

# Purification and Characterization of a Polygalacturonase-Inhibiting Protein from *Phaseolus vulgaris* L.<sup>1</sup>

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## ABSTRACT

Homogeneous endo-polygalacturonase (PG) was covalently bound to cyanogen-bromide-activated Sepharose, and the resulting PG-Sepharose conjugate was utilized to purify, by affinity chromatography, a protein from *Phaseolus vulgaris* hypocotyls that binds to and inhibits PG. Isoelectric focusing of the purified PG-inhibiting protein (PGIP) showed a major protein band that coincided with PG-inhibiting activity. PGIP formed a complex with PG at pH 5.0 and at low salt concentrations. The complex dissociated in 0.5 M Na-acetate and pH values lower than 4.5 or higher than 6.0. Formation of the PG-PGIP complex resulted in complete inhibition of PG activity. PG activity was restored upon dissociation of the complex. The protein exhibited inhibitory activity toward PGs from *Colletotrichum lindemuthianum*, *Fusarium moniliforme* and *Aspergillus niger*. The possible role of PGIP in regulating the activity of fungal PG's and their ability to elicit plant defense reactions are discussed.

The importance of microbial PG<sup>2</sup> in pathogenesis has been established not only in plant diseases characterized by rapid and extensive degradation of host cell walls, but also in some diseases where only a minimal breakdown of cell wall polysaccharides occurs during penetration and colonization of host tissue (8, 13–17). Evidence has been obtained that, in some cases, PG acts before other cell wall-degrading enzymes can attack their substrates (27), and it is the first polysaccharide-degrading enzyme secreted by pathogens cultured on isolated cell walls (26).

Cell walls of some plant tissues contain proteins (PGIP) that counteract cell wall-degrading microorganisms by inhibiting their PG activity (1–4, 6, 9–11, 21, 22, 24, 25). Molecular genetic techniques may lead to greater understanding of the function of PGIP in pathogenesis if sufficient quantities of PGIP could be purified. Toward that goal, we decided to attempt to develop a method for the purification of PGIP based on the affinity that PGIP exhibits for PG. This paper describes the affinity purification and the partial characterization of PGIP from hypocotyls of *Phaseolus vulgaris* L.

## MATERIALS AND METHODS

**Chemicals.** Sodium polypectate and D-galacturonic acid were obtained from NBC corporation. The 3MM chromatography

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<sup>2</sup> Abbreviations used: PG, endo-polygalacturonase (poly(1-4- $\alpha$ -D-galacturonide)-glycanohydrolase, EC 3.2.1.15); PGIP, polygalacturonase-inhibiting protein; RVU, relative viscosimetric units; RGU, reducing group activity unit

paper was from Whatman and ampholine carriers were from LKB. CNBr-activated Sepharose 4B was obtained from Pharmacia. Leupeptin sulfate, phenylmethylsulfonyl fluoride (PMSF), and pepstatin were obtained from Boehringer. Other chemicals used were reagent grade.

**Enzymes.** Homogenous PG was obtained from commercial preparations of *Aspergillus niger* pectinase (Sigma) by a method reported previously (14).

Homogenous PGs from *Fusarium moniliforme* and *Colletotrichum lindemuthianum* were prepared as reported by Salvi *et al.* (32) and Barthé *et al.* (7).

**Plant Culture.** French bean (*Phaseolus vulgaris* L., cv Cannelino) seeds were obtained from a local merchant and grown in vermiculite for 10 d as described previously (13).

**Enzyme assay.** PG activity was determined by measuring the decrease in relative viscosity at 30° C of a 0.6% (w/v) solution of sodium polypectate in 50 mM sodium acetate (pH 5.0), in a Cannon-Fenske No. 300 viscometer. One viscosimetric unit (RVU) was defined as the amount of PG enzyme causing in 1 min a 50% reduction in the relative viscosity of 6 ml of the reaction mixture under the standard assay conditions. Hydrolysis of glycosidic bonds was followed by the reducing end-group analysis as reported previously (14). One reducing group activity unit (RGU) was defined as the amount of enzyme producing 1  $\mu$ eq of reducing groups per min. The enzyme products were examined by descending paper chromatography, using the method of Nasuno and Starr (30), with 1-butanol/acetic acid/water (4:2:3, v:v:v) as eluent.

**PGIP Assay.** The inhibiting activity of PGIP was assayed with the standard PG assay (see above). One unit of inhibitor was defined as the amount of protein required to reduce the activity of 0.1 RVU of homogenous *A. niger* PG by 50%. PGIP and PG were usually preincubated at room temperature for 5 min before assay. Longer preincubation before adding substrate did not affect PGIP activity.

