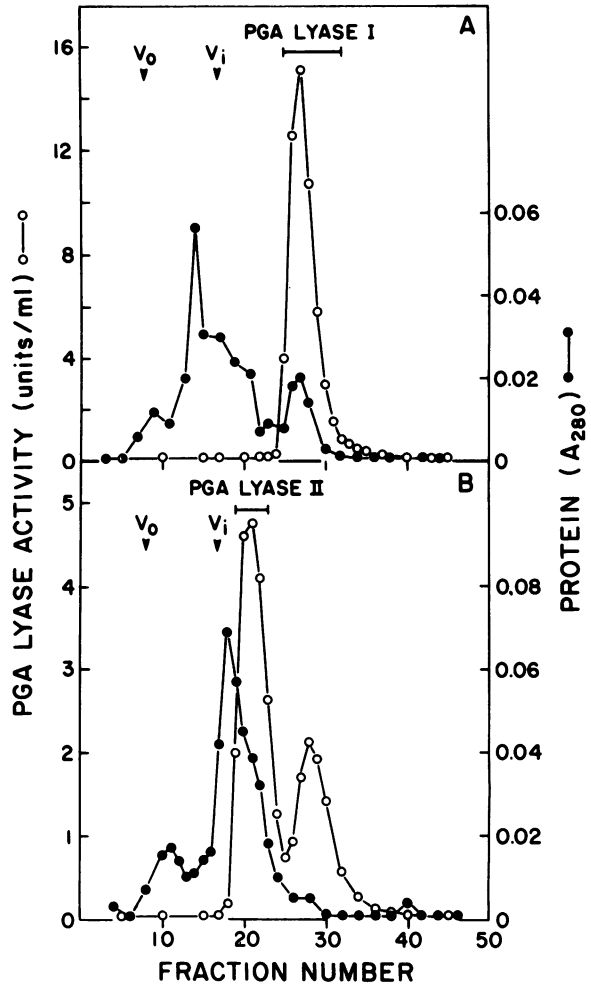

FIG. 2. Agarose-affinity chromatography of CM I and CM II on a Bio-Gel A-0.5m column. The concentrated and dialyzed CM I and CM II preparations were separately chromatographed on the Bio-Gel A-0.5m column (1.5 × 48.0 cm) equilibrated and the protein eluted with 50 mM Tris-HCl and 1 mM $CaCl_2$ at pH 8.5; 4.8-ml fractions were collected. Five-μl aliquots of selected fractions were assayed for PGA lyase activity, and 1:800 (v/v) dilutions were assayed for elicitor activity, as described in "Materials and Methods." A, Elution profile obtained when CM I was chromatographed on the agarose column. Fractions 25 thru 32, which contained most of the elicitor and PGA lyase activities, were pooled and represent agarose-purified PGA lyase I. B, Elution profile obtained when CM II was chromatographed on the agarose column. The earliest eluting peak of PGA lyase activity (pooled fractions 19 thru 23) represents agarose-purified PGA lyase II, whereas the second peak of lyase activity (fractions 27 thru 32) has the same elution volume as PGA lyase I. Heat-labile-elicitor activity was associated with the PGA lyase activities.

the following section. The response of maximally stimulated cotyledons by the two PGA lyases was estimated to be equivalent to 100 μg/ml glycinol. The glycinol concentration was calculated by taking the 0.08 absorbance value at maximal elicitation and converting to 0.8 (to account for 1:10 dilution of wound-droplet solution before absorbance measurement). A 0.8 absorbance is equivalent to 75 μg glycinol from standard graph (see Fig. 2 in Ref. 14). This value of 75 μg glycinol is equivalent to 5 μg/ml (75/15) in the wound-droplet solution. The concentration of glycinol in the wound droplets was calculated by correcting for the 20-fold dilution that occurs when the wound droplets are washed into 20 ml H_2O to prepare wound-droplet solutions. A concentration of 100 μg/ml glycinol has been shown to inhibit

Table I. *Comparison of the Purification of the PGA Lyases I and II with Heat-Labile Elicitor Activity*

Purification Procedure	Volume	Protein	PGA Lyase Activity			Elicitor Activity[a]		
			Specific activity	Relative purification	Yield	Specific activity (r)[b]	Relative purification	Yield
	ml	*μg/ml*	*units/mg*		%	*units/μg*		%
Freeze-thawed culture filtrate	3100	42.0	9.5	1.0	100	4.3 (0.974)	1.0	100
Acetone precipitation and dialysis	800	80.0	16.3	1.7	84	4.7 (0.973)	1.1	54
CM Sephadex cation exchange chromatography and concentration								
CM I	4.5	302.0	192.7	20.3	21	95.2 (0.985)	22.1	23
CM II	4.5	309.0	124.6	13.1	14	204.1 (0.878)	47.5	51
Agarose-affinity chromatography								
PGA lyase I	38.4	6.9	768.1	80.9	17	416.7 (0.974)	96.9	20
PGA lyase II	24.0	17.4	201.2	21.2	7	200.0 (0.997)	46.5	15

[a] The elicitor activity of the glucan controls varied by 18% or less in these experiments.
[b] Correlation coefficient of linear regression analysis.

the growth of a variety of microorganisms (33). PGA lyase I and II preparations boiled 30 min before application to the cotyledons did not elicit phytoalexin accumulation (Fig. 4).

