

# Elicitation of Necrosis in *Vigna unguiculata* Walp. by Homogeneous *Aspergillus niger* Endo-Polygalacturonase and by $\alpha$ -D-Galacturonate Oligomers<sup>1</sup>

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## ABSTRACT

Endo-polygalacturonase (PG) was purified from a commercial preparation of *Aspergillus niger* pectinase by means of carboxymethylcellulose chromatography, preparative isoelectric focusing, and gel permeation through Sephadex G-50. The enzyme was electrophoretically homogeneous and consisted of a single polypeptide chain with a molecular weight of 33,500. The enzyme exhibited a specific activity significantly higher than those of purified polygalacturonases from phytopathogenic fungi. Galacturonate oligomers with a degree of polymerization higher than four appeared quickly as products of the enzymic hydrolysis of Napolypolygalacturonate. The oligomers were later degraded to di- and monogalacturonate. The homogeneous enzyme and growing mycelium of *Aspergillus niger* separately elicited a necrotic response in cowpea (*Vigna unguiculata* Walp.) pods. Heat-inactivated PG and PG inactivated with specific antibodies did not elicit necrosis, suggesting that the catalytic activity of the enzyme is necessary for its function as an elicitor. The PG-released oligosaccharides from *Vigna* cell wall and the galacturonides with a degree of polymerization greater than four separately elicited necrosis, whereas di- and monogalacturonate did not.

lacturonides with a degree of polymerization that is characteristic of each system studied.

This paper shows that homogeneous *Aspergillus niger* endo-PG and/or enzymatic products of polygalacturonic acid hydrolysis are able to elicit a necrotic response in *Vigna unguiculata*.

## MATERIALS AND METHODS

**Chemicals.** Sodium polypectate and D-galacturonic acid were obtained from NBC Corp., Cleveland, OH. Citrus pectin and commercial preparations of *A. niger* pectinase (solution in 40% glycerol) were obtained from Sigma Chemical Co. CM-cellulose (sodium salt) was from BDH Chemicals, England; polyethylene glycol 4000, dinitrosalicylic acid, thiobarbituric acid, and Coomassie brilliant blue R-250 were from Merck, Germany; CM-cellulose CM-52 and 3MM chromatography paper were from Whatman Ltd., England; ampholine carriers were from LKB Inst., Sweden; SDS was from Sigma Co. and was ethanol-recrystallized. Acrylamide and *N,N'*-bis-methylene acrylamide were obtained from Bio-Rad Laboratories. Other chemicals used were reagent grade.

**Enzyme Assay.** PG activity was determined by measuring the decrease in relative viscosity of a 0.6% (w/v) solution of sodium polypectate in 50 mM sodium acetate (pH 5.0), in Cannon-Fenske No. 300 viscometers, at 30° C. One relative viscometric unit (RVU) was defined as the amount of enzyme causing a 50% reduction in the viscosity of 6 ml of the reaction mixture in 1 min under the conditions of the assay. Hydrolysis of glycosidic bonds was followed by reducing end-group analysis as reported in a previous study (9). One activity unit (RGU) was defined as the amount of enzyme producing 1  $\mu$ eq of reducing groups per min. The products formed by the action of PG on sodium polypectate were examined by descending paper chromatography, using the method of Nasuno and Starr (23). Endo- $\beta$ -1,4-glucanase activity was determined by measuring the decrease in relative viscosity of a 0.6% (w/v) solution of CM-cellulose, sodium salt. Lyase activity was measured by the thiobarbituric acid method as reported previously (26).

