

Endopolygalacturonase from *Fusarium oxysporum* f. sp. *lycopersici*: Purification, Characterization, and Production During Infection of Tomato Plants

Antonio Di Pietro and M. Isabel G. Roncero

Departamento de Genética, Facultad de Ciencias, Universidad de Córdoba, 14071 Córdoba, Spain.

This research was supported by the Comisión Interministerial de Ciencia y Tecnología (CICYT; grant BIO93-0923-CO2-01) and the D. G. XII of the European Commission (HCM CT93-0244). A. Di Pietro was supported by a postdoctoral fellowship from the European Commission (contract AIR1-CT94-8686).

We thank F. Cervone, G. De Lorenzo, and G. Salvi, Università "La Sapienza," Rome, for critical revision of the manuscript and providing purified *Fusarium moniliforme* and *Aspergillus niger* endoPG. We also thank M. Ruiz-Rubio and K. Lairini, Universidad de Córdoba, for helpful suggestions.

Accepted for publication 3 September 1996.

ABSTRACT

Di Pietro, A., and Roncero, M. I. G. 1996. Endopolygalacturonase from *Fusarium oxysporum* f. sp. *lycopersici*: Purification, characterization, and production during infection of tomato plants. *Phytopathology* 86:1324-1330.

Production of extracellular pectinolytic enzymes by *Fusarium oxysporum* f. sp. *lycopersici* on different carbon sources was analyzed by zymograms of isoelectric focusing (IEF) gels. Pectinolytic isoforms with pIs ranging from 4.0 to 9.0 were detected. A polygalacturonase (PG; EC 3.2.1.15) was purified by preparative IEF and denominated PG1. The enzyme consisted of one major unglycosylated isoform with pI 7.0 and a molecular weight (MW) of 35,000 and three major *N*-glycosylated isoforms with pIs 6.0, 6.5, and 6.75 and a MW of 37,500. Absence of PG1 from the filtrate after tunicamycin treatment indicated that *N*-glycosyla-

tion was essential for its secretion. PG1 was active as a monomer and apparently hydrolyzed polygalacturonic acid simultaneously in an endo- and exomanner; hydrolysis of substrate bonds was 3% at 50% viscosity reduction indicating random cleavage, whereas analysis of reaction products showed early appearance of galacturonic acid. pH and temperature optima were 4.0 and 37°C, respectively. Sequencing of 11 N-terminal amino acids showed 91% identity to a *Fusarium moniliforme* endoPG. PG1 was approximately twice as resistant to inhibition by purified tomato PG-inhibitor protein as endoPGs from *F. moniliforme* and *Aspergillus niger*. Zymograms revealed the presence of the main PG1 isoform (pI 7.0) in stems of tomato plants infected with *F. oxysporum* f. sp. *lycopersici*, while a pectate lyase (pI 8.7) was detected in roots during early stages of infection.

Plant pathogenic fungi produce an array of extracellular degradative enzymes that may be important in pathogenicity (38). Most attention has focused on enzymes that depolymerize pectin, a major component of the plant cell wall and middle lamella. Polygalacturonases (PGs; poly- α -1,4-galacturonide glycanohydrolase, EC 3.2.1.15) and other pectinolytic enzymes have been isolated from a wide variety of bacterial and fungal plant pathogens (10,11). Besides plant cell wall degradation, implication of pectinolytic enzymes in pathogenesis also includes the release of oligogalacturonides that can act as elicitors or suppressors of the plant defense response (15,28) and an interaction with plant proteins able to modulate PG activity (PG-inhibitor proteins [PGIPs]) (9).

Fusarium oxysporum Schlechtend.:Fr. is an economically important soilborne plant pathogen with a worldwide distribution that causes vascular wilt diseases on a wide variety of crops. The mechanisms of pathogenicity and symptom induction by this fungus remain poorly understood despite many ultrastructural, biochemical, and genetic studies (1). Production of pectinolytic enzymes by the tomato pathogen *F. oxysporum* f. sp. *lycopersici* (Sacc.) W.C. Snyder & H.N. Hans. has been reported previously, and their role in pathogenicity has been investigated (12,17,22, 25,29,36). However, conclusive evidence on their role in pathogenicity is still lacking. Moreover, it has not been determined how

many pectinolytic isozymes are produced by this pathogen and which of them are secreted during infection of the plant. In the present study, the pattern of pectinolytic isozymes secreted by *F. oxysporum* f. sp. *lycopersici* grown on different carbon sources and during infection of tomato plants was analyzed using zymograms of isoelectric focusing (IEF) gels. An endoPG produced in planta was purified and characterized, and its inhibition by purified PGIP from tomato was determined.

MATERIALS AND METHODS

Fungal strain and culture conditions. *F. oxysporum* f. sp. *lycopersici* strain 42-87 (race 2) was obtained from J. Tello, INIA, Madrid, and stored as a microconidial suspension in 30% glycerol at -80°C. The pathotype of the isolate was periodically confirmed by plant assays in a growth chamber (20). The fungus was grown in a synthetic medium (SM) containing 0.2 g of MgSO₄ · 7 H₂O, 0.4 g of KH₂PO₄, 0.2 g of KCl, 1 g of NH₄NO₃, 0.01 g of FeSO₄, 0.01 g of ZnSO₄, and 0.01 g of MnSO₄ in 1 liter of distilled water. Cultures were supplemented with 1% (wt/vol) of the appropriate carbon source(s) and incubated in Erlenmeyer flasks on a rotary shaker at 150 rpm at 28°C. Polygalacturonic acid (PGA) and pectin from citrus fruits were from Sigma Chemical Co. (St. Louis). Oat-spelt xylan was from United States Biochemical Corp. (Cleveland, OH).

Enzyme assays and protein determination. PG activity was routinely determined by measuring the release of reducing groups from PGA. The standard reaction mixture (100 μ l total volume) con-

Corresponding author: A. Di Pietro; E-mail address: ge2dipia@uco.es

tained 1% PGA (wt/vol), 50 mM sodium acetate buffer (pH 4.75), and various amounts of the enzyme preparation. After incubation at 30°C for 15 min, the reducing sugar content was determined by the method of Nelson (30) and Somogyi (34). Appropriate controls without either enzyme or substrate were run simultaneously. The quantity of reducing sugar released was calculated from standards of D-galacturonic acid. Enzyme activity was expressed in nanokatal (nkat), defined as the amount of enzyme that releases 1 nmol of D-galacturonic acid equivalent per second under the above conditions.

