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Host-Pathogen Interactions[1]

XXXIII. A Plant Protein Converts a Fungal Pathogenesis Factor into an Elicitor of Plant Defense Responses

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ABSTRACT

This paper describes the effect of a plant-derived polygalacturonase-inhibiting protein (PGIP) on the activity of endopolygalacturonases isolated from fungi. PGIP's effect on endopolygalacturonases is to enhance the production of oligogalacturonides that are active as elicitors of phytoalexin (antibiotic) accumulation and other defense reactions in plants. Only oligogalacturonides with a degree of polymerization higher than nine are able to elicit phytoalexin synthesis in soybean cotyledons. In the absence of PGIP, a 1-minute exposure of polygalacturonic acid to endopolygalacturonase resulted in the production of elicitor-active oligogalacturonides. However, the enzyme depolymerized essentially all of the polygalacturonic acid substrate to elicitor-inactive oligogalacturonides within 15 minutes. When the digestion of polygalacturonic acid was carried out with the same amount of enzyme but in the presence of excess PGIP, the rate of production of elicitor-active oligogalacturonides was dramatically altered. The amount of elicitor-active oligogalacturonide steadily increased for 24 hours. It was only after about 48 hours that the enzyme converted the polygalacturonic acid into short, elicitor-inactive oligomers. PGIP is a specific, reversible, saturable, high-affinity receptor for endopolygalacturonase. Formation of the PGIP-endopolygalacturonase complex results in increased concentrations of oligogalacturonides that activate plant defense responses. The interaction of the plant-derived PGIP with fungal endopolygalacturonases may be a mechanism by which plants convert endopolygalacturonase, a factor important for the virulence of pathogens, into a factor that elicits plant defense mechanisms.

Fungal endopolygalacturonases have important functions during the early stages of plant pathogenesis (25). These enzymes hydrolyze pectic components of plant cell walls, assisting in the colonization of plant tissues. Endopolygalacturonases are the first detectable enzymes secreted by phytopathogenic fungi when they are grown *in vitro* on plant cell walls (29). The products of the enzyme's action are thought to be used as nourishment by the fungi. Furthermore, pretreatment of plant cell walls with endopolygalacturonase appears to facilitate the ability of other fungus-secreted plant cell wall-degrading enzymes to attack their substrates (30).

Fungal endopolygalacturonases have also been shown to activate plant defense responses including phytoalexin accumulation (33), lignification (37), synthesis of proteinase inhibitors (39), and necrosis (10, 34). In these cases, evidence has been presented suggesting that the endopolygalacturonases activate plant-defense responses by releasing oligogalacturonides from the plant cell walls. The oligogalacturonides induce the plant defense responses. Linear α-(1→4)-linked oligogalacturonides of chain lengths varying between 10 and 13 have been shown to induce phytoalexin accumulation (24, 28, 36) and lignification (36). In these systems, oligogalacturonides shorter than 10 glycosyl residues had little or no biological activity (14, 15, 17, 24, 28, 36). Shorter oligogalacturonides have been shown to induce proteinase inhibitors (5), another plant defense response. *In vitro*, endopolygalacturonase converts polygalacturonic acid to mono-, di-, and, sometimes, trigalacturonic acid (21). Thus, while endopolygalacturonases are able to release biologically active oligogalacturonides, these enzymes are also able to rapidly convert them into inactive oligomers.

Fungal endopolygalacturonases have at least two functions in plant tissues: on the one hand, the endopolygalacturonases are pathogenicity factors as they disrupt the structure of plant cell walls, allowing fungal colonization of plant tissue and providing nourishment for the fungus. On the other hand, the endopolygalacturonases are potential avirulence factors, since they may activate plant defense responses by releasing plant cell wall fragments that signal the plant to defend itself.