**Isolation of Cell Walls and Carbohydrate Assay.** Cell walls were prepared from *Vigna* hypocotyls and leaves by grinding 5 g of fresh tissue in 50 ml of distilled water. The insoluble wall material (250 mg) was collected by filtration through a sintered-glass filter, washed with 50 ml of distilled water and with 50 ml of cold acetone, and then dried in a desiccator at 4°C. Total carbohydrate and uronic acid contents were determined in 10 mg of cell walls after hydrolysis in 2 N TFA for 60 min at 121°C in a sealed glass tube. After removing TFA by evaporation at 50°C, the residue was suspended in 2 ml of distilled water and centrifuged at 13,000g for 10 min. The supernatant was assayed

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PG<sup>2</sup> is the first cell wall-degrading enzyme synthesized by phytopathogenic fungi cultured on isolated cell walls (11). It has been reported that homogeneous polygalacturonase from *Rhizopus stolonifer* acts as an elicitor of the biosynthesis of casbene, an antifungal compound of castor bean (19, 20). The enzyme releases pectic fragments from castor bean cell wall, which act as "endogenous elicitors" (6, 13, 14).

Interest in the role of cell wall oligosaccharides as mediators of stress response in higher plants is growing. Cell wall oligosaccharides elicit natural plant defense mechanisms such as synthesis of phytoalexins (6, 17, 24.), lignin (27), proteinase inhibitors (3, 4, 33), and hydroxyproline rich glycoproteins (15, 28). The ability of oligosaccharides to elicit plant protection systems is an aspect of the general property of these molecules for regulation of gene expression (2, 22) and plant morphogenesis (32). Cell wall polysaccharide fragments that regulate gene expression or elicit defense responses include pectic fragments, e.g. oligo-1,4- $\alpha$ -D-ga-

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<sup>2</sup> Abbreviations used: PG, endo-polygalacturonase (poly(1,4- $\alpha$ -D-galacturonide)-glycanohydrolase, EC 3.2.1.15) RVU, relative viscosimetric unit; RGU, reducing group unit; CM-cellulose, carboxymethylcellulose; IgG, immunoglobulin G.

for reducing sugar by the arsenomolybdate method (30) and for uronic acid by the carbazole method (12).

**Isoelectric Focusing and Gel Electrophoresis.** Isoelectric focusing experiments were done at 4°C in a LKB column as reported previously (9). SDS-PAGE was performed in 9.25% acrylamide slab gels prepared according to Studier (31).

**Protein Determination.** Protein concentration was measured according to Lowry *et al.* (21) using BSA as standard. Protein content of an homogeneous preparation of PG was also determined with the biuret reagent (16) and by the Bradford (5) method in order to determine more accurately the extinction coefficient at 280 nm of pure PG.

**Immunology.** Rabbit antiserum against endo-PG was prepared by injecting 0.5 mg of *A. niger* homogeneous PG dissolved in 3 ml of Freund's complete adjuvant. An additional injection was made 1 month later and bleeding was done 2 months after the

first injection. Antiserum (diluted 1:8) reacted with homogeneous PG (0.2 mg/ml) in the double immunodiffusion tests performed according to Ouchterlony (25). IgG was purified from the serum by protein A-Sepharose chromatography (18). IgG concentration was estimated by absorbance at 280 nm, and the volume was adjusted to give a concentration of 10 mg of IgG per ml. IgG (40 µg) exhibited a complete inhibitory effect when incubated overnight at 25°C with 1.2 µg of homogeneous PG.

**Plant Material and Necrotic Response.** Pods (approximately 10-cm long) were detached from cowpea (*Vigna unguiculata* Walp.) plants grown under greenhouse conditions. Circular portions (2-mm diameter) of epidermis were removed from each pod with a scalpel. An effector (PG, pectic fragments, etc.) was applied in 6 µl of 50 mM acetate, pH 5.0, to the tissue after the epidermis was removed. *Vigna* pods were also inoculated with 6 µl of a suspension of *A. niger* mycelium which had been grown