The endo- or exomode of action of PG was determined by measuring the decrease in relative viscosity in a Cannon-Fenske viscosimeter (Atora, Spain) at 30°C. Reaction mixtures contained 13.5 nkat of PG enzyme in 6 ml of 1.5% (wt/vol) PGA solution in 50 mM sodium acetate buffer, pH 4.75. Simultaneously, the percentage of substrate hydrolysis was determined by the reducing group assay, assuming the substrate was 100% PGA with a molecular weight (MW) of 13,900.

Protein was determined according to the method of Bradford (3) using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis (PAGE) and IEF. PAGE was performed in a PhastSystem apparatus (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden) according to the manufacturer's instructions. Sodium dodecyl sulfate (SDS) (27) and native PAGE were conducted in 20% (wt/vol) discontinuous acrylamide gels (PhastGel, homogeneous 20; Pharmacia LKB Biotechnology Inc.). IEF gels had a pH range of 3 to 9. Gels were stained with AgNO₃ following the protocols supplied with the PhastSystem. MW and IEF markers were from Bio-Rad Laboratories and Pharmacia LKB Biotechnology Inc., respectively. MW and pI of purified proteins were estimated from a regression equation of the standard proteins versus distance migrated. The MW of the native enzyme was estimated by differences in relative mobility in different acrylamide concentrations following the method of Hedrick and Smith (24). Native MW standards were bovine serum albumin (66,000), egg albumin (45,000), and carbonic anhydrase (29,000) from Sigma Chemical Co.

Zymograms. Pectinolytic activity in IEF gels was detected using the substrate overlay technique described by Ried and Collmer (32). Concentrated culture filtrates and different enzyme fractions were applied to polyacrylamide IEF gels (PhastGel, IEF 3 to 9; Pharmacia LKB Biotechnology Inc.) and subjected to IEF under native conditions in a PhastSystem apparatus (Pharmacia LKB Biotechnology Inc.). Polyacrylamide gels were overlaid with ultrathin gels containing 1% (wt/vol) agarose and 0.1% (wt/vol) PGA in 50 mM sodium acetate, pH 4.2, and incubated at 100% humidity and 37°C for 15 to 30 min. Activity bands were visualized by staining the agarose overlay for 10 min in 0.05% (wt/vol) ruthenium red (Sigma Chemical Co.), followed by rinsing with distilled water.

Purification of PG1. All purification steps were carried out at 4°C unless otherwise stated. Cultures of *F. oxysporum* f. sp. *lycopersici* grown in SM supplemented with 1% citrus pectin were filtered through Whatman 3MM paper and Millipore membrane filters (pore size 0.22 µm), and the filtrate was transferred to dialysis tubing (10,000 MW cutoff) and concentrated 30-fold by placing the tubing in solid polyethylene glycol (MW of 35,000; Fluka Chemika-Biochemika, Buchs, Switzerland). The concentrated filtrate was dialyzed overnight against distilled water (10 liters per liter of culture filtrate) and subjected to preparative IEF on a Rotoform apparatus (Bio-Rad Laboratories). Preparative IEF was carried out in a total volume of 55 ml containing 2% (wt/vol) carrier ampholytes (Bio-Lyte 3/10; Bio-Rad Laboratories) at 10°C for 6 h at 12 W of constant power. Twenty fractions were collected and analyzed for PG activity. Fractions containing activity were pooled, the total volume was adjusted to 55 ml with distilled water, and the mixture was again subjected to preparative IEF.

Fractions of interest were concentrated by precipitation with 2.5 volumes of acetone, resuspended in 50 mM sodium acetate buffer, pH 4.75, and stored at -20°C.

Biochemical characterization of PG1. PG1 activity as a function of pH was assayed using a 100 mM citric acid and K₃PO₄ buffer mixture at pH values between 3.0 and 8.5 at 30°C. The effect of temperature on PG1 activity was determined in 50 mM sodium acetate buffer, pH 4.75, at 15 to 70°C.

For analysis of hydrolysis products, 100 µl of reaction mixture containing 1% (wt/vol) PGA in 50 mM sodium acetate buffer, pH 4.75, and 2.7 nkat of PG1 was incubated at 30°C. Aliquots of 10 µl were removed after 0, 10, 20, 30 min, 1, 2, 4, 8, and 24 h, boiled 5 min, and analyzed by ascending paper chromatography (Whatman 3MM paper) using *n*-butanol/acetic acid/water (2:1:2, vol/vol) as solvents and 0.04% bromophenol blue in 95% ethanol as detection reagent.

Removal of N-linked and both N- and O-linked carbohydrates was carried out, respectively, using *N*-glycosidase F or a combination of the former with *O*-glycosidase, neuraminidase, and *N*-acetyl-β-D-glucosaminidase (all from Boehringer GmbH, Mannheim, Germany). Deglycosylation protocols followed the instructions of the manufacturer.

The effect of tunicamycin on PG1 synthesis and secretion was studied by the addition of the antibiotic to cultures grown for 4 days in 0.1% glucose and induced by the addition of 0.5% (wt/vol) pectin. Tunicamycin (Sigma Chemical Co.) from a stock solution (10 mg ml⁻¹) in ethanol was added to each culture to a final concentration of 75 µg ml⁻¹ with the simultaneous addition of pectin. Occurrence of PG1 in the culture filtrate was monitored by IEF, followed by silver staining and activity stain of concentrated samples removed at the indicated intervals after pectin addition.

N-terminal sequencing of purified PG1 was performed by automated Edman degradation at the protein sequencing facility of the Universitat Autònoma de Barcelona on a Beckman LF3000 Sequencer (System Gold; Beckman Instruments, Inc., Palo Alto, CA).

Detection of PG isozymes in infected tomato roots. Ten-day-old tomato seedlings (cv. MoneyMaker) were inoculated with *F. oxysporum* f. sp. *lycopersici* strain 42-87 by dipping the roots for 30 min in a suspension containing 5 × 10⁶ microconidia ml⁻¹ water (20). Control plants were immersed in water. Seedlings were planted in minipots containing vermiculite and maintained in a growth chamber at 25°C with 14-h light and 10-h dark. Disease symptoms in aerial plant parts including epinasty and chlorosis of leaves, as well as wilting, were recorded periodically. At different time intervals postinoculation, 20 plants from each treatment were collected, washed carefully under running tap water to remove adhering vermiculite particles, and evaluated for root browning. Plantlets were then cut with a sterile scalpel to separate roots and the lower part of the stems that were then frozen in liquid nitrogen and stored at -80°C. Enzymes were extracted by grinding 0.1 g of tissue in a mortar under liquid nitrogen and transferring the frozen powder to 1 ml of 1 M NaCl. After mixing, samples were incubated 20 min on ice and centrifuged in an Eppendorf centrifuge 5 min at 16,000 × *g*. Supernatants were precipitated with 2.5 volumes of acetone and resuspended in 10 µl of distilled water. Zymograms of samples were performed as described above.