**Isoelectric Focusing and Gel Electrophoresis.** Isoelectric focusing experiments were done at 4° C in a LKB 8100 column containing 3 ml of 40% ampholine in a linear sucrose gradient (0–40%, w/v). Focusing was complete in 36 h when the initial voltage was 0.2 kV, and 1 kV was applied for the last 24 h. SDS-PAGE was performed in 9.25% acrylamide slab gels prepared according to Studier (33). Gels were stained by the silver method (5).

**Covalent Attachment of PG to Sepharose.** Homogenous *A. niger* PG (6 mg) was covalently bound to CNBr-activated Sepharose 4B (1 g) according to Eveleigh and Levy (20). The PG-Sepharose conjugate was suspended in 20 mM Na-acetate, pH 5.0, and packed in a column (0.7  $\times$  3.5 cm.) that was used for PGIP purification.

**Protein Determination.** Protein was measured according to Lowry *et al.* (29) using BSA as standard. Protein content of a purified preparation of PGIP was determined with the biuret reagent (23).

## RESULTS

Hypocotyls (100 g) from beans grown for 10 d were suspended in 200 ml of cold 20 mM Na-acetate (pH 5.0), containing 0.5 M NaCl and 4 mg of leupeptin sulfate. The hypocotyls were homogenized for 1 min in a Waring Blender at 4° C. The resulting suspension was filtered through a coarse sintered-glass funnel and the residue was homogenized once more with 100 ml of the same buffer. The extracts were combined and centrifuged at 13,000g for 30 min. The supernatant was dialyzed three times against 2 L of 20 mM Na-acetate (pH 5.0), centrifuged at 13,000g for 30 min, and assayed for PGIP activity. The supernatant (100 ml) was passed at a rate of 1 ml/min through a column (1.5 × 3.5 cm) containing 1 g of Sepharose 4B in order to remove proteins that bind to Sepharose 4B. PGIP was not adsorbed; all of the activity applied was recovered in the eluate (Fig. 1A). The eluate was passed through a column containing 1 g of PG-

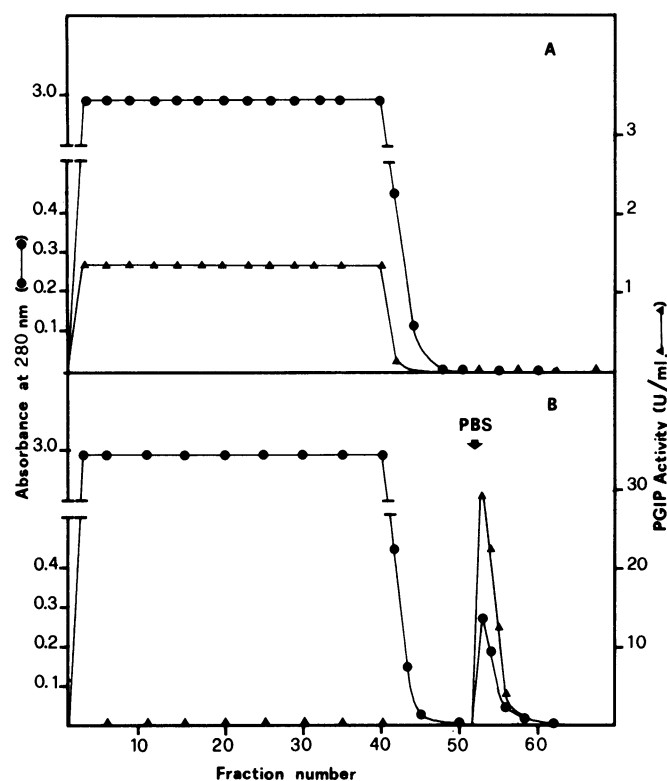


FIG. 1. (A) Elution profile of 100 ml of *P. vulgaris* extract passed through a column containing 1 g of Sepharose 4B. The elution buffer was 20 mM Na-acetate (pH 5.0). (B) Fractions 1 to 40 were pooled and passed through a column containing 1 g of PG-Sepharose conjugate. The elution buffer was 20 mM Na-acetate (pH 5.0) and PBS was added at Fraction 52 as indicated by the arrow. Fractions (2.5 ml) were collected and assayed for PGIP activity with the viscometric method (▲—▲) and *A* at 280 nm (●—●).

sepharose conjugate. PGIP adsorbed to the column. The activity was fully recovered by eluting with PBS (0.13 M NaCl, 7 mM Na<sub>2</sub>HPO<sub>4</sub>, 3 mM NaH<sub>2</sub>PO<sub>4</sub> [pH 7.3]) (Fig. 1B). The specific activities of PGIP before and after affinity chromatography are reported in Table I. PGIP was purified 2850-fold and concentrated to a volume of 10 ml.