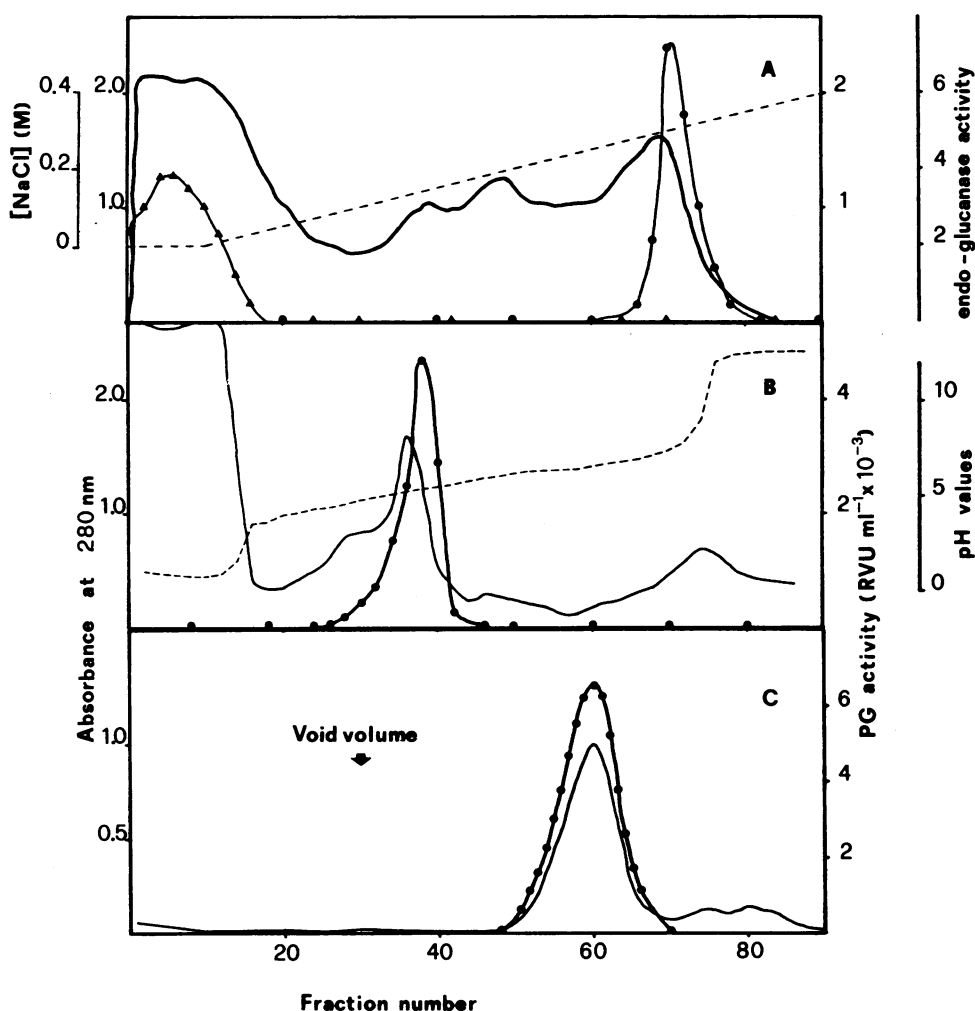


FIG. 1. (A) Elution profile of *A. niger* PG activity from a CM-cellulose column. Fractions (10 ml) were collected and assayed for activity by the viscometric method (●—●) PG activity; (▲—▲) cellulase activity; (—) *A* at 280 nm; (----) NaCl gradient. (B) Isoelectric focusing of PG. Fractions (2.5 ml) were collected and analyzed for PG activity (●—●) *A* at 280 nm (—), pH (----). (C) Elution profile of PG from a Sephadex G-50 column. Fractions (1 ml) were collected and analyzed for PG activity (●—●) and *A* at 280 nm (—).

Table I. Purification of *A. niger* Polygalacturonase

Fraction	Volume	Total Activity	Total Protein	Specific Activity	Yield	Purification
	ml	RVU × 10 <sup>-3</sup>	mg	RVU/mg	%	-fold
Commercial preparation	100	60.1	1360	44.1	100	0
CM-cellulose	60	53.4	52.3	1010	89	23
Electrofocusing	17.5	32.2	11.8	2730	53.6	62
Sephadex G-50	16	27.6	6.3	4370	46	99

in Czapek-Dox liquid medium for 10 d. Inoculated or treated pods were incubated in a humid chamber at 25°C in the dark, and 6  $\mu$ l of water were added at the inoculation point after 8 h. The appearance of the necrotic response was scored after 16 h of further incubation and was characterized by a visible browning detected at the inoculation point.