Purification and assay of tomato PGIP. PGIP was purified from 5-week-old tomato plants (cv. Vemar; Sluis & Groot Semillas, El Ejido, Spain) grown in pots containing vermiculite and maintained in a growth chamber at the conditions described above. Initial purification steps followed the protocol of Cervone et al. (7). All steps were carried out at 4°C. Briefly, 1,000 g of tissue from whole plants was homogenized in 1 liter of 1 M NaCl and centrifuged 20 min at 8,000 × *g*. The supernatant was filtered through Whatman 3MM paper, precipitated overnight with 75% (wt/vol) ammonium sulfate, and centrifuged 20 min at 8,000 × *g*. The pellet was thoroughly dissolved in 150 ml of 1 M NaCl and dialyzed twice against 5 liters of 20 mM sodium acetate, pH 4.75.

The dialyzed extract was then centrifuged 20 min at $8,000 \times g$, and the supernatant was subjected to preparative IEF in the pH range of 3 to 10 as described above. Inhibitory activity of the fractions against *Aspergillus niger* endoPG was assayed as described below. Fractions 14 to 20 from the first run showing a peak in PGIP activity were pooled and again focused by IEF. Fractions 16 to 20 from the second run containing most of the inhibitory activity were precipitated with 2.5 volumes of acetone, resuspended in 500 μ l of sodium acetate buffer, pH 4.75, and stored at -20°C . Purity of PGIP was checked by SDS-PAGE.

Inhibitory activity of purified tomato PGIP against different pectinolytic enzymes was assayed at 30°C by determining the release of reducing groups after 30 min in reaction mixtures (100 μ l total volume) containing 0.1 nkat of pectinolytic enzyme, 1% PGA in 100 mM sodium acetate, pH 4.75, and different quantities of tomato PGIP. Purified endoPGs from *F. moniliforme* and *A. niger* (6,18) were obtained from F. Cervone, Università "La Sapienza," Rome, Italy.

TABLE 1. Polygalacturonase (PG) activity in culture filtrates of *Fusarium oxysporum* f. sp. *lycopersici* grown on synthetic medium supplemented with different carbon sources

Carbon source	PG activity (nkat ml ⁻¹ culture filtrate ^a)
Glucose	0.8 \pm 0.2
Sucrose	0.6 \pm 0.2
Pectin	71.6 \pm 5.8
Pectin + glucose	7.8 \pm 1.1
Pectin + sucrose	7.1 \pm 0.8
Polygalacturonic acid (PGA)	12.3 \pm 1.6
PGA + glucose	2.7 \pm 0.5
PGA + sucrose	2.2 \pm 0.3
Xylan	0.7 \pm 0.1
Xylan + glucose	0.7 \pm 0.2
Xylan + sucrose	0.4 \pm 0.1

^a Data represent the mean values and standard deviations of results from two independent experiments.

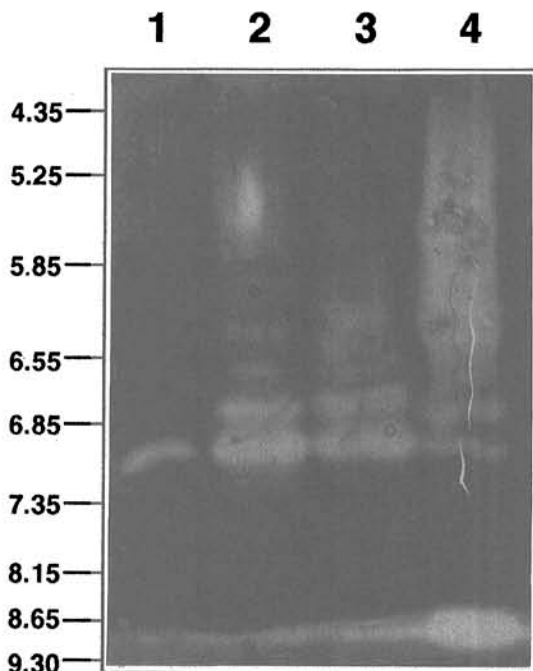


Fig. 1. Pectinolytic isozyme patterns from culture filtrates of *Fusarium oxysporum* f. sp. *lycopersici* grown on synthetic medium supplemented with 1% (wt/vol) of different commercial carbon sources. Four microliter samples were separated on an isoelectric focusing (IEF) gel (pH 3 to 9), followed by agarose overlay activity staining. Carbon sources were lane 1, glucose (filtrate 300X concentrated); lane 2, pectin (15X); lane 3, pectin + glucose (50X); and lane 4, polygalacturonic acid (40X). Positions and pIs of IEF markers are shown to the left of lane 1.

RESULTS

Production of pectinolytic isozymes on different carbon sources. Culture filtrates of *F. oxysporum* f. sp. *lycopersici* grown on SM supplemented with different carbon sources were analyzed for pectinolytic activity using the reducing sugar assay and IEF, followed by a substrate overlay. Total PG activity was at low constitutive levels in filtrates from *F. oxysporum* f. sp. *lycopersici* grown on glucose, sucrose, and xylan as the sole carbon source (Table 1). Total activity was about 100-fold and 20-fold induced on pectin and PGA, respectively, and catabolite repression by glucose or sucrose was approximately 90 and 80% on pectin and PGA, respectively. Zymograms of cultures grown on pectin as the sole carbon source showed a number of pectinolytic isozyme bands (Fig. 1). One isoform with pI 7.0 was the predominant isozyme produced on pectin (Fig. 1). Although the overall PG activity was reduced in filtrates from pectin + glucose and pectin + sucrose, isozyme patterns did not vary significantly, except for the activity bands between pIs 5.2 and 5.8 that were completely catabolite repressed (Fig. 1). In the filtrate of the fungus grown on glucose or sucrose alone or on oat-spelt xylan, only a faint activity band of the dominant isoform was visible even in highly concentrated samples. It remains unclear whether other pectinolytic isoforms were present at concentrations below the detection limit of the assay. On PGA as the sole carbon source, total PG activity in the filtrate after 4 days of growth was about six times lower than on pectin. The isozyme pattern showed a much stronger relative induction of the acidic (pIs 4.3 to 6.0) versus the neutral isozymes in PGA compared with pectin (Fig. 1). An additional activity band with pI 8.7 was observed on PGA. The latter activity band was completely repressed by glucose, whereas the other isozymes produced on PGA exhibited only partial catabolite repression (data not shown).