The dichotomy of the potential roles of endopolygalacturonase in plant-pathogen interactions raises the question of whether plants have a mechanism by which the balance between release of elicitor-active oligogalacturonides and the depolymerization of the active oligogalacturonides into inactive molecules could be altered to favor accumulation of elicitor-active molecules. PGIPs[3] have been isolated from a

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[3] Abbreviations: PGIP, endopolygalacturonase-inhibiting protein; PAHBAH, *p*-hydroxybenzoic acid hydrazide; FPLC, fast protein liquid chromatography.

variety of dicotyledonous plants, including beans (2, 11, 32), peas (26), green peppers (6), tomatoes (7), cucumbers (38), apples (22), pears (1), oranges (4), and alfalfa (19). PGIPs inhibit fungal endopolygalacturonases. PGIPs have not been found to inhibit fungal exopolygalacturonases (27), fungal pectin or pectate lyases, or bacterial and plant endopolygalacturonases (unpublished results of the authors). Albersheim and Anderson (2) reported that PGIP from *Phaseolus vulgaris* completely inhibited endopolygalacturonase activity. More recently, Cervone *et al.* (11) reported that, in the case of PGIP from *P. vulgaris* and the endopolygalacturonase from *Aspergillus niger*, the enzyme retained some residual activity even in the presence of an excess of PGIP. The suggestion was made that PGIP *in vivo* might affect the activity of fungal endopolygalacturonases such that the oligosaccharides these enzymes generate, that are active in eliciting plant defense responses, remain stable for longer periods of time (9, 16). We now show that the effect of *P. vulgaris* PGIP on the *in vitro* digestion of polygalacturonic acid by the endopolygalacturonases of either *A. niger* or *Fusarium moniliforme* leads to the prolonged existence, in the digestion mixture, of oligogalacturonides active in eliciting phytoalexins.

MATERIALS AND METHODS

Chemicals

PAHBAH, chlorhexidine, D-galacturonic acid, polygalacturonic acid (sodium salt), and *Aspergillus niger* pectinase were obtained from Sigma Chemical Co. (St. Louis, MO). Trigalacturonic acid was obtained from Carbohydrates International (Arlöv, Sweden). Decagalacturonic acid was chemically synthesized by Nakahara and Ogawa (35); its mol wt was confirmed by fast atom bombardment-mass spectrometry (36). Metahydroxybiphenyl was from Eastman Kodak (Rochester, NY).

Endopolygalacturonase Assay and Purification

Endopolygalacturonase activity was determined by reducing end-group analysis using the PAHBAH procedure (40). One activity unit (RGU) was defined as the amount of enzyme producing 1 microequivalent of reducing group/min at 30°C with 1% (w/v) polygalacturonic acid as substrate. Homogeneous endopolygalacturonase from *A. niger* was prepared from commercial *A. niger* pectinase as reported by Cervone *et al.* (10) and had a specific activity of 2,300 RGU/mg. Endopolygalacturonase from *Fusarium moniliforme* was prepared as reported by De Lorenzo *et al.* (18) and had a specific activity of 430 RGU/mg. Quantities of endopolygalacturonase given in pmoles were calculated from published apparent M_r values of 33,500 D for the *A. niger* enzyme (10) and 43,000 D for the *F. moniliforme* enzyme (18), respectively.

PGIP Purification

PGIP was purified 2850-fold from hypocotyls of *Phaseolus vulgaris* cv Cannellino by affinity chromatography through a Sepharose-endopolygalacturonase column and assayed as reported (11). Quantities of PGIP given in pmoles were calculated from the published apparent M_r of 41,000 D (11).

FPLC

Chromatography of oligogalacturonides by FPLC was performed on a 0.5 × 5 cm Mono Q column (Pharmacia Fine Chemicals, Sweden) equilibrated with distilled water. Samples containing ≃1 mg of oligogalacturonides dissolved in 500 μL of water were applied to the column and the column was then washed with 5 mL of water. Oligogalacturonides were eluted from the column at a flow rate of 1.0 mL/min with a linear 50 mL gradient of 0.2 to 1.0 M ammonium bicarbonate. Fractions (1 mL) were collected, and the uronic acid content of each fraction was determined by the *meta*-hydroxybiphenyl method (40).

Elicitor Assay

Elicitor activity of samples was determined by the cotyledon bioassay as described (24). Cotyledons from 8-d-old soybean plants (*Glycine max* L. cv Harper) were used.

RESULTS

The effect of varying the amount of *Aspergillus niger* endopolygalacturonase on the rate of depolymerization of polygalacturonic acid was determined by following the release of reducing groups (Fig. 1A). The average degree of polymerization of oligogalacturonides in the reaction mixture was determined as the molar ratio of uronic acids to reducing end groups. The time course of the depolymerization of polygalacturonic acid followed apparent first-order kinetics (Fig. 1B). The time required to reduce by 50% the average degree of polymerization of 1.44 μmol/mL of polygalacturonic acid varied between 230 min for 0.2 pmol/mL of endopolygalacturonase to 10 min for 10 pmol/mL of endopolygalacturonase.