### RESULTS AND DISCUSSION

A commercial pectinase solution contained 600 RVU/ml of PG and 65 RVU/ml of endo-glucanase. The solution did not contain detectable pectin or polygalacturonic acid lyase activity.

A solution (4 ml) of 0.5 M sodium acetate, pH 5.0, was added to 100 ml of commercial pectinase. The enzyme solution was adsorbed at 4°C on a 2.5  $\times$  30 cm CM-cellulose column equilibrated with 20 mM sodium acetate (pH 5.0). Endo-glucanase was not retained by the column and did not obviously damage the chromatographic material. The column was eluted at 4°C with 50 ml of 20 mM acetate (pH 5.0), followed by 400 ml of a linear 0 to 0.4 M NaCl gradient in the same buffer. A single peak of PG activity was eluted at about 0.3 M NaCl (Fig. 1A). The fractions with the highest specific PG activity were pooled, dialyzed at 4°C against 0.5% glycine and subjected to isoelectric focusing. A single peak of PG activity focused at pH 5.15  $\pm$  0.1. The accuracy of the pI value is given as the difference between the highest and lowest figure of five experiments (Fig. 1B). The fractions containing PG with the highest specific activity were pooled, dialyzed against distilled water, and concentrated to 5 ml by immersing the dialysis bag containing the enzyme in polyethylene glycol 4000. The concentrated enzyme was subjected to gel permeation chromatography through a 1.5  $\times$  60 cm Sephadex G-50 column equilibrated with distilled water. The PG activity eluted as a single peak at a relative elution volume of 2.0. The enzyme peak coincided with the absorbance at 280 nm (Fig. 1C). A summary of the PG purification from 100 ml of commercial pectinase is presented in Table I. PG was purified 99-fold with a recovery of 46%. Specific activity of the purified enzyme was 4,370 RVU/mg and 2,300 RGU/mg.

SDS-PAGE revealed that the purified PG was apparently

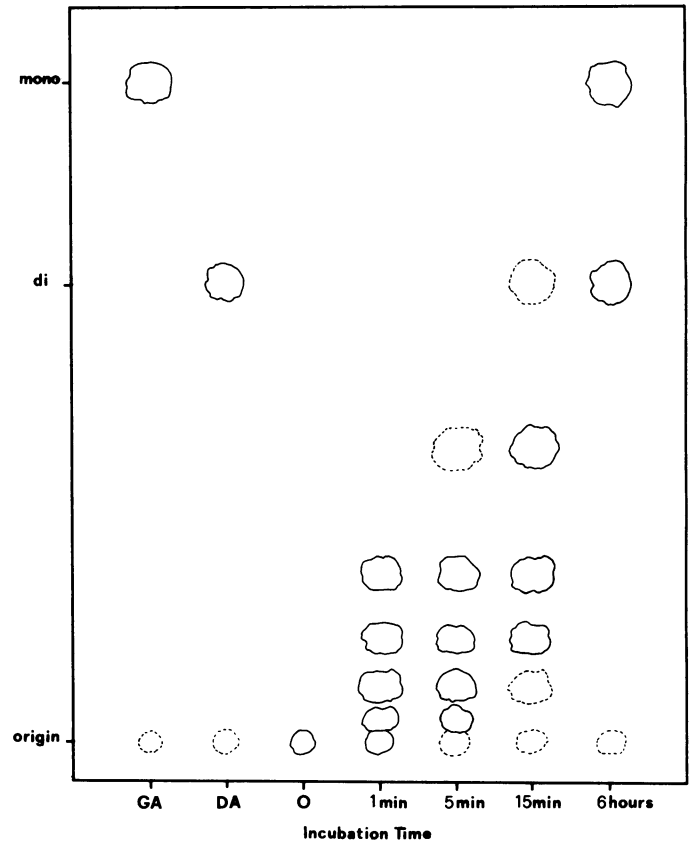


FIG. 3. Paper chromatogram of the reaction products of homogeneous PG with sodium polypectate. Incubation mixture contained 0.5% Na-polypectate, 50 mM Na-acetate (pH 5.0), and 5 RVU of PG. Aliquots (25  $\mu$ l) from the reaction mixture were boiled for 5 min and spotted on the chromatogram at the indicated times. GA and DA spots contained 25  $\mu$ l of 75 mM galacturonic acid and 75 mM digalacturonic acid, respectively.