Purification of PG1 consisting of differentially glycosylated isoforms. A summary of the purification procedure is presented in Table 2. Concentrated and dialyzed culture filtrate of *F. oxysporum* f. sp. *lycopersici* grown on SM with pectin was subjected to two consecutive runs of preparative IEF at a pH range of 3 to 9. Fractions from the second run showing a peak in PG activity were concentrated by acetone precipitation and analyzed by analytical IEF, followed by silver staining or activity overlay (Fig. 2A and B). Comparison of activity zymograms and silver stained gels of fractions showed that most of the major protein bands detected in the filtrate possessed pectinolytic activity. At least six major isozyme bands were discriminated, with estimated pI values of 5.4, 5.8, 6.0, 6.5, 6.75, and 7.0. Rotofor fractions 9 to 11, 14, 15, and 19 (Fig. 2, lanes 4, 5, and 6) containing mainly the four isoforms with pIs 6.0 to 7.0 were subjected to SDS-PAGE; only two major protein bands with a MW of 35,000 and 37,500 + 2,000 were visible (Fig. 3A). To determine whether the two bands were differentially glycosylated isoforms of the same protein, fraction 19 (Fig. 2, lane 6) was treated either with *N*-glycosidase F or a combination of the former with *O*-glycosidase, neuraminidase,

TABLE 2. Purification of endopolygalacturonase PG1 from culture filtrates of *Fusarium oxysporum* f. sp. *lycopersici* grown on synthetic medium supplemented with 1% (wt/vol) citrus pectin^a

Step	Total protein (mg)	Total activity (μ kat)	Specific activity (μ kat/mg)	Purification (fold)	Yield (%)
Culture filtrate	64.2	91.3	1.4	1	100
Dialysate	7.2	112.4 ^b	15.7	11	123
Rotofor 1	3.9	93.9	23.8	17	103
Rotofor 2 ^c	0.2	5.9	27.0	19	6.4

^a Data reflect one representative purification run.

^b The increase in total activity compared with the crude culture filtrate may reflect removal of inhibitory substances by dialysis.

^c Fraction 19 (described in text).

and *N*-acetyl- β -D-glucosaminidase. Both treatments produced a single protein band with a MW of 35,000, indicating that the fraction contained pure enzyme, denominated PG1, and that the 37,500-MW band was a *N*-glycosylated isoform of the 35,000-MW protein (Fig. 3B). Comparison of banding intensities in different fractions of the IEF and SDS gels suggested that the non-glycosylated isoform (Fig. 3A, main band in lane 3) corresponded to the major activity band with pI 7.0, while the glycosylated form corresponded to the three activity bands with pI values of 6.0, 6.5, and 6.75. The MW of the native protein was estimated to be 35,000 \pm 5,000, indicating that the enzyme was active as a monomer.

Effect of tunicamycin. Activity bands corresponding to PG1 isoforms were detected in the filtrate of *F. oxysporum* f. sp. *lycopersici* 8 h after the addition of pectin, but not in cultures treated with 75 μ g ml⁻¹ tunicamycin (Fig. 4). The same was true after 24 h, whereas at 48 h, all PG1 isoforms were present in both culture filtrates, most likely because of the inactivation of tunicamycin. The absence of PG1 protein from the filtrates was confirmed by silver staining (data not shown), indicating that the lack of PG1 activity was not because of the presence of an inactive form of the enzyme. Tunicamycin at the applied concentration did not have any detectable effect on mycelial growth of *F. oxysporum* f. sp. *lycopersici*.

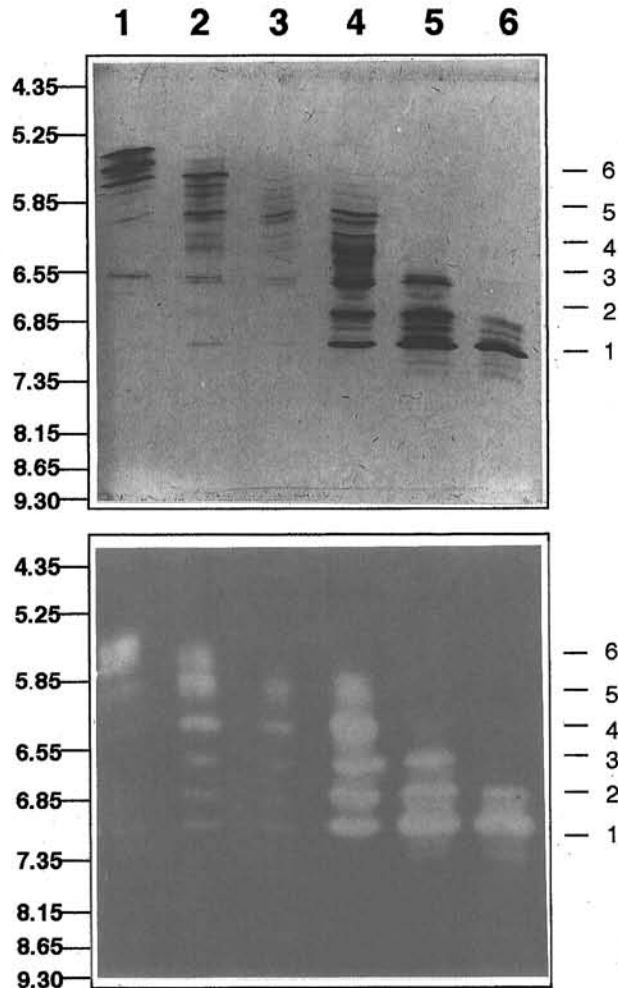


Fig. 2. Analytical isoelectric focusing (IEF) of concentrated and dialyzed culture filtrates of *Fusarium oxysporum* f. sp. *lycopersici* grown on synthetic medium supplemented with 1% (wt/vol) citrus pectin. Samples from different preparative IEF fractions were separated on an IEF gel (pH 3 to 9) followed by **A**, silver staining or **B**, pectinolytic activity staining. Lane 1, IEF fractions 3 to 4; lane 2, fraction 5; lane 3, fraction 7; lane 4, fractions 9 to 11; lane 5, fractions 14 and 15; and lane 6, fraction 19. Major pectinolytic isoforms are labeled 1 to 6 to the right of lane 6. Positions and pIs of IEF markers are shown to the left of lane 1.