Analysis of the Agarose-Purified PGA Lyases by SDS-PAGE. Analysis of agarose-purified PGA lyases I and II by SDS-PAGE (Fig. 5) demonstrated that PGA lyase I was homogeneous with respect to size with an apparent mol wt of 42,500. The PGA lyase II fraction contained at least three minor components and one major component with an apparent mol wt of 41,000. Because the agarose-purified PGA lyase I preparation appeared to be homogeneous with respect to size and charge (Fig. 3), it was chosen for use in the following experiments.

Catalytic Properties of PGA Lyase I. The apparent K_m value of PGA lyase I for polygalacturonic acid (grade III) was found to be 1.7 mg/ml by measuring the initial reaction rates at substrate concentrations of 0.75 to 2.5 mg/ml in the presence of 50 mM Tris-HCl and 1 mM $CaCl_2$ at pH 8.5. PGA lyase I exhibited non-Michaelis-Menten kinetics at substrate concentrations greater than 2.5 mg/ml. For example, the enzyme exhibited 74% of maximum activity at 5.0 mg/ml polygalacturonic acid. This decreased activity could have been due to substrate inhibition or a lowering of the free calcium concentration by chelation with polygalacturonic acid.

PGA lyase I exhibited a sharp pH optimum at pH 8.5 when measured over a pH range of 5.0 to 10.0 at 0.5 pH unit increments. This enzyme had undectable PGA lyase activity at pH 5.0 to 6.5 and 45% of maximum activity at pH 10.0. PGA lyase I did not exhibit any activity in the absence of $CaCl_2$ and exhibited optimal activity in the presence of 0.5 to 1.5 mM $CaCl_2$. The activity in the presence of 1 mM $CaCl_2$ was totally inhibited by 1 mM EGTA. The properties of PGA lyase I and PGA lyase II (data not presented) were consistent with these enzymes being the extracellular and intracellular PGA lyases, respectively, described by Moran *et al.* (23).

Determination of Whether PGA Lyase I Cleaves PGA in an Endo- or Exo-Fashion. The following experiment was performed to determine whether PGA lyase I is an endo- or exoenzyme. PGA lyase I was incubated with a sodium-polypectate reaction mixture. Aliquots were removed after 1 and 19 h of incubation and chromatographed on a Sephadex G-15 gel-filtration column (Fig. 6). After 1 h of incubation, approximately 17% of the available galacturonosyl linkages were cleaved. The majority of the 235-nm absorbing products in the 1-h digest voided the Sephadex G-15 column, indicating that they had a mol wt of 1500 or larger. Smaller amounts of 235-nm absorbing products had elution volumes consistent with di- and trigalacturonosyl acid residues. After 19 h of incubation, approximately 39% of the available galacturonosyl linkages were cleaved. The elution volumes of most of the 19-h products were consistent with di-, tri-, tetra-, and pentagalacturonosyl acid residues. A smaller amount of 235-nm absorbing products still voided the column. The absence of monogalacturonic acid and the relatively low abundance of di- and trigalacturonic acid in the 1-h digest demonstrated the PGA lyase I was an endoenzyme. The same experimental procedures were used to show that PGA lyase II was also an endoenzyme (data not presented).

Comparison of the Heat Lability of PGA Lyase I and the Associated Elicitor Activity. The following experiment was performed to test whether or not the observed elicitor activity of PGA lyase I was dependent on the catalytic activity of the enzyme. Agarose-purified PGA lyase I was heated at 60°C in a water bath. At selected time intervals, aliquots of the enzyme solution were removed and assayed for both PGA lyase and elicitor activity (Fig. 7). Both the enzyme and the elicitor activities were lost in logarithmic fashion, as would be expected for heat inactivation of an enzyme. The loss of elicitor activity closely paralleled the loss of enzyme activity during the first 2 min of incubation. The elicitor activity appeared to be slightly more stable between 2 and 15 min of incubation, while both the enzyme and elicitor activities were totally inactivated between 15 and 20 min of incubation. The elicitor and enzyme activities at each time interval were compared by linear regression analysis (units PGA lyase/ml versus μg endogenous elicitor eq/cotyledon). A correlation coefficient (r) of 0.973 was obtained, showing a strong correlation between the inactivation of the enzyme and elicitor activities.