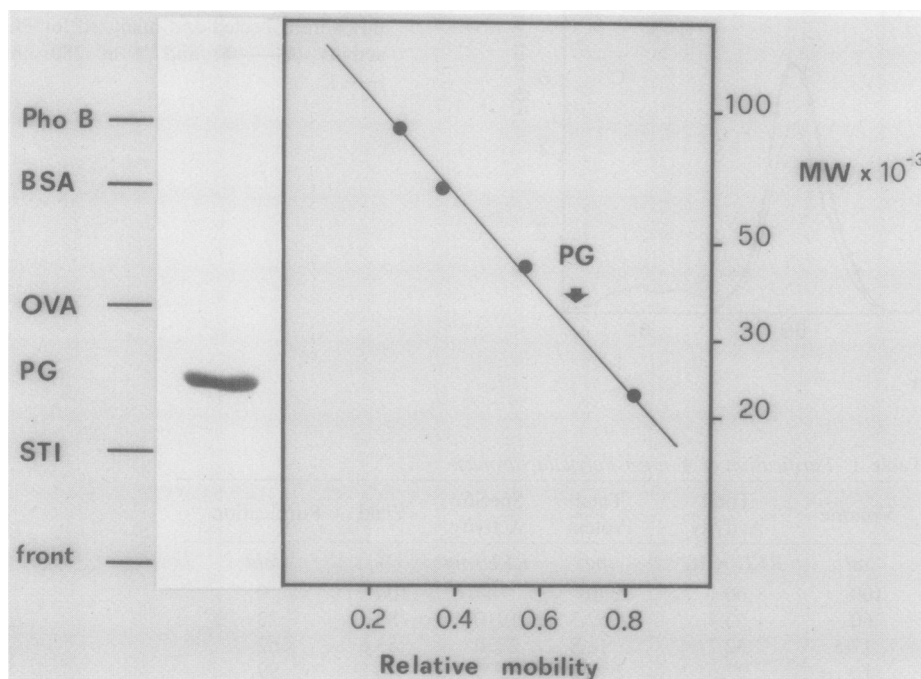


FIG. 2. SDS-PAGE and mol wt determination of purified *A. niger* polygalacturonase. Proteins were mixed with SDS-sample buffer, boiled for 3 min and loaded on a stacking gel containing 3.1% acrylamide. The electrophoresis buffer, formed by 6.0 g of Tris base plus 28.8 g of glycine dissolved in 1 L of distilled water, contained 0.1% SDS and had a final pH of 8.3. Gels were stained overnight with 0.5% Coomassie brilliant blue R-250, destained and photographed with a red filter. Mol wt standards were phosphorylase *b* (92,500), BSA (66,200), ovalbumin (45,000) and soybean trypsin inhibitor (21,500).

homogeneous and consisted of a single polypeptide chain with an apparent mol wt of 33,500 (Fig. 2). The extinction coefficient at 280 nm of the homogeneous PG ( $E_{280}^{1\%}$ ) was 11.9.

The homogeneous enzyme hydrolyzed the glycosidic bonds of the substrate in a typical 'endo' fashion. The galacturonate oligomers produced by the action of homogeneous PG on Na-polypectate were analyzed by descending paper chromatography. Oligomers with a degree of polymerization higher than four were released rapidly in the incubation mixture and were later fragmented to a shorter length. The final products of the enzyme hydrolysis were mono- and digalacturonate (Fig. 3). The action