PGIP purified by affinity chromatography was subjected to isoelectric focusing in the pH range 9 to 11. One major protein peak, corresponding to PGIP activity, focused at pH 9.5 (Fig. 2). The same isoelectric point for the *P. vulgaris* PGIP has been reported by Lafitte *et al.* (28). The mol wt of PGIP, determined by Sephadex G-100 permeation chromatography (data not shown), was 45,000, very close to that reported by Lafitte *et al.* (28) and by Albersheim and Anderson (3). A slightly lower mol wt (41,000) was determined by SDS-PAGE (Fig. 3). Heat-stability curves showed that PGIP activity was lost at temperatures above 55° C (Fig. 4). A very similar heat-stability has been reported for PGIP characterized by Albersheim and Anderson (3). Selected protease inhibitors were ineffective in inhibiting PGIP activity (Table II). All these results strongly suggested that the PGIP we have purified is likely to be the same protein characterized by other authors (3, 28).

Purified PGIP was tested against PGs purified to homogeneity from three fungi: *A. niger* (Fig. 5A), *F. moniliforme* (Fig. 5B), and *C. lindemuthianum* (Fig. 5C). The relative specific activity of PGIP toward PG from each fungus was calculated from Figure 5. The calculated values are reported in Table III.

The effect of salt concentration and pH on the interaction between PG and PGIP was investigated by diluting samples (300 μl) of purified PGIP to 3 ml with 20 mM Na-acetate (pH 5.0), and reabsorbing the PGIP on the PG-Sepharose column. The column was then irrigated with 5 ml of buffers at various pH and ionic strength. The PG-PGIP complex was almost completely dissociated by 500 mM Na-acetate, pH 5.0. PGIP was also dissociated at pH values lower than 4.5 and higher than 6.0 (Fig. 6).

The capacity of PGIP to inhibit PG activity was measured at various pH and ionic strength (Fig. 7). The results clearly indicated that maximum inhibition of PG activity occurred at pH values and salt concentrations that favor the formation of the PG-PGIP complex.

Apparently neither PG nor PGIP was irreversibly modified by formation of the complex. This was demonstrated by allowing the two proteins to interact for 30 min and then subjecting them to isoelectric focusing in the range of pH 4 to 11. This treatment dissociated the complex and allowed the two separated proteins to focus at the same pH values they focused when subjected to isoelectric focusing separately. The dissociated proteins also maintained their catalytic and inhibitory properties (Fig. 8).

To determine whether PGIP can interact with PG in the presence of substrate and whether substrate can dissociate the enzyme-inhibitor complex once it had formed, incubation mixtures were prepared in which (a) substrate was added to a mixture of PG and PGIP, (b) PG was allowed to degrade polypectate in the absence of inhibitor, (c) PGIP was added to a mixture of PG and substrate, and (d) PG was added to a mixture of PGIP and

Table I. Purification of *P. vulgaris* PGIP

Fraction	Volume ml	Total activity	Total protein	Specific activity	Yield %	Relative Purification -fold
		inhibitor units	mg	inhibitor units/ mg		
<i>P. vulgaris</i> extract	100	133	4250	0.031	100	1
Sepharose column	100	128	4160	0.031	96	1
Sepharose-PG column	10	115	1.3	88.5	86	2850