Solubilization of Heat-Stable Elicitors from Purified Soybean Cell Walls by PGA Lyase I. It has been demonstrated in recent work that oligogalacturonide elicitors can be released from soybean cell walls by acid hydrolysis (14, 25). The following experiment was performed to see whether similar elicitors could be solubilized from purified soybean cell walls by PGA lyase I.

Reaction mixtures consisted of 300 mg cell walls suspended in 30 ml filter-sterilized buffer containing 5 mM Tris-HCl and 1 mM $CaCl_2$ at pH 8.5. Boiled or unboiled PGA lyase I (0.8 units) was added to each reaction mixture and incubated at 30°C. At the time intervals indicated in Figure 8A, the reaction mixtures were vortexed to obtain an even suspension of the insoluble cell

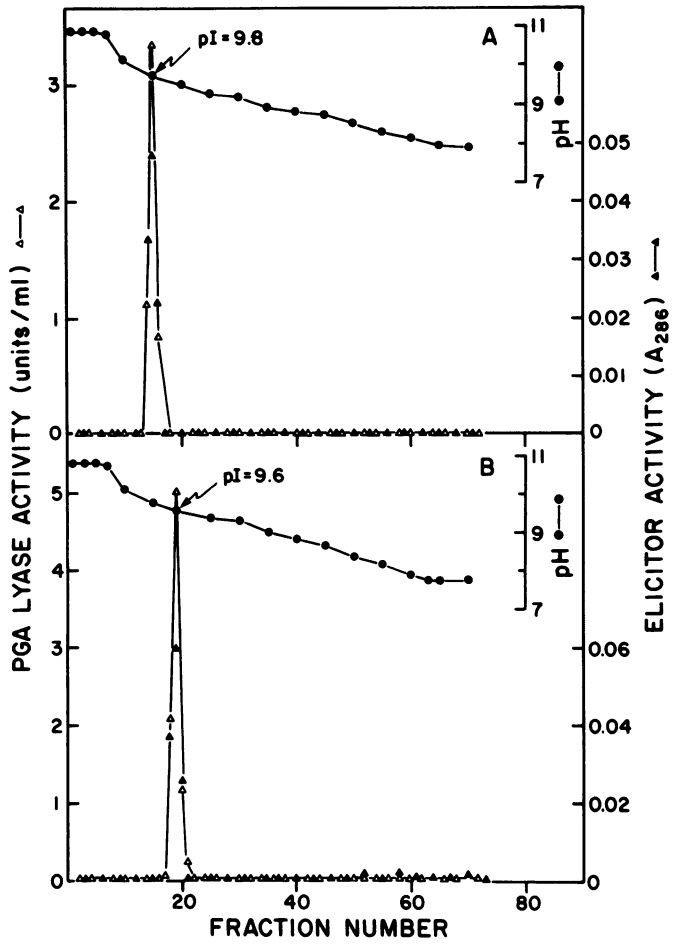

FIG. 3. Column chromatofocusing of the agarose-purified PGA lyase I and II elicitors. Chromatofocusing was performed, and PGA lyase and elicitor activity measured, as described in "Materials and Methods," except that the lyase activity of PGA lyase II was determined at a $CaCl_2$ concentration of 0.25 mM, the optimal $CaCl_2$ concentration for this enzyme. Ten-μl aliquots were assayed for enzyme activity, and 5-μl aliquots were assayed for elicitor activity. A, Elution profile obtained when 26.5 units of PGA lyase I was subjected to column chromatofocusing. B, Elution profile obtained when 38.7 units of PGA lyase II was subjected to column chromatofocusing.

walls, and 5 ml was immediately removed. The walls were removed from each aliquot by filtration through 1.2-μm Millipore filters (type RA). The filtrate was collected in a test tube immersed in an ice bath to stop further enzyme reaction. Aliquots were removed, rapidly brought to pH 5.2–5.5 with 1 M HCl, and boiled 30 min to inactivate the enzyme. The samples were frozen at −20°C until assayed for heat-stable elicitor activity.

The elicitor activity of the PGA lyase I solubilized cell wall material is illustrated in Figure 8A. Elicitor activity was released from the insoluble walls as early as 20 min after addition of PGA lyase I. Increasing amounts of heat-stable elicitors continued to be released over the 4-h incubation period. The samples obtained from the boiled enzyme control also exhibited some elicitor activity. However, after 4 h, this activity was 10-fold less than that released from walls treated with active enzyme and was similar to that obtained by extracting cell walls with the Tris-HCl, $CaCl_2$ buffer used in these experiments (data not shown).