Biochemical characterization of PG1. K_m of purified PG1 on PGA was 0.3 mg ml⁻¹ and V_{max} was 12.5 nkat μ g⁻¹. The end products of enzymatic hydrolysis of PGA were analyzed using ascending paper chromatography. Galacturonic acid was the first degradation product detected after 10 min, but intermediate products appeared later during hydrolysis. The final degradation products were mono- and digalacturonic acid. These results suggest an exomode of action of PG1. However, measurement of the decrease in relative viscosity of a PGA solution showed only 3% hydrolysis at 50% viscosity reduction, values typical of an endo-acting enzyme. It, therefore, appears from our results that PG1 displays both endo- and exoPG activity.

PG1 exhibited 75 to 100% of its maximum activity in the pH range of 3.5 to 5.0, with an optimum at pH 4.0. Activity decreased sharply above pH 5.5. The temperature optimum for PG1 activity was 37°C, with 80 to 100% of maximum activity between 30 and 43°C, about 25% at 15°C, and a sharp drop in activity above 60°C.

Amino acid sequence analysis of the N-terminus of the PG1 isoforms in fraction 19 gave a single, 11-residue sequence, D-P-C-S-V-T-D-Y-S-G-L, thereby confirming that they consisted of the

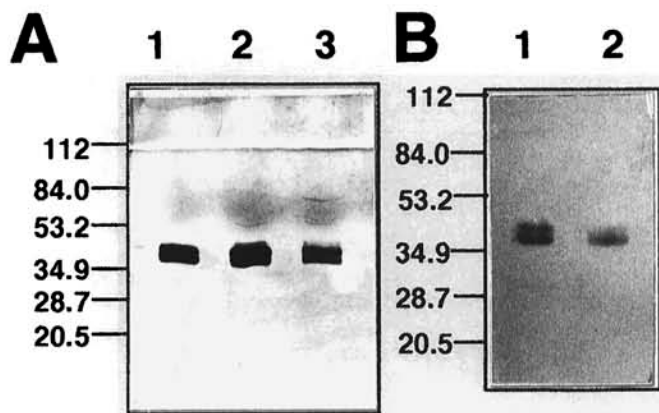


Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining of **A**, isoelectric focusing fractions 19 (lane 1), 14 and 15 (lane 2), and 9 to 11 (lane 3); and **B**, fraction 19 containing pure polygalacturonase 1 (PG1) from *Fusarium oxysporum* f. sp. *lycopersici*. Lane 1, 0.5 μ g of non-glycosylated PG1; and lane 2, 0.5 μ g of PG1 treated with *N*-glycosidase F. Positions and molecular weights of size markers are shown to the left of lane 1.

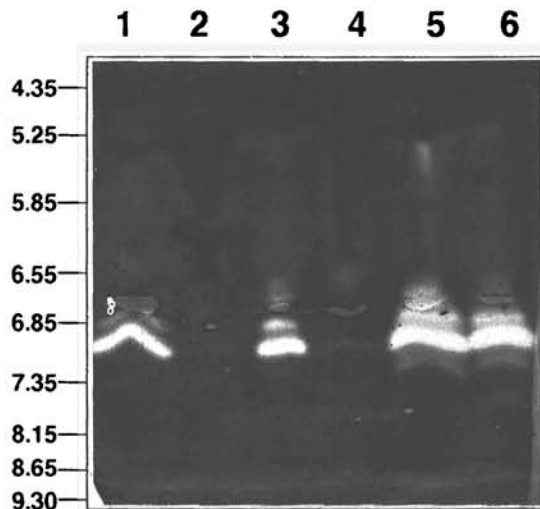


Fig. 4. Pectinolytic isozyme patterns from cultures of *Fusarium oxysporum* f. sp. *lycopersici* induced by the addition of 0.5% (wt/vol) citrus pectin and incubated with 75 μ g ml⁻¹ tunicamycin (lanes 2, 4, and 6) or without tunicamycin (lanes 1, 3, and 5). Samples were removed 8 h (lanes 1 and 2), 24 h (lanes 3 and 4), and 48 h (lanes 5 and 6) after the addition of pectin. Positions and pIs of isoelectric focusing markers are shown to the left of lane 1.

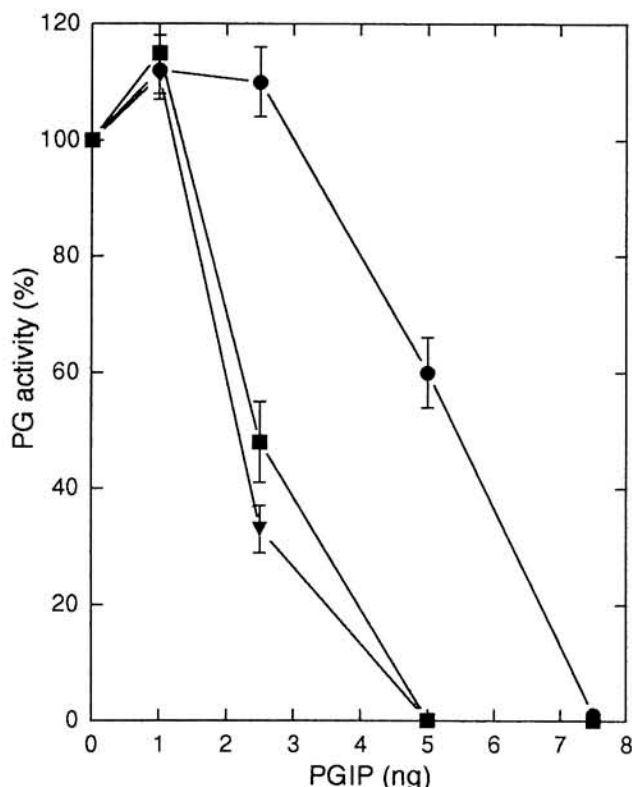


Fig. 5. Inhibition of endopolygalacturonases from *Fusarium oxysporum* f. sp. *lycopersici* (●), *F. moniliforme* (■), and *Aspergillus niger* (▲) by increasing amounts of purified tomato polygalacturonase-inhibitor protein (PGIP). Activity was determined by measuring the release of reducing groups by the Nelson-Somogyi assay after 30 min at 30°C, in reaction mixtures (100 µl total volume) containing 0.1 nkat of pectinolytic enzyme, 1% polygalacturonic acid in 100 mM sodium acetate, pH 4.75, and different quantities of tomato PGIP. Error bars represent standard deviations of the means of two experiments.

same protein. This sequence was almost identical to the N-terminus of the endoPG from *F. moniliforme* with the only exception of the substitution of an Asp for a Glu at position seven (5).