Endopolygalacturonase hydrolyzed polygalacturonic acid much more slowly in the presence of 250 pmol/mL of PGIP (Fig. 2A). The time required to decrease the average degree of polymerization of polygalacturonic acid by 50% in the presence of PGIP was on the order of hours, even at a concentration of endopolygalacturonase as high as 25 pmol/mL. The time course of hydrolysis in the presence of PGIP followed apparent first-order kinetics (Fig. 2B).

The size distribution of the oligogalacturonides produced by endopolygalacturonase treatment of polygalacturonic acid, in the presence or absence of PGIP, were evaluated by FPLC on a Mono Q anion exchange column. In the presence of 25 pmol/mL endopolygalacturonase, polygalacturonic acid was hydrolyzed to intermediate-sized galacturonides in less than 1 min (Fig. 3B). Further incubation resulted in continued hydrolysis yielding oligogalacturonides with a degree of polymerization of less than six after 15 min (Fig. 3C) and oligogalacturonides with a degree of polymerization of less than four after 90 min (Fig. 3D). FPLC analysis of the oligogalacturonides generated from polygalacturonic acid by endopolygalacturonase in reactions identical to those of Figure 3, except in the presence of 250 pmol/mL of PGIP, showed that the degree of polymerization was reduced at a much slower rate (Fig. 4). It required 24 h (Fig. 4C) rather than about 1 min (Fig. 3B) to convert most of the polygalacturonic acid substrate to intermediate size oligomers (Fig. 4C).

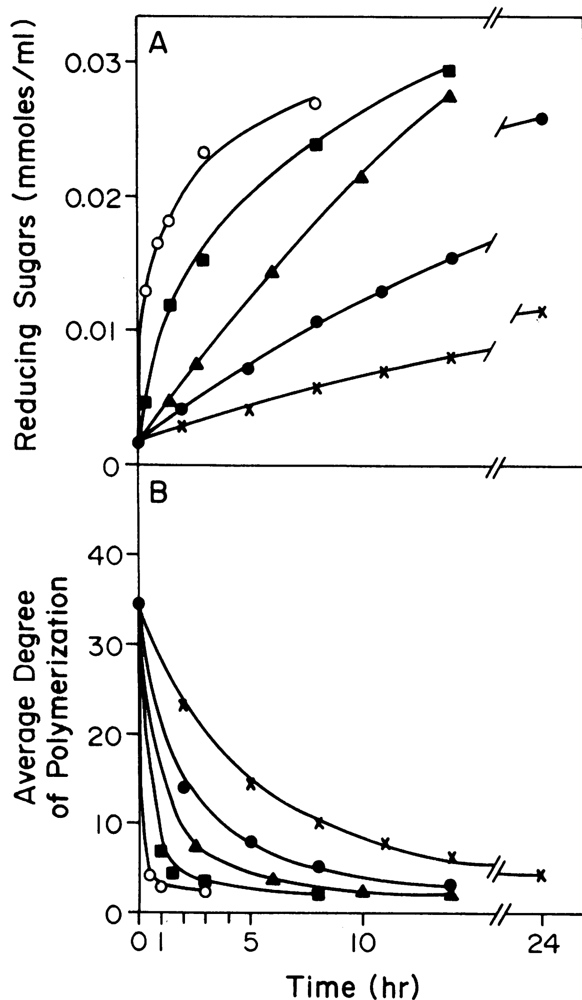

Figure 1. Time-course of digestion of polygalacturonic acid by *A. niger* endopolygalacturonase. A, Release of reducing groups and B, average degree of polymerization of oligogalacturonides generated. The 1 mL reaction mixtures in 50 mM sodium acetate (pH 5.0), contained 10 mg polygalacturonic acid and the following quantities of endopolygalacturonase: 0.25 pmol (×); 0.5 pmol (●); 1.0 pmol (▲); 2.5 pmol (■); 10 pmol (○). When less than 1.0 pmol of endopolygalacturonase was used, 0.1 mg of BSA was added to stabilize the enzyme.