The release of heat-stable elicitor activity by PGA lyase I was correlated with an increase in the *A* at 235 nm (due to the unsaturated reaction products) and with solubilization of materials containing uronic acid. Approximately 77 μg/ml of galacturonic acid equivalents were solubilized after 20 min of incubation. This increased to 277 μg/ml galacturonic acid equivalents after 4 h of incubation. The specific activity (μg endogenous elicitor eq/cotyledon/μg galacturonic acid equivalents) of the lyase-released heat-stable elicitors after 4 h of incubation was 13-fold higher than cell wall elicitors extracted with the boiled enzyme control used in the experiment. The most active heat-stable elicitor fraction obtained with enzymic treatment (4 h) of cell walls contained 90% (w/v) uronic acids, 10% (w/v) neutral sugars, and less than 0.5% (w/v) protein. These results demonstrated that PGA lyase I released uronic acid-rich heat-stable elicitors from insoluble soybean cell walls.

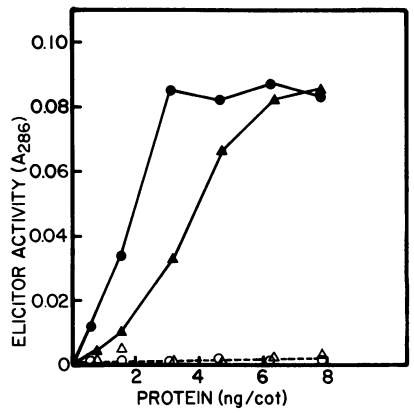

FIG. 4. Response of soybean cotyledons treated with increasing amounts of agarose-purified PGA lyases I (●, ○) and II (▲, △). Test solutions containing increasing amounts of boiled or unboiled PGA lyases I and II were assayed for elicitor activity as described in "Materials and Methods." Each data point represents the average of 20 cotyledons. The elicitor activity of a water control was subtracted from the data in this figure (A_{286} of 0.018 and 0.017 for PGA lyases I and II, respectively). The closed symbols (●, ▲) represent unboiled enzyme, and the open symbols (○, △), boiled enzyme. The maximum elicitor activity shown is equivalent to approximately 15 μg EE eq/cotyledon (EE = QAE-purified endogenous elicitor).

Release of Heat-Stable Elicitors from Citrus Pectin and Sodium Polypectate. The previous experiment demonstrated that PGA lyase I could solubilize heat-stable elicitors from purified soybean cell walls. The solubilized heat-stable elicitor preparation was found to be rich in uronic acids, suggesting that the elicitors may be present in the pectic fraction of the cell wall. The following experiments were performed to determine if heat-stable elicitor activity could be released from commercial preparations of citrus pectin and sodium polypectate.

Boiled or unboiled PGA lyase I (0.27 units) was added to 10 ml of a filter-sterilized reaction mixture containing 5 mM Tris-HCl, 1 mM $CaCl_2$, and 0.1% (w/v) citrus pectin or sodium polypectate (grade II) at pH 8.5. The reaction mixtures were incubated at 30°C. At selected time intervals, 1.0-ml aliquots were removed and placed in an ice bath to stop further enzyme reaction. The aliquots were immediately brought to pH 4.5 to 5.0 with 1 M HCl and placed in a boiling water bath to inactivate the enzyme. A portion of each aliquot was then assayed for elicitor activity, while the remainder was frozen at −20°C for further analysis.

Heat-stable elicitors were released from both citrus pectin and sodium polypectate. The amount of heat-stable elicitor activity released from citrus pectin did not increase significantly after 0.5 h (Fig. 8B). The *A* at 235 nm had increased to 0.59 after 5 h. This was equivalent to the cleavage of approximately 7% of the available galacturonosyl linkages. The maximum amount of elicitor activity released from sodium polypectate (Fig. 8C) was approximately 10-fold greater than that observed from citrus

