Detection of Cutinases and Pectic Enzymes During Infection of Tomato by *Pseudomonas syringae* pv. *tomato*

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ABSTRACT

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Cutinolytic and pectinolytic activities were detected in leaves of either susceptible or resistant tomato cultivars within 48 hr after infection with *Pseudomonas syringae* pv. *tomato*. These activities markedly decreased at later stages of disease development. The enzymes involved appeared to be constitutive, the activities being only slightly enhanced in the presence of their respective substrates. The cutinolytic activity seemed to be of bacterial origin whereas the pectinolytic (polygalacturonase) activity was partially of bacterial origin and partially evolved from the pathogenic interaction between the pathogen and its host. Inoculation with high numbers of pathogen propagules (10^9 colony-forming units per milliliter) enhanced cutinolytic and pectinolytic activities and resulted in higher disease severity. Neither proteolytic nor cellulolytic activities seemed to play a role during the first 48 hr of infection. It was suggested that the pathogen-produced cutinases and pectic enzymes may be involved in the primary stages of disease development in tomato cultivars susceptible to bacterial speck.

Additional key words: bacterial speck of tomato, *Lycopersicon esculentum*, phytopathogenic pseudomonads.

On tomato, *Pseudomonas syringae* pv. *tomato* causes a leaf disease characterized by small brown-black specks, 1–3 mm in diameter, with a yellow chlorotic halo. The main mechanism of penetration by *P. s.* pv. *tomato* has been shown to be through open stomata, wounds, and broken glandular hairs (7,24). The multiplication of the pathogen occurs on both susceptible and resistant cultivars. In susceptible plants, the pathogen population increases with time; in the resistant plants the population decreases after a short period of multiplication (7). Free water on the leaves enhances infectivity. However, infection occurs in the absence of free water or when the waxy outer layer of the leaf is partially removed by chemicals (4).

It is generally accepted that phytopathogenic bacteria invade the plant tissue passively through natural openings and wounds (13). Although degradation of leaf cuticle due to enzymes produced by bacterial pathogens has not been reported so far, active invasion of roots has been suggested for *P. solanacearum* (15).

An active leaf-penetrating system might include cutinolytic, pectolytic, cellulolytic, and proteolytic enzymes (8). Pectic enzymes play a role in pathogenesis by some phytopathogenic bacteria (e.g., the Erwiniae and pseudomonads [20,32]). However, direct penetration through the cuticle based on the above enzymes has not been reported.

The purpose of this study was to assess the activities of hydrolytic enzymes that develop in the infected plant and to determine their possible role in degradation of the leaf tissue.

MATERIALS AND METHODS

Organisms, media and growth conditions, and experimental design and statistical analysis. *P. s.* pv. *tomato* (WT-1) isolated from infected tomato (*Lycopersicon esculentum* Mill.) plants (4) and plants of tomato cultivars VF-198 (highly susceptible to bacterial speck) and Rehovot 13 (resistant) (33) were used in all experiments.

Growth conditions, inoculum preparation, inoculation procedures, mist chamber, pathogenicity tests, and disease index (D.I.) were as described previously (4,33). Inoculation of plants was carried out by using an inoculum level of 10^5 colony-forming units (CFU) per milliliter. Bacteria were grown in yeast-peptone broth (YP). For enzymatic analysis, the medium contained asparagine and galactose as a sole nitrogen and carbon source (BOH) (6). In some instances, specific substances were added to the synthetic medium to test for enzyme activity. For cutinolytic activity, cucumber cuticle, tomato cuticles extracted from resistant or susceptible plants, sucrose, and fructose were added. For pectinolytic activity, Na-polypectate, pectin, carboxymethylcellulose, chlorogenic acid, and either catechol (Sigma), fructose, sucrose or NH_4Cl were added.

In one experiment, bacteria were grown on tomato leaf extract prepared by homogenization of 10 g of tomato leaves (cultivar VF-198) in an Omni-Mixer (Sorvall) in 0.06 M phosphate buffer, pH 6.8. The homogenate was filtered through cheesecloth, centrifuged (10,000 *g*, 30 min), and sterilized by filtration through a 0.45-μm Millipore filter.

All experiments were carried out two or three times with 5–10 replicates each, with 5–20 plants or reaction mixtures or Erlenmeyer flasks per replicate, according to the experiments. Results are from a representative experiment. Significance is given at $P \leq 0.05$.