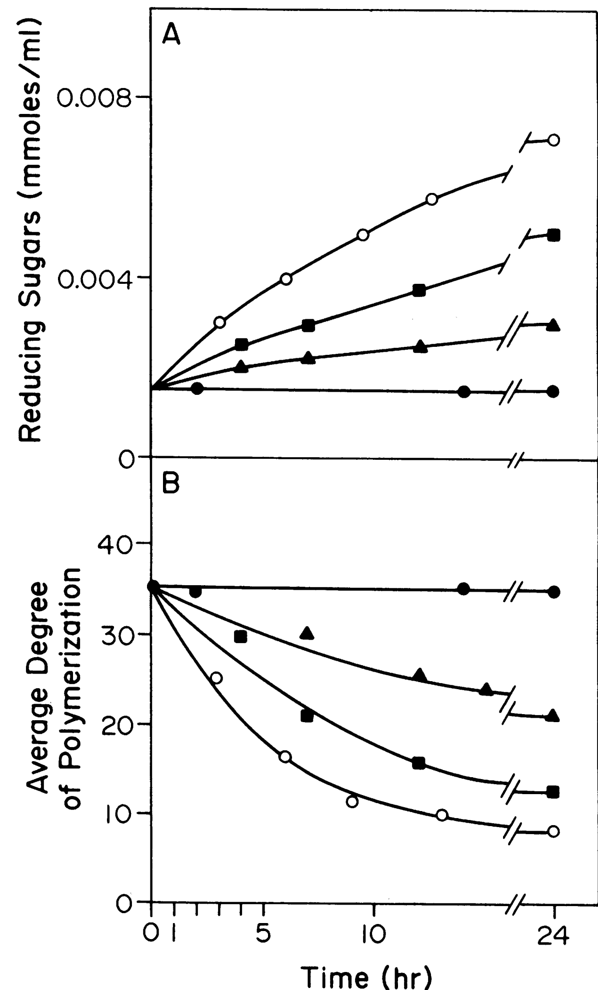

Figure 2. Time-course of digestion of polygalacturonic acid by *A. niger* endopolygalacturonase in the presence of *P. vulgaris* PGIP. A, Release of reducing groups and B, average degree of polymerization of oligogalacturonides generated. The 1 mL reaction mixtures in 50 mM sodium acetate (pH 5.0), contained 10 mg polygalacturonic acid, 250 pmol PGIP and the following quantities of endopolygalacturonase: 0 pmol (●); 2.5 pmol (▲); 10 pmol (■); 25 pmol (○).

Short oligomers (length < 6) became predominant after 48 h of digestion (Fig. 4D).

The phytoalexin-inducing activity of the digestion mixtures obtained from polygalacturonic acid that had been treated with endopolygalacturonase either in the presence (Fig. 5) or absence (Fig. 5, inset) of PGIP was tested with the soybean cotyledon bioassay. The elicitor activity of the digestion products of polygalacturonic acid treated with endopolygalacturonase in the absence of PGIP was transient, only the sample boiled immediately after adding the endopolygalacturonase to the polygalacturonic acid exhibited appreciable elicitor activity. Boiling polygalacturonic acid had no effect on either its elution from the Mono Q column or its elicitor activity (data not shown). In contrast, when polygalacturonic acid was treated with endopolygalacturonase in the presence of PGIP, the elicitor activity of the products reached a maximum after 13 h of incubation. The elicitor activity of the digestion products remained high for at least 24 h, decreasing to a low level only after 48 h of incubation (Fig. 5).

An estimation was made of the sizes of oligogalacturonides in the digestion mixtures that were active as elicitors. Polygalacturonic acid was treated with endopolygalacturonase in the presence of PGIP for 24 h, and the digestion products were fractionated by FPLC as before. The oligogalacturonide-containing fractions were divided into four pools, and the elicitor activity of each pool was determined (Table I). The results clearly demonstrated that those oligogalacturonides eluting in pool II were most active. This result was expected, inasmuch as previous work had demonstrated that oligogalacturonides with a degree of polymerization between 10 and 13 were most active in inducing phytoalexin accumulation (14, 15, 24, 28, 36). The next larger class of oligogalacturonides