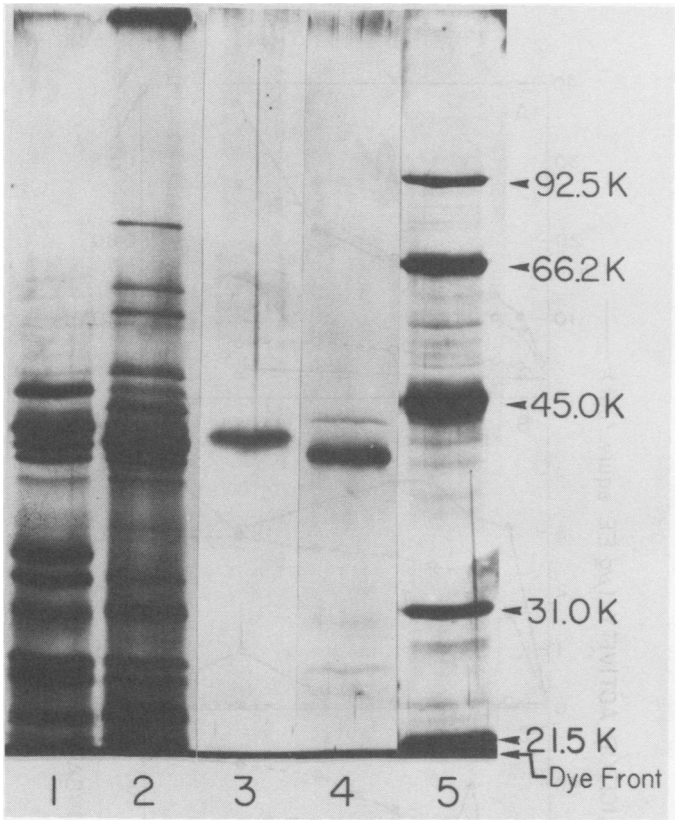

FIG. 5. Analysis of agarose-purified PGA lyase I and II elicitors by SDS-PAGE. Electrophoresis was performed with 0.75-mm-thick acrylamide slab gels (4.5% stacking gel and 10% separation gel) as described by Laemmli (17). Samples were run at a constant current of 17.5 mamp until the bromophenol blue marker dye was approximately 1 cm from the bottom of the gel (~3.5 h). Gels were fixed and silver-stained with reagents obtained from Bio-Rad according to the manufacturer's instructions. Lane 1, Fraction CM I (approximately 3 μg protein). Lane 2, Fraction CM II (approximately 3 μg protein). Lane 3, PGA lyase I (approximately 75 ng protein). Lane 4, PGA lyase II (approximately 75 ng protein). Lane 5, Low-mol-wt standard containing approximately 200 ng each of soybean trypsin inhibitor (mol wt 21,500), carbonic anhydrase (mol wt 31,000), ovalbumin (mol wt 45,000), BSA (mol wt 66,200), and phosphorylase B (mol wt 92,500).