Cuticle and wax extractions. Published extraction techniques (14,27) were used. To facilitate the separation of the cuticle layer, tomato leaves were cut near the edges, and leaf area was determined with an area-meter (Hayashi Denko, Tokyo). The leaves were then transferred to a solution of 60% $ZnCl_2$ dissolved in concentrated HCl and incubated for 5 days at room temperature (4.5 ml of solution per square centimeter of leaf). The separated cuticles were washed more than 20 times during 10 days to remove traces of acid. The extracted cuticles were lyophilized, milled to powder 595 μm (300-mesh), and stored at −20 C.

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Wax was extracted from detached leaves incubated at 30 C for 24 hr. During incubation, stomata were closed and the leaves wilted. Each leaflet was successively washed four times in purified chloroform (~100%) for exactly 20 sec. Solutions were combined and the chloroform was evaporated at 70 C.

Cuticle staining. Saturated boiled Sudan black III in 60% ethanol was cooled, double filtered, and placed on the sample for 30 min at room temperature (3). Samples were observed under a light microscope.

Enzyme extraction. Bacteria from 24-hr liquid cultures were collected by centrifugation at 10,000 *g* for 10 min at 4 C. The pellet was washed three times in 0.06 M potassium phosphate buffer, pH 7.0; resuspended in 5 ml of buffer; and sonicated with an MSE ultrasonic disintegrator at 1.5 A (60 sec × 3, with a 60-sec interval in an ice bath between each treatment). The homogenate obtained was centrifuged at 30,000 *g* for 30 min at 4 C. Partial purification of proteins was done by gel filtration on a column of Sephadex G-25 and then on a column of G-200 (61 × 2.5 cm, elution rate 0.15 − 0.2 ml/min, 0.06 M phosphate buffer, pH 7.0). Fractions showing enzymatic activities were lyophilized and redissolved in phosphate buffer before being tested.

Leaves were first homogenized in an ice bath with an Ultra Turrax (Janke and Kunkel, FRG), then in an Omni-Mixer (Sorvall), again with an Ultra Turrax, and finally with a fine homogenizer (Elda, Israel). The homogenate obtained was centrifuged at 12,000 *g* for 10 min, and the supernatant was subjected to two further centrifugations at 30,000 *g* for 30 min each. Proteins from the last supernatant were then partially purified as above.

Enzyme assays. *Polygalacturonase.* To test for polygalacturonase (PG), the cultures were grown in BOH synthetic medium supplemented with 0.1% Na-polypectate (Sigma). Activity was tested in the supernatant after 24 hr of growth at 30 C and after removal of bacterial cells (10^9 CFU/ml). Enzyme activity from diseased leaves was tested by dissolving lyophilized leaf extracts (after Sephadex partitioning) in 0.04 M sodium citrate buffer, pH 4.8. This pH was found to be the optimum for PG activity between pH 4 and pH 8. Reaction mixtures contained 0.5 ml of Na-polypectate (20 mg/ml); 0.3 ml of 0.04 M citrate buffer, pH 4.8; and 0.2 ml of enzyme preparation (approximately 0.2 mg of protein equivalent) to a final volume of 1 ml. Increase in reducing end groups after 4 hr of incubation at 35 C was tested by the dinitrosalicylic acid method (31). In addition, the enzyme was tested by using a viscosimetric method as described later. An enzyme unit was defined as micrograms of reducing end groups liberated in 1 hr per milligram of protein (18).

Pectin lyase. Cultures were grown as described previously, and the medium was supplemented with 0.1% apple pectin (grade 250, BDH). The reaction mixture contained: 0.5 ml of pectin (20 mg/ml), 0.3 ml of 0.02 M tris-HCl buffer, pH 8.0, supplemented with 0.001 M $CaCl_2$ and 0.2 ml of enzyme preparation to a final volume of 1 ml. The reactions were stopped by adding 0.2 ml of 9% $ZnSO_4 \cdot 7H_2O$, mixing well, and centrifuging at 10,000 *g* for 10 min). Changes in absorbance were recorded at 235 nm. An enzyme unit was defined as a change of 0.01 absorbance unit in 1 hr per milligram of protein (18).

Pectate lyase. The reaction mixture contained: 1 ml of 0.5% solution of Na-polypectate; 0.5 ml of 0.05 M tris-HCl buffer, pH 8.5, supplemented with 0.001 M $CaCl_2$; and 1 ml of enzyme preparation. The reaction products were scanned in a scanning spectrophotometer (Varian 635) over the range 200–400 nm. Activity was measured as maximum absorbance of products at 235 nm. An enzyme unit was defined as described for pectin lyase (18).