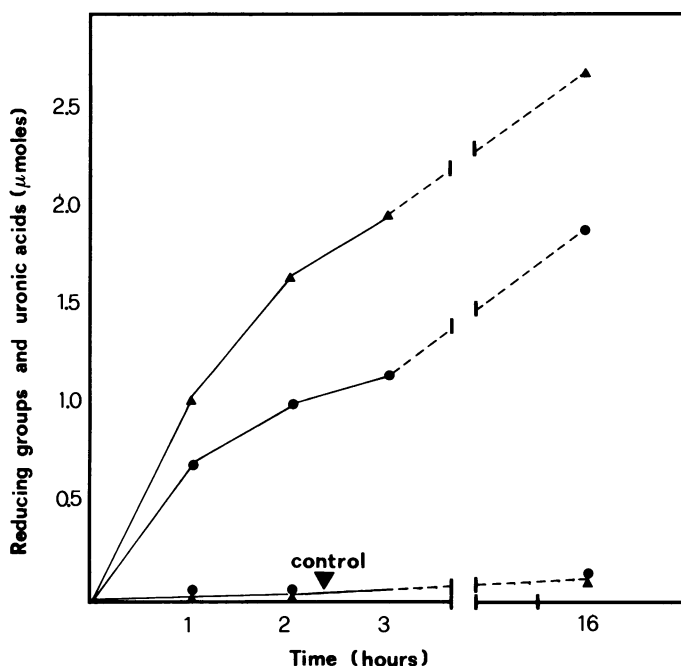


FIG. 4. Oligosaccharides released from isolated *Vigna* cell walls by *A. niger* PG. Cell wall material (15 mg) was suspended in 2 ml of 0.1 M Na-acetate (pH 5.0), containing 1 RVU of homogeneous PG and incubated at 30° C. After centrifugation at 12,000g for 30 min, the supernatant was collected and boiled for 5 min to inactivate PG. Oligosaccharides enzymatically solubilized from cell walls were determined in the supernatant by measuring reducing sugars (●—●) and uronic acids (▲—▲). Boiled PG was used as a control.

of PG was also tested on cell walls isolated from hypocotyls of *Vigna unguiculata*. The release from the wall of soluble reducing sugars and uronic acids was detected rapidly upon incubation, showing that purified PG was able to attack glycosidic bonds of isolated cell walls. After 16 h of enzymatic hydrolysis, approximately 7% of reducing sugars of the wall and 37% of uronic acids of the wall had been released (Fig. 4).

*Vigna* pods were treated with homogeneous PG and incubated at 25° C as described in "Materials and Methods." The treatment produced a necrotic response which was easily detected after 24 h of incubation. Enzymatic activity of PG was necessary for the eliciting activity. Enzyme inactivated at 100° C for 5 min or PG that was inactivated with rabbit anti-PG IgG did not provoke the necrotic response. A mixture of  $\alpha$ -D-galacturonate oligomers with a degree of polymerization higher than four, produced by a partial PG digestion of Na-polypectate, elicited a necrotic response in *Vigna* pods that was indistinguishable from that elicited by native PG. D-Galacturonic acid and the final PG degradation products of Na-polypectate (di- and mono-galacturonic acid) did

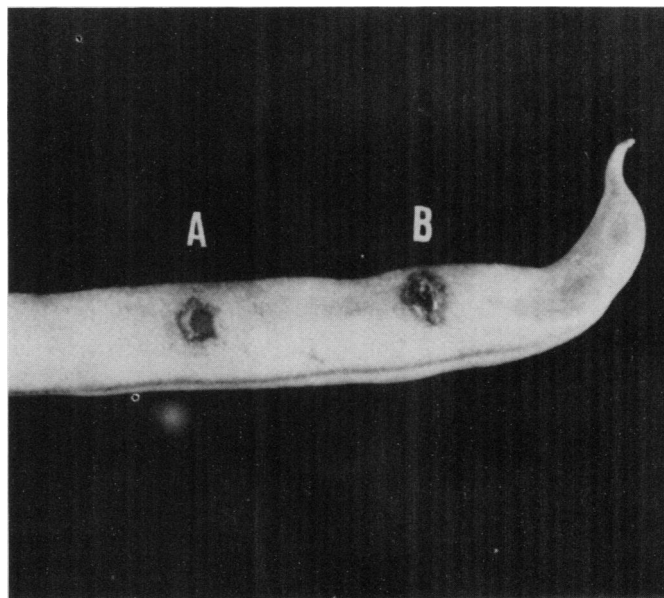


FIG. 6. Necrotic response induced in *V. unguiculata* pods by: (A) 3  $\mu$ g of homogeneous PG and (B) *A. niger* mycelium.