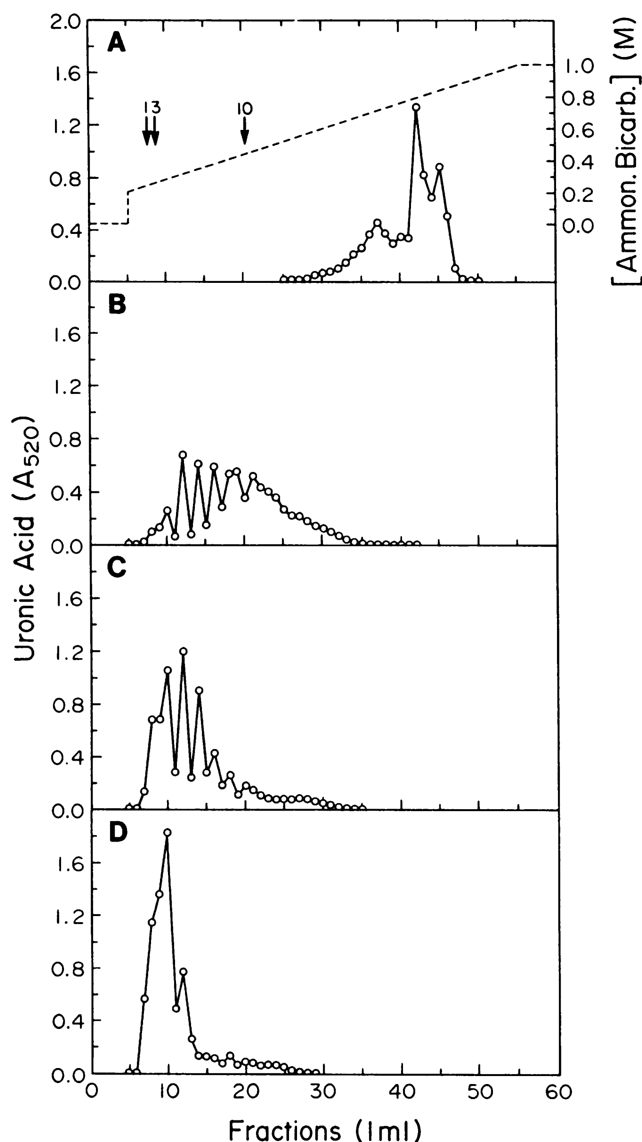

Figure 3. FPLC of oligogalacturonides generated by *A. niger* endopolygalacturonase treatment of polygalacturonic acid. The reaction mixture (1 mL) contained 10 mg of polygalacturonic acid and 25 pmol of endopolygalacturonase. The reactions were carried out in H_2O to permit bioassay of aliquots of the reaction mixture (Fig. 5). The pH of polygalacturonic acid dissolved in H_2O was ≈5. At the indicated times, aliquots (100 μL) were removed from the digestion mixture, boiled for 10 min, diluted to 500 μL with distilled water and subjected to FPLC on a Pharmacia Mono Q anion exchange column as described in "Materials and Methods." A, Untreated polygalacturonic acid; B, polygalacturonic acid mixed with endopolygalacturonase and immediately boiled; C, 15 min digestion; and D, 90 min digestion. Arrows in A indicate the elution volumes of mono-, tri-, and decagalacturonic acid.