Inhibition by purified tomato PGIP. Tomato PGIP was purified and consisted of diffuse protein band with a MW between 35,000 and 41,000 upon SDS-PAGE (data not shown) as described previously (35). PGIP was tested for inhibitory activity against endoPGs from *F. oxysporum* f. sp. *lycopersici*, *F. moniliforme*, and *A. niger* (6,18). ED₅₀ for inhibition of 0.1 nkat endoPG from *F. moniliforme* and *A. niger* were 2.2 and 2.5 ng of tomato PGIP, respectively (Fig. 5). In contrast, ED₅₀ for *F. oxysporum* f. sp. *lycopersici* endoPG1 was 5 ng of PGIP.

Production of pectinolytic enzymes in infected tomato plants. The presence of pectinolytic isozymes in root and stem extracts of tomato plants infected with *F. oxysporum* f. sp. *lycopersici* was analyzed by zymograms (Fig. 6). Neither disease symptoms nor pectinolytic activity were detected in uninfected control plants (data not shown). Symptoms in infected plants included browning of roots (starting at day 7 postinoculation), epinasty and chlorosis of leaves (day 10), and wilting (day 14). A pectinolytic activity band with pI 8.7 was observed in infected roots at 4, 7, and 10 days post-inoculation (Fig. 6), but not at 14 days postinoculation (data not shown). This band corresponded to a pectate lyase (PL; EC 4.2.2.2) whose purification and characterization has been described elsewhere (19). The PL activity band was absent from stem samples, but at day 14 postinoculation, an activity band was detected corresponding to the main PG1 isoform with pI 7.0. The appearance of the PG1 activity band coincided with the onset of the first wilt symptoms on the aerial parts of infected plants (Fig. 6).

The production of pectinolytic enzymes has been investigated in a wide range of plant pathogens (10,11). Because of its peculiar infection mechanism, the vascular wilt fungus *F. oxysporum* has been subject to particularly intense studies (1). Since the first report on the production of pectinases by *F. oxysporum* f. sp. *lycopersici* and their possible role in pathogenesis (33), numerous authors have contributed to the increasing knowledge on pectinolytic enzymes from this pathogen. However, in spite of these considerable research efforts, it is still unclear how many pectinolytic isozymes are produced and which of them are secreted during plant infection (1). It also remains unknown whether different isozymes correspond to different proteins or if they are because of variations in the carbohydrate moieties of the same enzyme (36).

In the present study, a complex pattern of >10 pectinolytic activity bands was detected in culture filtrates of *F. oxysporum* f. sp. *lycopersici* grown on different carbon sources. This complexity was unexpected from previous reports, in which, at most, two PG isoforms were described for this pathogen (36). The high number of isozymes observed was probably because of the superior resolving capacity and sensitivity of the activity staining technique that allowed clear detection even of closely spaced bands. Similarly complex results have been obtained with this technique for bacterial pectinolytic enzymes (32). To allow detection of the complete range of pectinolytic isozymes secreted, the assay conditions in our study did not strictly discriminate between PG and PL activity. Although the buffer pH (4.2) was optimal for PGs, no EDTA was added to prevent activity of calcium-dependent PLs (32). In fact, the activity band with pI 8.7 specifically induced on PGA, corresponded to a PL (19). All pectinolytic activities were subject to substrate inducibility and catabolite repression. Differential induction of pectinolytic enzymes depending on substrates, as well as catabolite repression, have been described previously in vascular wilt pathogens (13). In the present study, the relative amounts of each isozyme varied greatly when the fungus was grown either on citrus pectin or on PGA, and the extent of catabolite repression differed considerably between distinct isozymes. This indicates that secretion of different pectinolytic enzymes in *F. oxysporum* is regulated independently.

Although isolation of individual pectinolytic isoforms was extremely difficult because of their closely spaced pI values, preparative IEF allowed separation into major isozyme groups. At least four major and a number of minor isozyme bands corresponded to differentially glycosylated isoforms of a single endoPG, denominated PG1, that was purified to homogeneity. The enzyme consisted of an apparently unglycosylated form with a MW of 35,000 and of three differentially glycosylated forms with a MW of 37,500. Four differentially glycosylated PG isoforms encoded by a single gene have also been reported in the fungal plant pathogen *F. moniliforme* (4), although, in that case, the isoforms differed in MW rather than in pI and no unglycosylated isoform was detected. Treatment with tunicamycin, a specific inhibitor of glycosylation, completely prevented PG1 activity in the culture filtrate, indicating that glycosylation of this enzyme is essential for its secretion. This confirms a previous report on a glycosylated xylanase from *Trichoderma viride* (16) and on a PL in the same pathogen, *F. oxysporum* (19), and provides further evidence for the importance of N-linked glycans in the secretory pathway (23). Our results suggest that PG1 was secreted into the medium in a glycosylated form and that the major, apparently unglycosylated isoform was probably a product of postsecretional deglycosylation, although this hypothesis has to be confirmed by further studies. The biological function of the modifications in the glycosidic part of the protein and their possible role in enzyme-plant interactions remain to be investigated.

Temperature and pH optima of PG1 and its activity as a monomer were similar to other fungal endoPGs (18,31,36,39). The prop-

erties of PG1, such as pI, MW, and modes of action, closely resembled those of two endoPG isozymes from *F. oxysporum* f. sp. *lycopersici* described by Strand et al. (36). However, these authors stated that their isomers were indistinguishable by pI and MW, whereas we could clearly discriminate at least four charge and two size isoforms of PG1. Strand et al. (36) suggested that differences in covalently linked carbohydrates might be responsible for electrophoretic mobilities of the isomers, a fact that was confirmed by our studies. Their observation that the PG isomers seemed to act simultaneously in an endo- and an exomanner is also corroborated by our work. Although analysis of degradation products by paper chromatography resolves smaller products (mono, di, and trimer) better than oligomers with a higher degree of polymerization, the early appearance of the monomer as the first visible degradation product strongly suggests an additional exoactivity of PG1. Because of the apparent purity of the PG1 enzyme preparation upon SDS-PAGE, it seems unlikely that our sample was contaminated with exoPG. A combined exo- and endo-activity of purified PGs has also been described in other fungal plant pathogens (21,37).