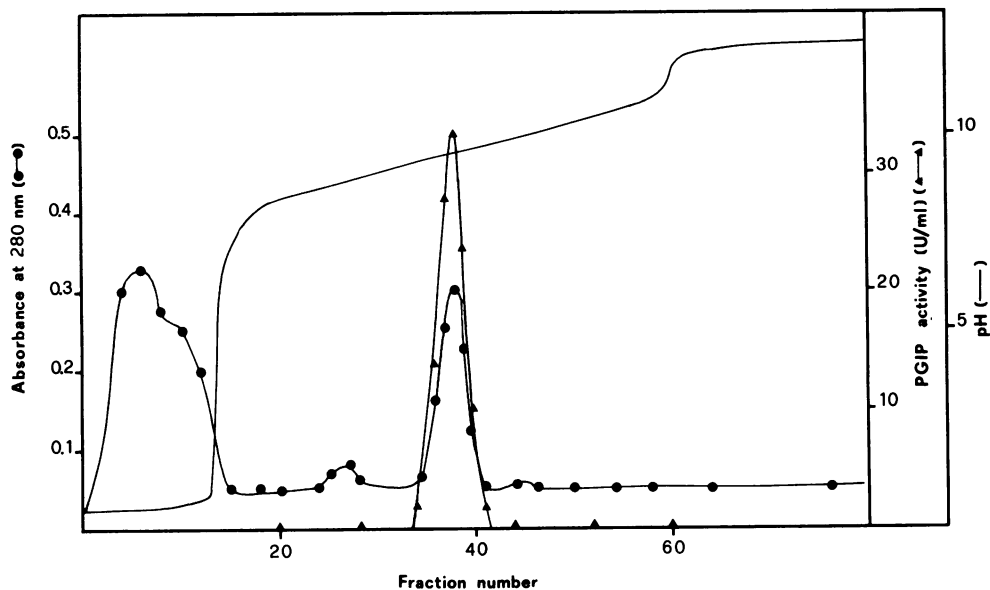


FIG. 2. Isoelectric focusing (pH range 9–11) of affinity purified PGIP. Fractions (1.4 ml) were assayed for PGIP activity with the viscosimetric method. (▲—▲). *A* at 280 nm (●—●) and pH (—).

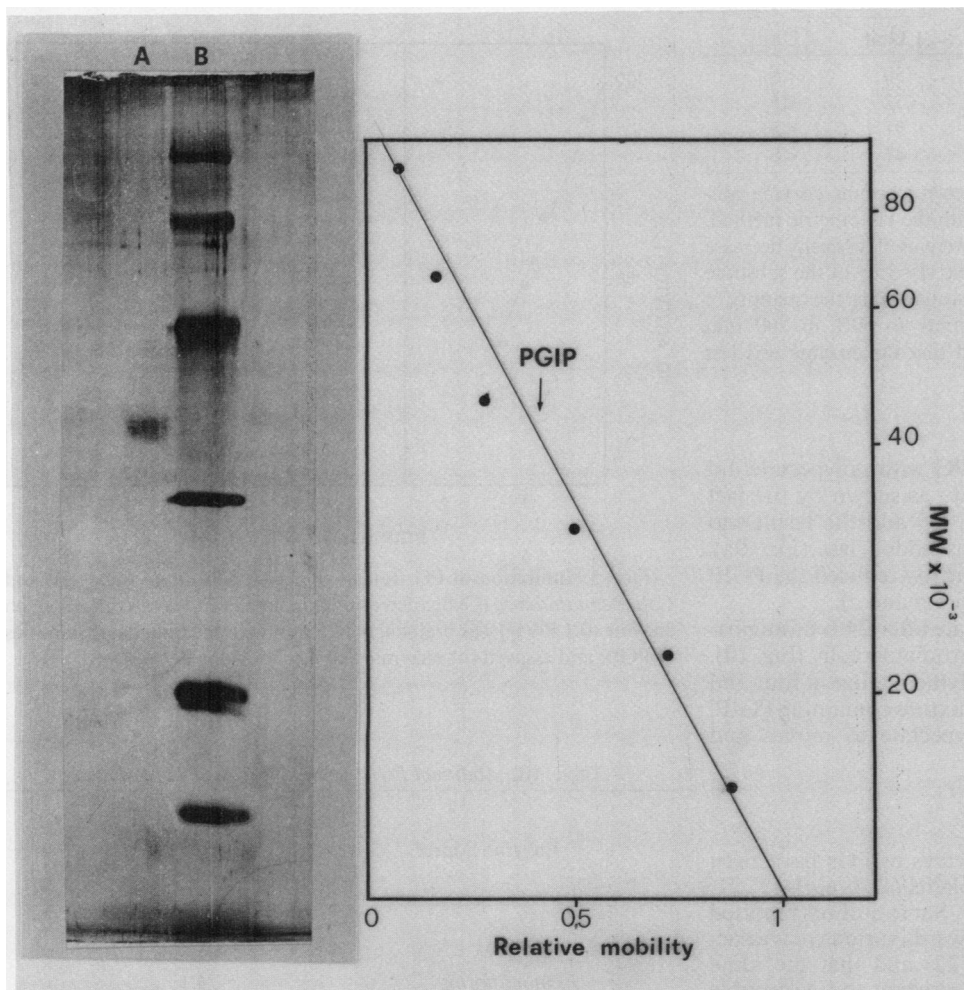


FIG. 3. SDS-PAGE and mol wt determination of purified PGIP. PGIP (lane A) from isoelectric focusing reported in Figure 2 and standard proteins (lane B) were mixed with SDS-sample buffer, boiled for 3 min and loaded on a stacking gel containing 3.1% acrylamide. The electrophoresis buffer, formed by 6.0 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 with silver nitrate. Molecular weight standards were phosphorylase *b* (92,500), BSA (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme 14,000).