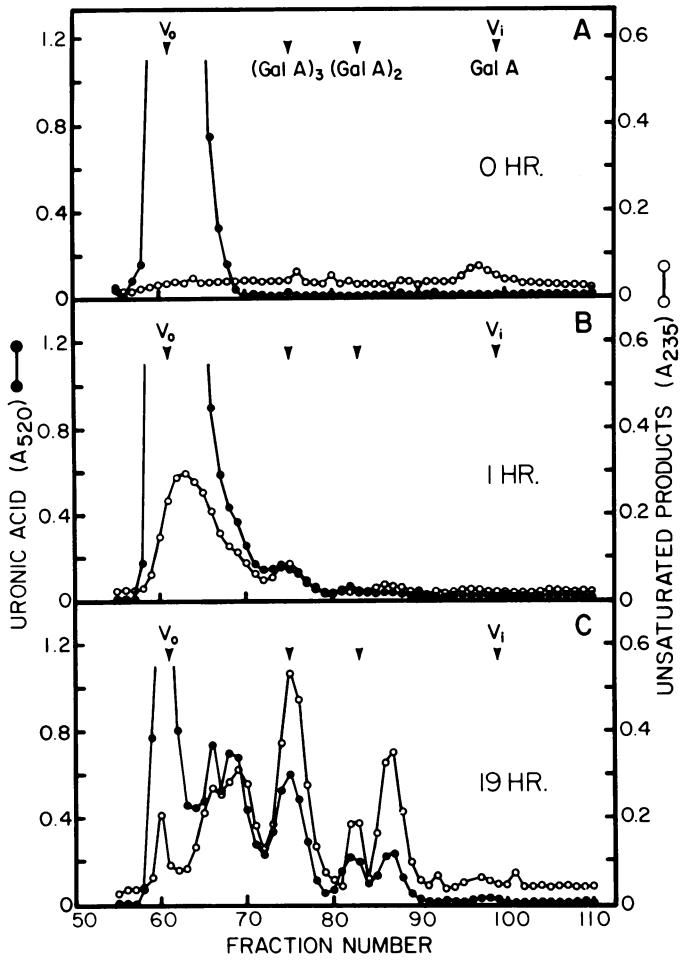

FIG. 6. Determination of the mode of lyitic cleavage, endo- or exo-, by PGA lyase I. A 5-ml reaction mixture containing 2.5 mg/ml sodium polypectate 50 mM Tris-HCl, 1 mM $CaCl_2$, and 200 μg/ml streptomycin sulfate at pH 8.5 was treated with 0.1 units of PGA lyase I. The reaction mixture was incubated at 30°C. A 1-ml aliquot was removed after 1 h, and again after 19 h, of incubation. The 1-ml aliquots were brought to pH with 1 M HCl, and boiled 30 min to inactivate the enzyme. These aliquots were chromatographed separately on a Sephadex G-15 gel filtration column (1.5 × 114 cm) equilibrated with 50 mM sodium phosphate at pH 7.0. Fractions of 1.2 ml were collected at a flow rate of 10 ml/h and assayed for uronic-acid content and A at 235 nm. The void volume (elution volume of 10,000 mol wt dextran) and the elution volumes of mono-, di-, and trigalacturonosyl acid were determined in a separate run. A, Elution profile of the sodium-polypectate reaction mixture before addition of PGA lyase I. B, Elution profile of the sodium-polypectate reaction mixture after 1 h of incubation with PGA lyase I. C, Elution profile of the NaPP reaction mixture after 19 h of incubation with PGA lyase I.

pectin (Fig. 8B). Detectable elicitor activity was released within 0.5 h. The maximum amount of elicitor activity was released after 1 h and coincided with an increase in the A at 235 nm of 0.96. This corresponded to the cleavage of approximately 11% of the available galacturonosyl linkages. The elicitor activity decreased at incubation times of more than 1 h, reaching the value of boiled enzyme controls after 3 h (A_{235nm} = 3.17). This corresponded to the cleavage of approximately 36% of the available galacturonosyl linkages. Reaction mixtures containing either citrus pectin or sodium polypectate and boiled PGA lyase I had detectable elicitor activity. This elicitor activity was 4-fold and 40-fold less than the maximum elicitor activities obtained from pectin and sodium-polypectate reaction mixtures, respectively, treated with boiled enzyme (Fig. 8, B and C).

These results demonstrated that elicitor activity could be released from commercial preparations of pectic polysaccharides that had been isolated from citrus cell walls. This release of elicitor activity was accomplished when 11% or less of the total available galacturonosyl linkages in the polymers were cleaved.

DISCUSSION

The results presented in this paper demonstrated that PGA lyases isolated and purified from *E. carotovora* elicited phytoalexin accumulation in soybean cotyledons. Heat-labile elicitor activity present in *E. carotovora* culture filtrates co-purified with PGA lyase activity throughout a purification procedure that included cation-exchange and agarose-affinity chromatographies (Table I). The relative purification and recovery of PGA lyase I and the associated heat-labile elicitor activity were very similar. The 2-fold difference between the relative purification and recovery of PGA lyase II and the associated heat-labile elicitor activity (Table I) can be explained by the observation that the PGA lyase II activity presented in Table I was underestimated by a factor of at least two. This was because PGA lyase II exhibited maximum enzyme activity in the presence of 0.25 mM

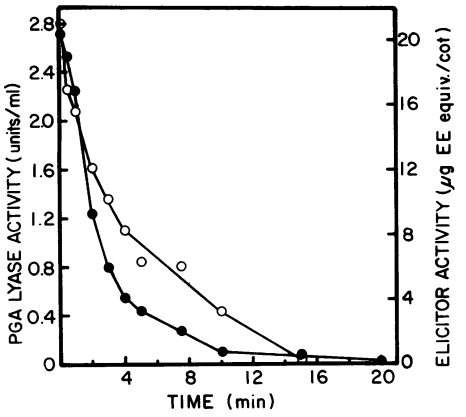

FIG. 7. The heat lability of PGA lyase I and the associated elicitor activity. A 1-ml aliquot of agarose-purified PGA lyase I (5.3 units enzyme activity) was placed on a hot water bath at 60°C ± 0.3°C. At the time intervals indicated in the figure, 75-μl aliquots were removed and placed in test tubes immersed in an ice bath. At the end of the experiment, 5 μl from each aliquot was assayed for PGA lyase activity (●) and 10 μl for elicitor activity (○), as described in "Materials and Methods." The enzyme activity shown was the average of two determinations, and the elicitor activity shown was the average obtained from 20 cotyledons (EE = QAE-purified endogenous elicitor).