The dual mode of action of PG1 might provide a possible explanation for its increased resistance observed against inhibition by tomato PGIP compared with PGs from the cereal pathogen *F. moniliforme* and the saprophyte *A. niger*, which both exhibit typical endomodes of action (6,18), since it has been shown previously that exoacting PGs are not inhibited by PGIP (8). However, in the latter study, no difference was found between inhibitory activity of bean PGIP against endoPGs from different fungi (*A. niger*, *A. japonicum*, *Colletotrichum lindemuthianum*, and *F. moniliforme*). Therefore, the increased resistance of *F. oxysporum* f. sp. *lycopersici* endoPG as compared with the *F. moniliforme* enzyme was somewhat surprising considering that both pathogens belong to the same genus, and particularly taking into account the strong homology of N-termini displayed by the two enzymes. In spite of this homology, the divergent modes of action and differential sensitivities to PGIP suggest the presence of crucial structural and functional differences between the endoPGs from the dicot and the monocot pathogen.

In the present study, we detected production of PG1 on commercial substrates including citrus pectin, PGA, glucose, sucrose, and xylan. Before proceeding to further studies on PG1 such as gene isolation and disruption, its production in planta or at least in cultures supplemented with plant extracts should be confirmed, since specific pectinolytic isozymes may be produced exclusively during growth on SM or in planta (26). Recently, secretion of PG1, together with a PL, was detected during growth of *F. oxysporum* f. sp. *lycopersici* in liquid medium supplemented with tomato vascular tissue as the sole carbon source (19). In the present work, both enzymes were found to be secreted in tomato plants infected with the pathogen. Although PG activity in plants infected with *F. oxysporum* f. sp. *lycopersici* has been described previously (2,17,22), this is the first report in which pectinolytic activity in planta could be assigned to specific isozymes of a vascular wilt pathogen. PL activity was detected only in roots during early stages of infection, even before any macroscopic disease symptoms were visible (4 to 10 days postinoculation), whereas PG1 activity only became apparent in stems at 14 days postinoculation, coinciding with the onset of the characteristic wilt symptoms. It is, therefore, tempting to speculate that PL mainly plays a role in penetration of the roots and entry into the vascular elements, whereas PG1 is involved primarily in the phase of colonization of the vascular system and expression of wilt symptoms. The presence of PG1 in stems at later stages of infection (14 days) coincides with an earlier report in which an increase in PG activity was detected in infected tomato stems at 15 days after inoculation with *F. oxysporum* f. sp. *lycopersici* (17). In the vascular pathogen *Verticillium albo-atrum*, peaks of endoPL and endoPG activities in infected tomato plants were detected at 3 and

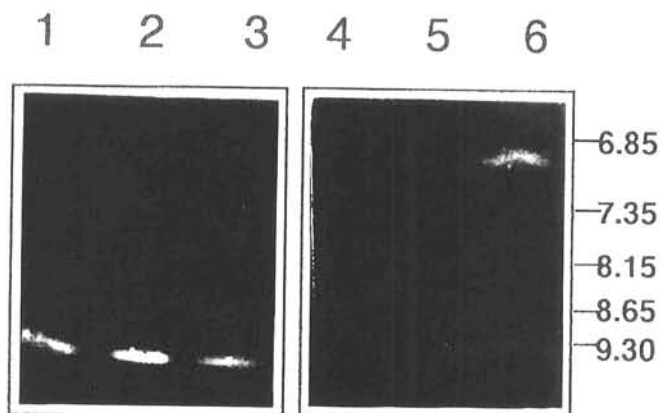


Fig. 6. Pectinolytic isozyme patterns from extracts of tomato plants infected with *Fusarium oxysporum* f. sp. *lycopersici*. Roots and stems of infected plants were ground under liquid nitrogen and extracted in 1 M NaCl. Proteins were concentrated 100X by precipitating with acetone and resuspending in distilled water. Four microliter samples were separated on an isoelectric focusing (IEF) gel (pH 3 to 9), followed by agarose overlay activity staining. Lanes 1 to 3, extracts from infected roots at 4, 7, and 10 days postinoculation, respectively. Lanes 4 to 6, extracts from infected stems at 7, 10, and 14 days postinoculation, respectively. Positions and pIs of IEF markers are shown to the left of lane 1.

6 days, respectively, confirming the sequence of appearance of enzyme activities observed in the present study (14). It has to be taken into account that in the zymogram technique used in the present study, the detection limit for a PG activity band is considerably higher than in the enzyme assays used to measure endoPG activity in the above reports. Moreover, partial inactivation by plant compounds such as phenolics or PGIPs may account for a reduction of PG activity detected in the extracts. Therefore, it cannot be excluded that PG1 might be secreted in planta at low concentrations during earlier stages of infection or that additional pectinolytic isozymes are produced at concentrations below the detection level. The fact, however, that PG1 and PL are secreted during plant infection makes these enzymes interesting candidates for further studies on their role in pathogenicity of *F. oxysporum*.

LITERATURE CITED

1. Beckman, C. H. 1987. The nature of wilt diseases of plants. The American Phytopathological Society, St. Paul, MN.
2. Biehn, W. L., and Dimond, A. E. 1971. Effect of galactose on polygalacturonase production and pathogenesis by *Fusarium oxysporum* f. sp. *lycopersici*. *Phytopathology* 61:242-243.
3. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
4. Caprari, C., Bergmann, C., Migheli, Q., Salvi, G., Albersheim, P., Darvill, A., Cervone, F., and de Lorenzo, G. 1993. *Fusarium moniliforme* secretes four endopolygalacturonases derived from a single gene product. *Physiol. Mol. Plant Pathol.* 43:453-462.
5. Caprari, C., Richter, A., Bergmann, C., Lo Cicero, S., Salvi, G., Cervone, F., and de Lorenzo, G. 1993. Cloning and characterization of a gene encoding the endopolygalacturonase of *Fusarium moniliforme*. *Mycol. Res.* 97:497-505.
6. Cervone, F., de Lorenzo, G., Degrà, L., and Salvi, G. 1987. Elicitation of necrosis in *Vigna unguiculata* Walp. by homogeneous *Aspergillus niger* endo-polygalacturonase and by α -D-galacturonate oligomers. *Plant Physiol.* 85:626-630.
7. Cervone, F., de Lorenzo, G., Degrà, L., Salvi, G., and Bergami, M. 1987. Purification and characterization of a polygalacturonase-inhibiting protein from *Phaseolus vulgaris* L. *Plant Physiol.* 85:631-637.
8. Cervone, F., de Lorenzo, G., Pressey, R., Darvill, A., and Albersheim, P. 1990. Can *Phaseolus* PGIP inhibit pectic enzymes from microbes and plants? *Phytochemistry* 29:447-449.
9. Cervone, F., Hahn, M. G., de Lorenzo, G., Darvill, A., and Albersheim, P. 1989. Host-pathogen interactions. XXXIII. A plant protein converts a fungal pathogenesis factor into an elicitor of plant defense responses. *Plant Physiol.* 90:542-548.
10. Collmer, A., and Keen, N. T. 1986. The role of pectic enzymes in plant