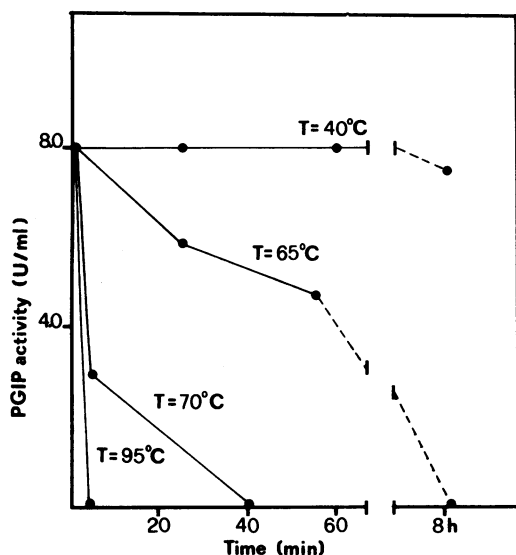


FIG. 4. Heat-stability of PGIP. PGIP was incubated in PBS at various temperatures and assayed at the indicated times.

Table II. Inhibition of *A. niger* PG by PGIP

Enzyme	Percent of Enzyme Activity after Incubation with PGIP <sup>a</sup>	
	1 Unit	4 Units
PG (0.1 RVU)	50	<5
PG (0.1 RVU) + leupeptin (20 $\mu$ g/ml)	52	<5
PG (0.1 RVU) + PMSF (2 mM)	51	<5
PG (0.1 RVU) + Pepstatin (50 $\mu$ M)	47	<5

<sup>a</sup> Percent of enzyme activity refers to the corresponding enzyme control without added PGIP. PG was assayed with the viscometric method. When saturating amounts of PGIP (4 units) were used, viscosity decrease was followed for 200 min. After this time, the viscosity of the substrate was still much higher than 50% of the initial value. Since the quantity of PG that reduces the viscosity of the substrate to 50% in 200 min corresponds to 0.005 RVU, it was assumed that the enzyme was less than 5% of the control.

substrate (Fig. 9). Previous contact of PG with polypectate did not prevent the inhibition of PG activity as shown by the fact that inhibitor could be added last (Fig. 9c) and the result was almost the same as when enzyme was added last (Fig. 9a). Preincubation of PGIP with substrate slightly reduced the PGIP inhibitory activity (compare Fig. 9, d, with a and c).

Degradation products of Na-polypectate after 24 h of incubation with PG were analyzed by paper chromatography (Fig. 10). Oligogalacturonides with a degree of polymerization of four and higher were detected in the incubation mixture containing PGIP, while PG alone degraded the Na-polypectate to mono- and digalacturonate.

## DISCUSSION

Proteins (PGIP) that inhibit the activities of PGs have been found in the cell walls of a variety of plants (1-4, 6, 9-11, 21, 22, 24, 25). Their role *in vivo* is not clear. Some authors reported that PGIP does not exhibit specificity towards various polygalacturonase from different pathogens (4, 22) and that the same concentration of PGIP is present in both resistant and susceptible cultivars of *P. vulgaris* (28). Our data show that PGIP retards

the PG-catalyzed hydrolysis of polypectate and leads to the formation of oligomers with a degree of polymerization greater than four. Such oligomers elicit active defense mechanisms in plants (12, 14, 18, 19, 31, 34), whereas mono- and digalacturonate, the products of PG action on polypectate when PGIP is not present, are not elicitors. Therefore, we suggest that the action of PGIP *in vivo* may be to counteract fungal invasion by causing fungal polygalacturonases to increase their elicitation of plant defense responses.

We have developed a method for obtaining mg quantities of nearly pure PGIP by exploiting the affinity of the protein for PG. PG, purified to homogeneity from a commercial preparation of pectinase, was immobilized by covalently linking some of its amino groups to CNBr-activated Sepharose. Since the amino groups of PG can be modified without reducing its capacity for forming complexes with PGIP, the PG-Sepharose conjugate was successfully used to purify PGIP. PGIP was specifically adsorbed to PG-Sepharose and desorbed by increasing pH or ionic strength. The dissociation of PG-PGIP complex by pH and salt reversed the inhibition of PG, establishing that the inhibition results from formation of the complex. Physiological changes in the cell wall pH during infection would affect PG activity by regulating the interaction between PG and PGIP.