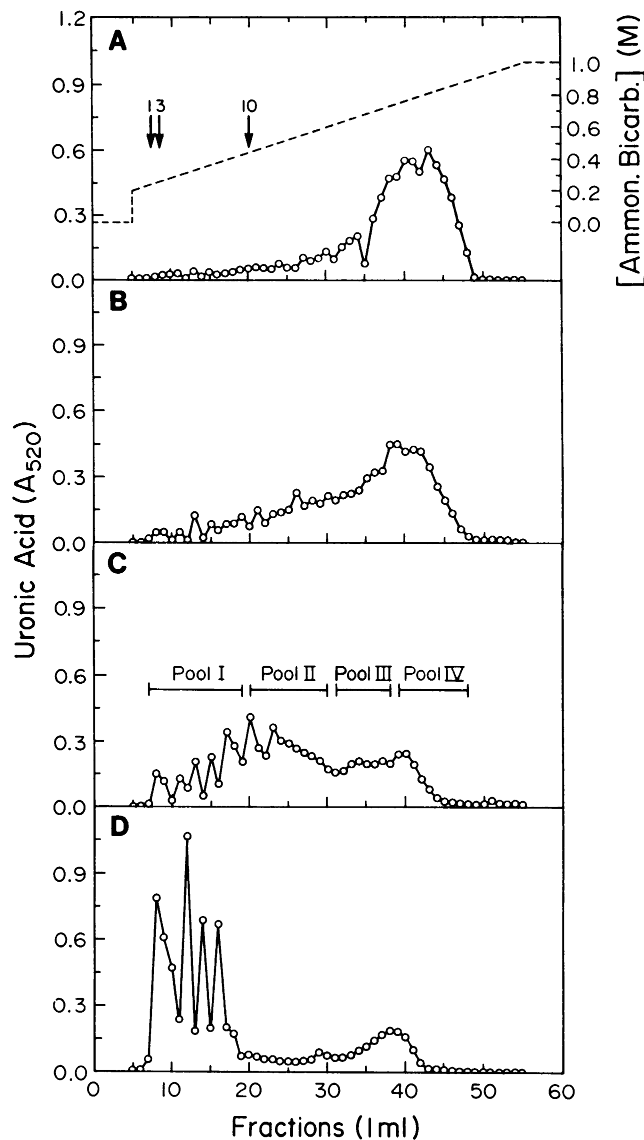

Figure 4. FPLC of oligogalacturonides generated by *A. niger* endopolygalacturonase treatment of polygalacturonic acid in the presence of PGIP. The incubation mixture (1 mL) was the same as that described in Figure 3 except for the addition of 250 pmol of *P. vulgaris* PGIP and 0.1 mg/mL chlorhexidine as an antibiotic. The presence of the antibiotic had no effect on the activity of either endopolygalacturonase or PGIP (data not shown). Other experimental details were the same as described for Figure 3. Fractions indicated in C were pooled as follows for determination of elicitor activity (see Table I): pool I, fractions 7–19; pool II, fractions 20–30; pool III, fractions 31–38; pool IV, fractions 39–48. Digestion times were (A) 6 h 30 min; (B) 13 h; (C) 24 h; and (D) 48h.

(pool III) had, on average, half of the activity of those in pool II.

The effect of *P. vulgaris* PGIP on an endopolygalacturonase from *Fusarium moniliforme* was examined in order to determine whether the observed effect of PGIP on the *A. niger* endopolygalacturonase is a general phenomenon with fungal endopolygalacturonases, as would be expected from earlier observations (23). *F. moniliforme* endopolygalacturonase alone or in the presence of *P. vulgaris* PGIP digested polygalacturonic acid in a manner that was not apparently different from that of the *A. niger* endopolygalacturonase (Fig. 6). In the absence of PGIP, *F. moniliforme* endopolygalacturonase hydrolyzed polygalacturonic acid to short oligogalacturonides within 30 min. In the presence of a 25-fold molar excess of

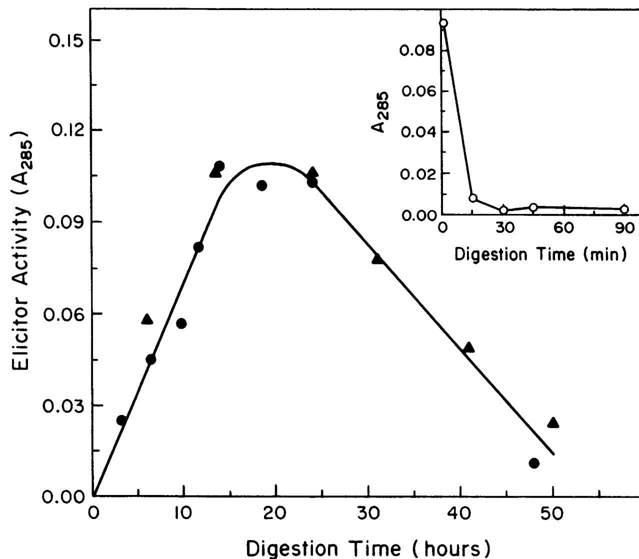

Figure 5. Elicitor activity of oligogalacturonides produced by *A. niger* endopolygalacturonase treatment of polygalacturonic acid in the absence (inset) and presence of a 10-fold molar excess of *P. vulgaris* PGIP. The incubation mixtures with and without PGIP were as reported in Figures 3 and 4, respectively. At various times, aliquots (100 μL) were taken from the digestion mixture, heated to 100°C for 10 min, diluted to a uronic concentration of 130 μg/mL (±10%), and tested for elicitor activity in the soybean cotyledon bioassay (24). Elicitor activity was measured as the A_{285} of the wound-droplet solution. The A_{285} is linearly correlated with the actual phytoalexin content of the wound-droplet solutions (3, 24). Each data point is the average of two replicates (each replicate is the average of ten cotyledons). The results with PGIP are for two separate experiments (●, ▲).