$CaCl_2$, and was inhibited 64% at 1 mM $CaCl_2$ (unpublished results), a $CaCl_2$ concentration at which PGA lyase I was maximally active and which was present in the PGA lyase assay used during the purification procedure. It is possible that other heat-labile elicitors were present in the initial culture filtrate and were removed during the purification procedure. However, this is unlikely because if the heat-labile elicitor activity is corrected for the loss of PGA lyase I and II during the purification procedure, essentially 100% of the heat-labile elicitor activity in the freeze-thawed culture filtrate was due to the PGA lyases. This calculation assumes that PGA lyase I and II were the only PGA lyases present in the freeze-thawed culture filtrate and includes the correction for the 2-fold underestimation of PGA lyase II activity.

The agarose-purified PGA lyase I elicitor was maximally active as an elicitor in the soybean cotyledon assay at a concentration of 0.03 units/ml PGA lyase activity. This is similar to the elicitor activity of an endopolygalacturonase from *Rhizopus stolonifer*, which has been shown to be near maximally active as an elicitor of casbene synthetase in castor bean (*Ricinus communis*) at 0.05 units/ml enzyme activity (7). This suggested that the two enzymes may elicit by similar mechanisms. The elicitor activity of these endo- pectin-degrading enzymes was correlated with the ability of the enzymes to depolymerize pectic polymers and was not dependent on the mechanism of depolymerization, *i.e.*, lyitic *versus* hydrolytic.

The close correlation between the loss of PGA lyase I and the associated elicitor activity during heat treatment suggested that the enzyme activity was necessary for elicitor activity. This was supported by the observation that heat-stable elicitors were obtained by the lyitic degradation by PGA lyase I of purified soybean cell walls, citrus pectin, and sodium polypectate. The elicitor-active fraction solubilized from the soybean cell walls was rich in uronic acid, as was an acid-released endogenous elicitor previously described (14, 25). This elicitor-active fraction probably contains many different components; the composition and structure of the active components remain to be determined.

The heat-stable elicitors released from sodium polypectate were degraded during longer incubation times with PGA lyase I. This demonstrated that the elicitor-active molecules contained α-1,4-galacturonosyl linkages that were necessary for elicitor activity. The observation that the heat-stable elicitors released