The possible regulatory effect of pH, as well as of ions or other cell wall molecules, on the formation of the PG-PGIP complex can now be investigated in further detail. The PG-Sepharose conjugate can also be used for investigating the distribution and the concentration of PGIP in different plants and tissues. It may also be possible to immobilize PGIP and use it for rapid purifi-

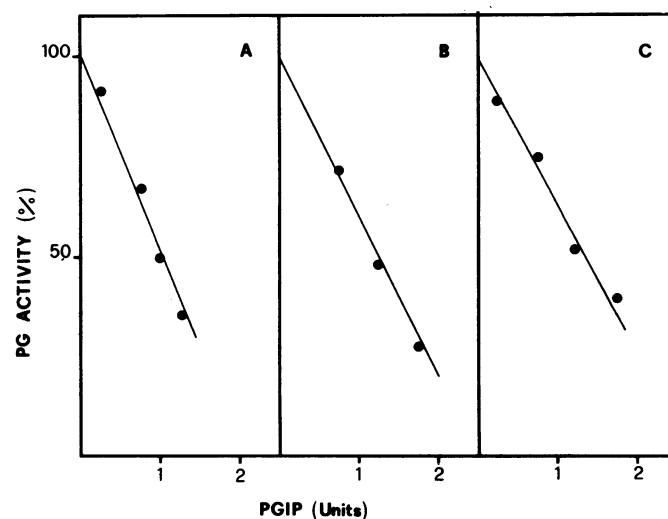


FIG. 5. Inhibition of PG from *A. niger* (A), *F. moniliforme* (B) and *C. lindemuthianum* (C) by increasing amounts of *P. vulgaris* PGIP. Each enzyme (0.1 RVU) was preincubated 5 min with the indicated quantities of PGIP and assayed for enzymic activity.

Table III. Ability of PGIP to Inhibit PGs of Three Fungi

Enzyme Source	Comparative Specific Activity of PGIP Toward PG of Three Fungi
	units/mg
<i>A. niger</i>	92.6
<i>F. moniliforme</i>	74.1
<i>C. lindemuthianum</i>	67.7

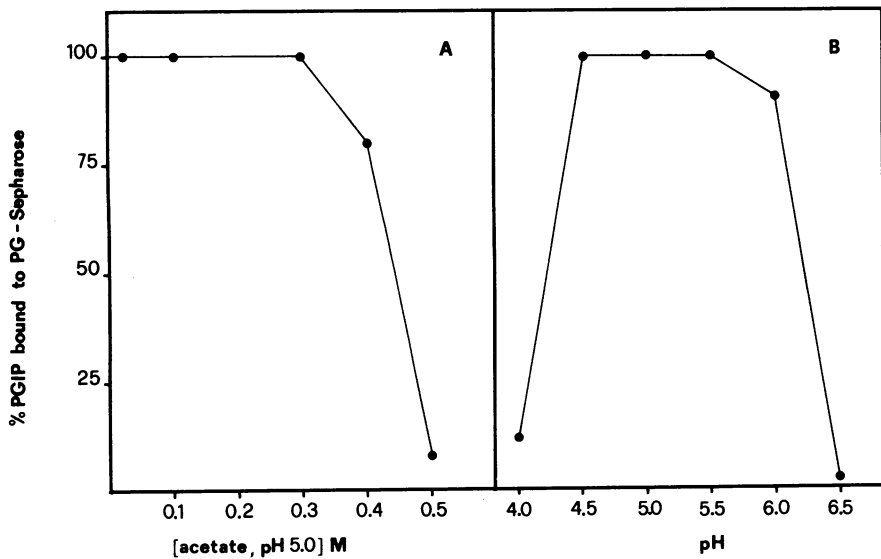


FIG. 6. Effect of ionic strength (A) and pH (B) on the relative quantity of PGIP bound to PG-Sepharose. Each experiment was performed with 300  $\mu$ l (3.5 units) of purified PGIP. PGIP was diluted to 3 ml with 20 mM acetate, pH 5.0, absorbed on the PG-Sepharose column and eluted with 5 ml of acetate (pH 5.0), at various concentrations (A) or with 100 mM acetate at different pH (B). The eluted PGIP was then dialyzed against 20 mM acetate, pH 5.0, and assayed for PG inhibitory-activity with the viscometric method. The column was washed extensively with PBS after each experiment.