Table I. *Elicitor Activity of Oligogalacturonides Collected from FPLC (see Fig. 4C)*

Oligogalacturonide Pool[a]	Elicitor activity (A_{285})[b]
I (Fractions 7–19 eluting between 0.2 and 0.42 M bicarbonate)	0.01
II (Fractions 20–30 eluting between 0.42 and 0.6 M bicarbonate)	0.07
III (Fractions 31–38 eluting between 0.6 and 0.72 M bicarbonate)	0.04
IV (Fractions 39–48 eluting between 0.72 and 0.87 M bicarbonate)	0.00

[a] 1 mg of polygalacturonic acid was digested for 24 h with 25 pmol of *A. niger* endopolygalacturonase in the presence of a 10-fold molar excess of *P. vulgaris* PGIP as reported in the legend of Figure 4. The products were subjected to FPLC as described in Figure 4. Fractions (1 mL) were collected and pooled as indicated. Each pool was lyophilized and redissolved in water. The lyophilization was repeated twice more to remove residual ammonium bicarbonate. Each pool was dissolved in water and the concentration of uronic acid in each pool was adjusted to 80 μg/ml. [b] Elicitor activity was measured with the soybean cotyledon bioassay as described in Figure 5.

PGIP, hydrolysis of polygalacturonic acid occurred slowly, with kinetics very similar to that observed with *A. niger* endopolygalacturonase (*cf.* Figs. 2 and 6). Fractionation, by FPLC, of the oligogalacturonides generated from polygalacturonic acid by *F. monoliforme* endopolygalacturonase in the presence or absence of PGIP yielded elution profiles that were similar to those obtained with products of *A. niger* endopolygalacturonase digestion (data not shown). Small amounts of phytoalexin-eliciting activity were detected in the *F. moniliforme* endopolygalacturonase digestion products of polygalacturonic acid incubated for 2 min in the absence of PGIP, but no elicitor activity was detected after longer incubation times (Table II). In the presence of PGIP, the elicitor activity of the digestion products increased during the 55-h incubation period examined (Table II).

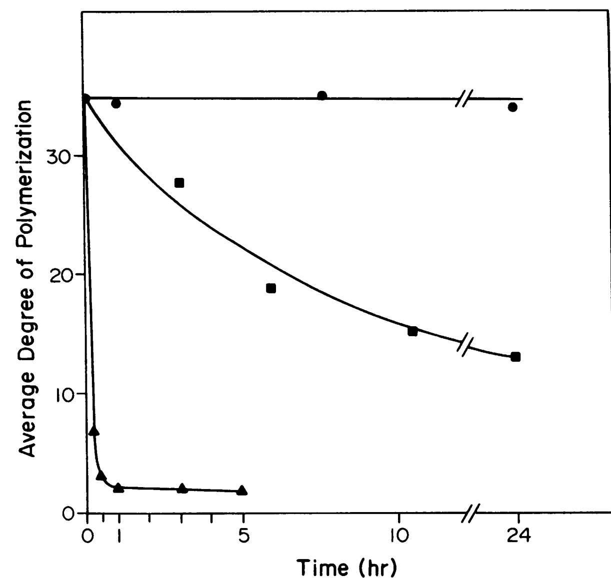

Figure 6. Time-course of digestion, in the absence (▲) and in the presence of *P. vulgaris* PGIP (■), of *F. moniliforme* endopolygalacturonase-treated polygalacturonic acid. The 1 mL reaction mixture in 50 mM sodium acetate (pH 5.0), contained 10 mg polygalacturonic acid and either 10 pmol of endopolygalacturonase (▲) or 10 pmol endopolygalacturonase plus 250 pmol of PGIP (■).

Table II. *Elicitor Activity of the Digestion Products, in the Absence and Presence of P. vulgaris PGIP, of F. moniliforme* Endopolygalacturonase-treated polygalacturonic acid.

Treatment Time[a]	Elicitor Activity (A_{285})[b]
Undigested	0.00
+Endopolygalacturonase	
2 min	0.02
30 min	0.00
90 min	0.01
+Endopolygalacturonase +PGIP	
6 h	0.01
13.5 h	0.02
24 h	0.06
55 h	0.10

[a] Enzyme digestions were carried out as described in Figure 6. [b] Elicitor activity of the digestion mixtures was assayed as described in Figure 5.