- pathogenesis. *Annu. Rev. Phytopathol.* 24:383-409.
11. Cooper, R. M. 1984. The role of cell wall-degrading enzymes in infection and damage. Pages 13-27 in: *Plant Diseases: Infection, Damage and Loss*. R. K. S. Wood and G. J. Jellis, eds. Blackwell Scientific Publications, Oxford.
 12. Cooper, R. M., and Rankin, B. 1978. Cell wall-degrading enzymes of vascular wilt fungi. II. Properties and modes of action of polysaccharidases of *Verticillium albo-atrum* and *Fusarium oxysporum* f.sp. *lycopersici*. *Physiol. Plant Pathol.* 13:101-134.
 13. Cooper, R. M., and Wood, R. K. S. 1973. Induction of synthesis of cell wall-degrading enzymes in vascular wilt fungi. *Nature (Lond.)* 246:309-311.
 14. Cooper, R. M., and Wood, R. K. S. 1980. Cell wall degrading enzymes of vascular wilt fungi. 3. Possible involvement of endo pectin lyase in *Verticillium albo-atrum* wilt of tomato. *Physiol. Plant Pathol.* 16:285-300.
 15. Davis, K. R., Lyon, G. D., Darvill, A. G., and Albersheim, P. 1984. Host-pathogen interactions. XXV. Endopolygalacturonic acid lyase from *Erwinia carotovora* elicits phytoalexin accumulation by releasing plant cell wall fragments. *Plant Physiol.* 74:52-60.
 16. Dean, J. F. D., and Anderson, J. D. 1991. Ethylene biosynthesis-inducing xylanase. Purification and physical characterization of the enzyme produced by *Trichoderma viride*. *Plant Physiol.* 95:316-323.
 17. Deese, D. C., and Stahmann, M. A. 1962. Pectic enzymes in *Fusarium*-infected susceptible and resistant tomato plants. *Phytopathology* 52:255-260.
 18. de Lorenzo, G., Salvi, G., Degrà, L., D'Ovidio, R., and Cervone, F. 1987. Induction of extracellular polygalacturonase and its mRNA in the phytopathogenic fungus *Fusarium moniliforme*. *J. Gen. Microbiol.* 133:3365-3373.
 19. Di Pietro, A., and Roncero, M. I. G. Purification and characterization of a pectate lyase from *Fusarium oxysporum* f.sp. *lycopersici* produced on tomato vascular tissue. *Physiol. Mol. Plant Pathol.* In press.
 20. Elias, K. S., and Schneider, R. W. 1991. Vegetative compatibility groups in *Fusarium oxysporum* f. sp. *lycopersici*. *Phytopathology* 81:159-162.
 21. English, P. D., Maglothlin, A., Keegstra, K., and Albersheim, P. 1972. A cell wall-degrading endopolygalacturonase secreted by *Colletotrichum lindemuthianum*. *Plant Physiol.* 49:293-297.
 22. Ferraris, L., Garibaldi, A., and Matta, A. 1974. Polygalacturonase and polygalacturonate *trans*-eliminase production in vitro and in vivo by *Fusarium oxysporum* f.sp. *lycopersici*. *Phytopathol. Z.* 81:1-14.
 23. Fiedler, K., and Simons, K. 1995. The role of *N*-glycans in the secretory pathway. *Cell* 81:309-312.
 24. Hedrick, A. L., and Smith, A. J. 1968. Size and charge isomer separation and estimation of molecular weights of proteins by disc gel electrophoresis. *Arch. Biochem. Biophysiol.* 126:155-164.
 25. Jones, T. M., Anderson, A. J., and Albersheim, P. 1972. Host-pathogen interactions. IV. Studies on the polysaccharide degrading enzymes secreted by *Fusarium oxysporum* f.sp. *lycopersici*. *Physiol. Plant Pathol.* 2:153-166.
 26. Kelemu, S., and Collmer, A. 1993. *Erwinia chrysanthemi* EC16 produces a second set of plant-inducible pectate lyase isozymes. *Appl. Environ. Microbiol.* 59:1756-1761.
 27. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.
 28. Lu, H., and Higgins, V. J. 1993. Partial characterization of a non-proteinaceous suppressor of non-specific elicitors from *Cladosporium fulvum* (syn. *Fulvia fulva*). *Physiol. Mol. Plant Pathol.* 42:427-439.
 29. Mann, B. 1962. Role of pectic enzymes in the *Fusarium* wilt syndrome of tomato. *Trans. Brit. Mycol. Soc.* 45:169-178.
 30. Nelson, N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. *J. Biol. Chem.* 153:375-380.
 31. Pérez Artés, E., and Tena, M. 1990. Purification and characterization of pectic enzymes from two races of *Fusarium oxysporum* f.sp. *ciceri* differing in virulence to chickpea (*Cicer arietinum* L.). *Physiol. Mol. Plant Pathol.* 37:107-124.
 32. Ried, J. L., and Collmer, A. 1985. Activity stain for rapid characterization of pectic enzymes in isoelectric focusing and sodium dodecyl sulfate-polyacrylamide gels. *Appl. Environ. Microbiol.* 50:615-622.
 33. Scheffer, R. P., and Walker, J. C. 1953. The physiology of *Fusarium* wilt of tomato. *Phytopathology* 43:116-125.
 34. Somogyi, M. 1952. Notes on sugar determination. *J. Biol. Chem.* 195:19-23.
 35. Stotz, H. U., Contos, J. J. A., Powell, A. L. T., Bennett, A. B., and Labavitch, J. M. 1994. Structure and expression of an inhibitor of fungal polygalacturonases from tomato. *Plant Mol. Biol.* 25:607-617.
 36. Strand, L. L., Corden, M. E., and MacDonald, D. L. 1976. Characterization of two endopolygalacturonase isozymes produced by *Fusarium oxysporum* f.sp. *lycopersici*. *Biochim. Biophys. Acta* 429:870-883.
 37. Urbanek, H., and Zalewska-Sobczak, J. 1975. Polygalacturonase of *Botrytis cinerea* E-200 Pers. *Biochim. Biophys. Acta* 377:402-409.
 38. Walton, J. D. 1994. Deconstructing the cell wall. *Plant Physiol.* 104:1113-1118.
 39. Walton, J. D., and Cervone, F. 1990. Endopolygalacturonase from the maize pathogen *Cochliobolus carbonum*. *Physiol. Mol. Plant Pathol.* 36:351-359.