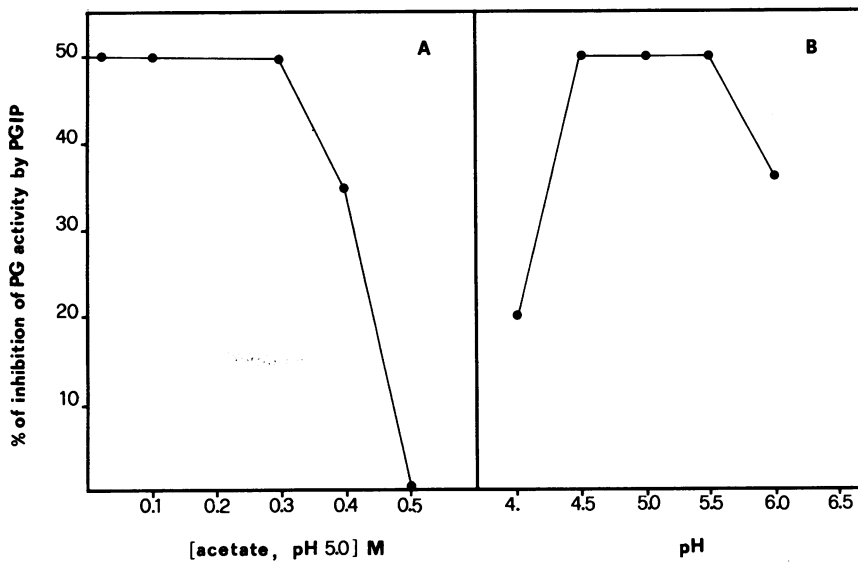


FIG. 7. Effect of ionic strength (A) and pH (B) on PGIP activity. *A. niger* PG (0.1 RVU) was assayed with the reducing end-group assay after incubation with 1 unit of PGIP. A blank contained PG without PGIP. PGIP activity at pH 6.5 could not be determined because *A. niger* PG is not measurably active at that pH.

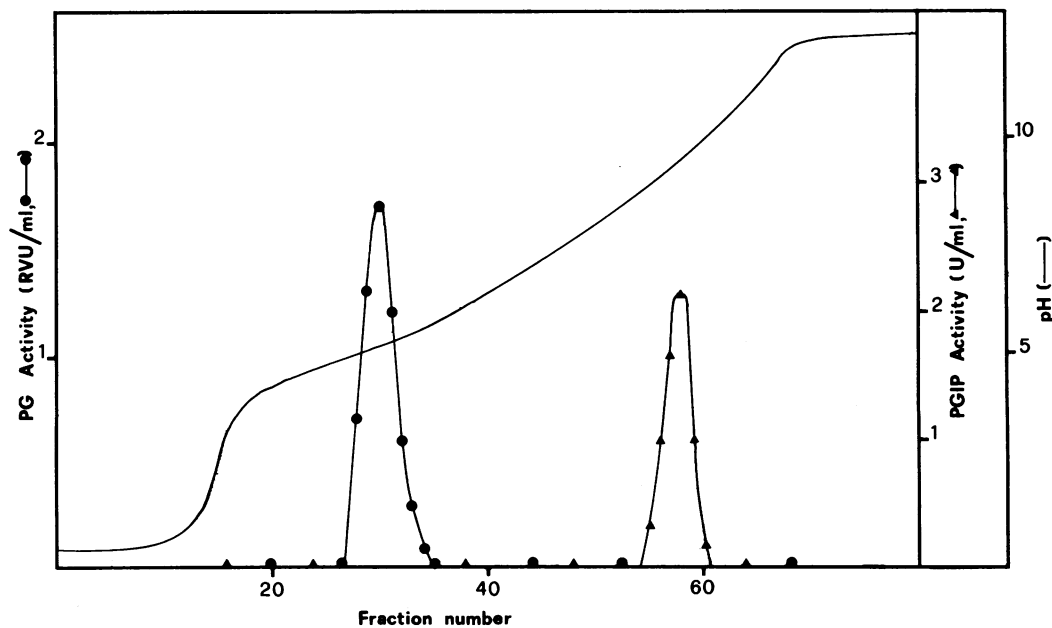


FIG. 8. Isoelectric focusing (pH range 4-11) of the PG-PGIP complex. Homogeneous PG (8 RVU) was incubated with 10 units of purified PGIP for 30 min at 30° C in 50 mM acetate (pH 5.0), and the resulting complex was subjected to isoelectric focusing. Fractions (1.4 ml) were collected and assayed for PG (●—●), PGIP (▲—▲), and pH (—).