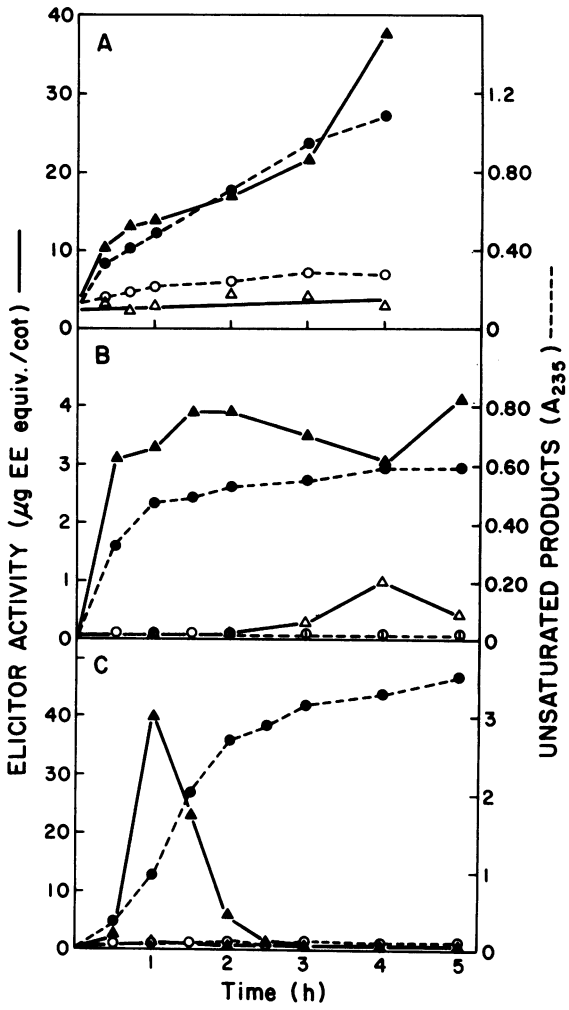

FIG. 8. Solubilization of HS elicitors from soybean cell walls, citrus pectin, and sodium polypectate. Reaction mixtures contained substrate (either 10 mg/ml cell walls, or 1 mg/ml citrus pectin or sodium polypectate), 5 mM Tris-HCl, 1 mM $CaCl_2$, and 0.027 units/ml PGA lyase I. Controls included an equivalent amount of PGA lyase I that had been boiled for 30 min before addition to the reaction mixtures. Reaction mixtures were incubated at 30°C with constant shaking. Aliquots were removed at the time intervals indicated in the figure and processed as described in "Results." A 1-ml portion of each aliquot from the cell wall experiment and 0.25-ml portions of each aliquot from the citrus pectin and sodium-polypectate experiments were diluted with sterile H_2O to a total volume of 2.0 ml and assayed for elicitor activity, as described in "Materials and Methods." The elicitor activity (▲, △) shown was the average obtained from 20 cotyledons. The *A* at 235 nm (●, ○) for each aliquot was determined from a 1:10 dilution into deionized H_2O. The closed symbols (▲, ●) represent data obtained with unboiled enzyme, and the open symbols (△, ○), data obtained with boiled enzyme. A, Solubilization of heat-stable elicitors from soybean cell walls. B, Solubilization of heat-stable elicitors from citrus pectin. The elicitor activity of a citrus-pectin reaction mixture without enzyme (2.2 μg EE eq/cotyledon) has been subtracted from each data point. C, Solubilization of heat-stable elicitors from sodium polypectate. The elicitor activity of a sodium-polypectate reaction mixture without enzyme (3.1 μg EE eq/cotyledon) has been subtracted from each data point (EE = QAE-purified endogenous elicitor).

from citrus pectin were not degraded at longer incubation times established that the elicitor-active molecules were different from those obtained from sodium polypectate. This difference was most likely due to the presence of methyl-esterified galacturonosyl residues in the pectin-derived, but not in the sodium-polypectate-derived, elicitor. The presence of the methyl-esterified residues would have protected the elicitor-active molecules from further degradation by PGA lyase I. This is consistent with the observation that the *A* at 235 nm of the citrus-pectin reaction mixture increased very slowly after the 1st h of incubation and never reached the levels obtained in the sodium-polypectate reaction mixture.

The variations in the ability of PGA lyase I to degrade citrus pectin and sodium polypectate may also explain why the maximum elicitor activity obtained from citrus pectin was less than that obtained from sodium polypectate. The structural characterization of heat-stable elicitors obtained by partial acid hydrolysis of soybean cell walls and citrus pectin has shown that only oligogalacturonides containing approximately 9 to 13 galacturonosyl residues have elicitor activity (25). Because citrus pectin is a poor substrate for PGA lyase, it is likely that the elicitors released from citrus pectin by PGA lyase were present in the reaction mixture in lower concentrations than the elicitors released from sodium polypectate. Also, the elicitors obtained from citrus pectin may not have been the correct size for maximum elicitor activity. Another possibility is that the presence of methyl-esterified galacturonosyl residues in the elicitor-active components derived from citrus pectin decreased their elicitor activity.

The results presented in this paper strongly suggest that PGA lyase elicits phytoalexin accumulation by releasing elicitor-active, galacturonic acid-rich oligosaccharides from the pectic polymers of soybean cell walls. These results are analogous to those obtained by Lee and West (19, 20). They have demonstrated that a highly purified endopolygalacturonase from the fungus *Rhizopus stolonifer* is an elicitor of casbene synthetase in castor bean seedlings. It has also been shown that this *R. stolonifer* pectin-degrading enzyme can release heat-stable elicitors from citrus pectin, polygalacturonic acid, and a particulate fraction obtained from castor bean seedlings (7). The results with the *R. stolonifer* and *E. carotovora* enzymes, considered in conjunction with the observation that pectic oligosaccharide elicitors can be obtained from several different types of plant cell walls by partial acid hydrolysis (14), suggests a general concept of elicitation by microbial pectin-degrading enzymes (7, 14, 34). Briefly, this concept proposes that plants have evolved a defense mechanism that responds to pectin-degrading enzymes in a manner that does not depend on recognizing the enzyme molecules themselves. Rather, the plant cells recognize a pectic oligosaccharide (endogenous elicitor) released by partial depolymerization of pectic polymers present in the primary cell walls of plants; such depolymerization appears to be necessary for microorganisms to infect plants. In this hypothesis, the endogenous elicitor serves as a regulatory molecule that initiates the synthesis and accumulation of phytoalexins in infected tissues.

It is apparent from recently published results (7, 19, 20) and those in this manuscript that pectin-degrading enzymes are likely to be physiologically important elicitors of phytoalexin accumulation. One question that relates to the role of pectin-degrading, phytoalexin-eliciting enzymes is, how do some microorganisms that secrete these enzymes successfully infect plants despite their phytoalexin-elicitor activity? There are likely to be several factors that allow such infections to occur. These may include the observed ability of many phytopathogens to detoxify the phytoalexins of their hosts (32), and the well-known ability of some phytopathogens to secrete phytotoxins that may injure or kill enough of the cells of its host at the site of infection to prevent the host from mounting a successful defense (31). Another possibility is that microorganisms may secrete enzymes that so rapidly degrade the endogenous elicitor that it cannot accumulate to effective levels. Other factors that may lead to successful infections by such pectic enzyme secreting microorganisms are discussed in a review of this subject (9).

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