DISCUSSION

Plant-cell-wall-derived oligogalacturonides, with a degree of polymerization higher than nine, are an important class of

elicitors of phytoalexin accumulation in plants (24, 28, 36). The release of the phytoalexin-inducing oligogalacturonides *in vivo* at the onset of a fungal attack, appears to be a mechanism by which the presence of the pathogen is signaled to the plant and defense responses are activated. Endopolygalacturonase, which is secreted by fungal pathogens before they secrete other plant-cell-wall-degrading enzymes (13, 29), is thought to be involved in the release of the phytoalexin-inducing oligogalacturonides. However, endopolygalacturonases rapidly degrade polygalacturonides into oligomers too short to possess elicitor activity (24, 36).

The results presented in this paper suggest a mechanism by which the activity of fungal endopolygalacturonases is modified by a constitutive plant protein such that the half-life of elicitor-active oligogalacturonides released by the endopolygalacturonases is dramatically increased. The endopolygalacturonases retained about 0.3% of their initial activity in the presence of excess PGIP (Figs. 2, 4, and 6), a plant cell-wall-derived protein previously thought to completely inhibit fungal endopolygalacturonases (2). The residual activity is sufficient to form elicitor-active oligogalacturonides from polygalacturonic acid (Fig. 5; Table II), yet limited enough to only slowly depolymerize the active oligogalacturonides to oligogalacturonides too short to possess elicitor activity (Fig. 5). As a consequence of the presence of PGIP, the elicitor-active oligogalacturonides are maintained in the reaction mixture for many hours (Figs. 4, 5, and 6) rather than a few minutes (Figs. 3 and 5, inset).

Comparison of the digestion of polygalacturonic acid by very low amounts of endopolygalacturonase in the absence of PGIP (Fig. 1) with digestion by larger amounts of the enzyme in the presence of excess PGIP (Fig. 2) suggested that the digestion proceeded in a similar way in both cases. This was confirmed by the FPLC experiments (Figs. 3 and 4) that showed that comparable oligogalacturonide profiles resulted from very short reaction times in absence of PGIP (Fig. 3B) and long digestion times in the presence of the inhibitor protein (Fig. 4C). Thus, it appears that PGIP does not alter the mode of action of endopolygalacturonase on polygalacturonic acid. Therefore, depolymerization of polygalacturonic acid by endopolygalacturonase in the presence of PGIP is probably due to the enzyme activity of residual free endopolygalacturonase in equilibrium with the endopolygalacturonase-PGIP complex. Assuming that the enzyme fraction engaged in a theoretical biomolecular endopolygalacturonase-PGIP complex is fully inactive, dissociation constants can be calculated from the data in Figure 2 using published values for the mol wt (10) and specific activity (8) of *A. niger* endopolygalacturonase and the mol wt of PGIP (11). The average of the dissociation constants calculated for three ratios of endopolygalacturonase to PGIP was $1.1 \pm 0.16 \times 10^{-9}$ M.

"Recognition" in animal cells often involves binding of signals originating from one cell to a specific receptor on another cell. Such signal molecules bind specifically, reversibly, saturably, and with high affinity ($K_d \simeq 10^{-8}$ M) to a receptor protein often located on the surface of the target cell. The interaction between the signal and the receptor initiates the accumulation of other signals that lead to altered gene expression in the target cell. Endopolygalacturonase and PGIP may function, respectively, as a signal molecule and its receptor protein in the recognition that must occur between plants and their potential pathogens. Endopolygalacturonase and PGIP form a specific, reversible, saturable, high-affinity complex that leads to higher concentrations of oligogalacturonides that activate plant defense responses by regulating gene expression (12, 20).

The results described in this paper indicate that PGIPs of plants may have an important function in plant-pathogen interactions. Fungal phytopathogens that utilized endopolygalacturonase to invade and colonize plant tissue would not be expected to lose the function of these enzymes and remain phytopathogens. We suggest that PGIP converts endopolygalacturonase, an essential pathogenicity factor of the pathogen, into a trigger of plant defense responses. Experiments are in progress to investigate the *in vivo* role of PGIP, endopolygalacturonase, and oligogalacturonides in host-pathogen interactions.

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