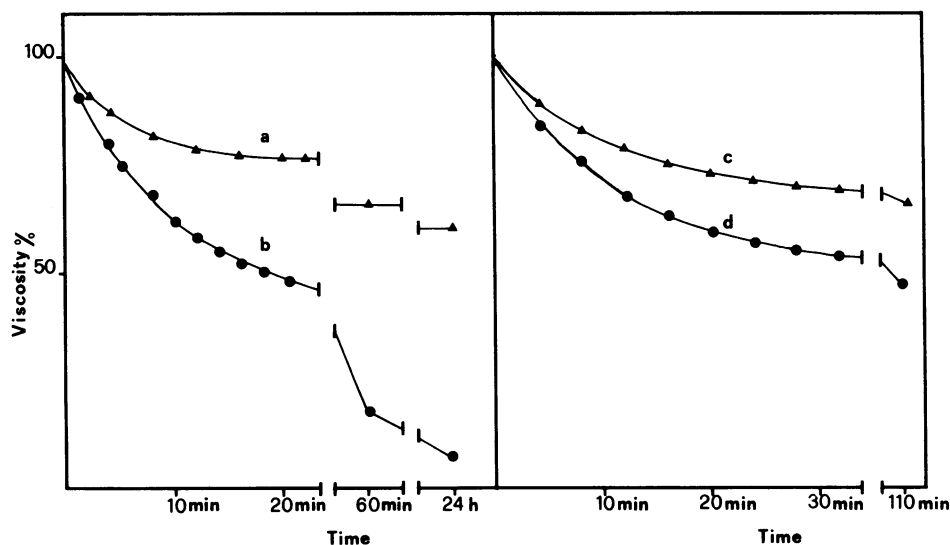


FIG. 9. Viscosity changes of sodium polypectate incubated with: (a) homogeneous PG (0.05 RVU) preincubated with 1 unit of purified PGIP; (b) homogeneous PG (0.05 RVU); (c) homogeneous PG (0.05 RVU) added to the viscometer 10 s before adding 1 unit of purified PGIP; and (d) purified PGIP (1 unit) added to the viscometer 2 min before adding PG (0.05 RVU).

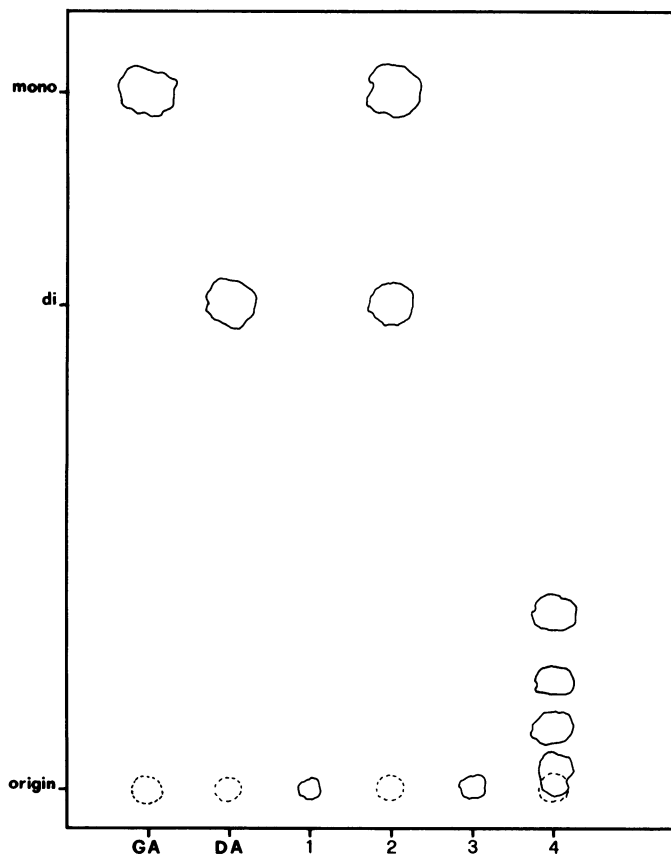


FIG. 10. Paper chromatogram of the products of reaction of homogeneous PG (columns 1 and 2) or PG plus purified PGIP (columns 3 and 4) with sodium polypectate. Mixtures were incubated at 30° C and contained, in 1 ml, 0.5% Na-polypectate, 50 mM acetate buffer, pH 5.0, and 0.5 RVU of PG (1 and 2) or 0.5 RVU of PG plus 10 units of purified PGIP (3 and 4). Aliquots (25  $\mu$ l) from the reaction mixture were boiled for 5 min and spotted on the chromatogram at 0 time (1 and 3) and after 24 h of incubation (2 and 4). The columns marked GA and DA contained 25  $\mu$ l of 75 mM galacturonic acid and 75 mM digalacturonic acid, respectively.

cation of PG from culture filtrates of phytopathogenic fungi. Finally, the purified PGIP can be used for preparing specific antisera that can be used to select cDNA clones containing nucleotide sequences coding for PGIP in a *P. vulgaris* expression library.

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