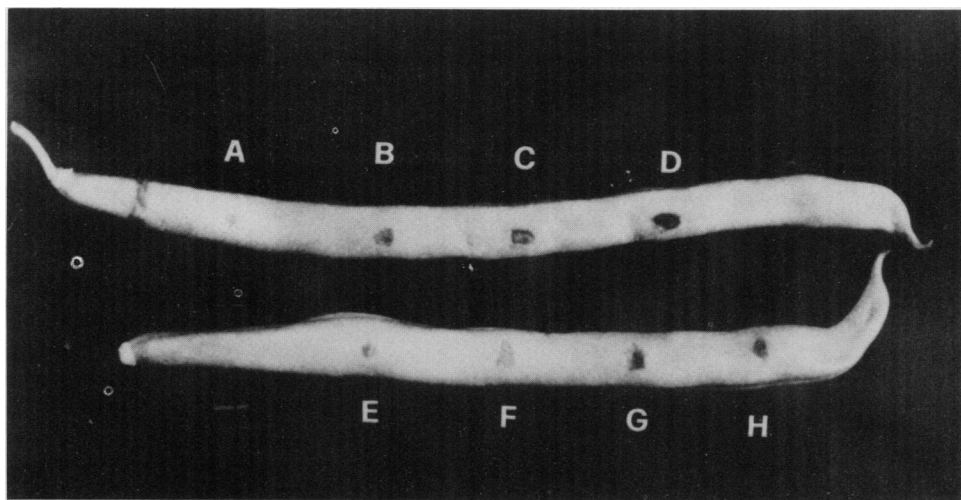


FIG. 5. Necrotic response induced in *V. unguiculata* pods by: (A) 50 mM Na-acetate, pH 5.0 (control); (B) 0.6  $\mu$ g of homogeneous PG; (C) 1.8  $\mu$ g of homogeneous PG; (D) 3  $\mu$ g of homogeneous PG; (E) galacturonic acid 0.5%; (F) Na-polypectate 0.5% incubated 6 h with 5 RVU of homogeneous PG; (G) Na-polypectate 0.5% incubated for 5 min with 5 RVU of homogeneous PG; (H) supernatant of *Vigna* cell wall material incubated for 1 h with homogeneous PG.

not elicit a necrotic response. Oligosaccharides released from *Vigna* cell walls by partial digestion (1 h) with PG were also active elicitors of the necrotic response (Fig. 5). Responses elicited by homogeneous PG, by the degradation products of Na-polypectate, and by isolated cell wall fragments were similar in appearance to the non-watersoaked necrosis induced in *Vigna* pods by inoculation with *A. niger* mycelium (Fig. 6).

In conclusion, *A. niger* PG was shown to act as an endoglycanase and to efficiently hydrolyze polygalacturonate. The characteristics of *A. niger* PG are similar to those of other fungal PGs (8). Nevertheless, the enzyme exhibited a specific activity considerably higher than those of PGs purified to homogeneity from phytopathogenic fungi that successfully attack and colonize living plants (7, 10, 29). It seems, therefore, that an apparent paradox exists: a fungus (*A. niger*) with a highly efficient PG acts only in post-harvest decays and is not able to attack field plants (1), while fungi with less efficient PGs are capable of attacking field plants. The ability of *A. niger* PG to release cell wall pectic fragments and to elicit a necrotic response in *Vigna* provides a possible explanation to this paradox. A very efficient enzyme rapidly forms fragments which, as demonstrated by several authors (6, 13, 17, 24), are capable of eliciting a resistance response. A less efficient enzyme on the contrary may delay elicitation of a resistance response allowing colonization of the tissue. If this is true, an evolution of PG toward less efficient enzymatic forms may have occurred from saprophytes to facultative parasites to obligate parasites.

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