Pectin methylesterase. Production in culture was similar to that of pectin lyase. The reaction mixture contained 1 ml of 0.1 M phosphate buffer supplemented with 1% apple pectin and 0.15 M NaCl and 1 ml of enzyme preparation. Activity was measured by titration of the reaction with 0.1 N NaOH with methyl-red as an indicator. An enzyme unit was defined as equivalent to the titration of 0.01 ml of 0.1 N NaOH for 1 hr per milligram of protein (18).

Incubation conditions for all the pectinases were 4 hr at 35 C. This temperature was optimum for all assays. During incubation, a drop of toluene was added on the surface of all the reaction mixtures (including the controls) to prevent bacterial contamination. Reaction mixtures without enzyme or substrate were used as controls as were reactions in which the enzyme was added just before testing. In addition, the activity of all pectinases was determined viscosimetrically with an Ostwald 300 viscosimeter at 35 C (21).

Cellulase. Cellulase activity was determined according to the method described by Benefield (9).

Cutinases. Activities of these enzymes were indirectly measured either by following the decrease of the pH in the reaction mixture, by titration of liberated free fatty acids with 0.1 N NaOH using phenolphthalein as an indicator and oleic acid as a standard, or by thin-layer chromatography of the reaction products. The reaction mixture contained: 2 ml of 0.001M potassium phosphate buffer, pH 6.0; 1 ml of homogenized extracted cuticle (10 mg/ml); and 0.2 mg (protein equivalent) of dried enzyme preparation. The reaction mixture was incubated at 30 C for 24 hr with one drop of toluene. One enzyme unit was defined as the amount (in micrograms) of free fatty acids liberated in 24 hr per milligram of protein. Controls were mixtures that contained boiled enzyme or mixtures which lacked the enzyme or the substrate (26).

Proteases. Proteases were determined as described by Rinderknecht et al (22).

Wax degradation. The reaction mixture contained 1 ml of 0.001 M phosphate buffer at pH 5.5, 7.5, or 8.5; 1 ml of leaf extract preparation (100 mg of protein per milliliter); and leaf wax (10 mg/ml). Incubation time, testing methods, and controls were as for cutinases. The following enzyme sources were used for wax degradation: bacterial suspension grown in synthetic medium supplemented with wax, and washed for 24 hr supernatant of sonicated bacterial cells, extract of severely diseased leaf tissue, and extract of healthy tissue. In addition, the pathogen was grown in synthetic medium (BOH, without asparagine and galactose) with wax as a sole carbon source and KNO_3 as nitrogen source for 5 days. Proteins in all enzyme assays were determined by the Coomassie blue method (25) and glucose by the Glucostat (Sigma) method.

Thin-layer chromatography (TLC). *TLC of PG activity products.* Published analytical methods (1,28) were used to test for PG activity products. TLC plates (20 × 20 cm) were covered with microcrystalline cellulose (No. 19, Sigma). Samples (100-fold concentration of the reaction mixture, 0.5 ml of solution for each spot applied as 10-μl droplets) were chromatographed by using water:glacial acetic acid:ethyl acetate (2:1:2) as solvents. Spots were developed by spraying with $AgNO_3$ solution (prepared from 0.1 ml saturated $AgNO_3$ in 20 ml of acetone with some drops of water to clear the solution), and subsequently spraying with 0.5 N NaOH dissolved in 50% ethanol. Galacturonic acid was used as a marker.

TLC of cutinases activity products. The plates were covered with silica gel H. Samples were dissolved in petroleum-ether, run by using a petroleum-ether:diethyl-ether:glacial acetic acid (80:20:1) solvent system (17) with cholesterol, oleic acid, lecithin, and s,n-1,2,3,-tri-*O*-acyl glycerol as markers. Spots were developed in three successive steps, whereas visible spots were marked after each step. The plates were either placed in a concentrated iodine atmosphere and excess iodine was removed by exposure to air for 5 min; sprayed with 0.2% 2′,7′-dichlorofluorescein dissolved in absolute ethanol and observed under UV illumination; or sprayed with concentrated sulphuric acid (30).

Electrophoresis and isozyme staining. Electrophoresis was done on 7% polyacrylamide gels by using slight modifications of the method of Davis (12). After a prerun (1 mA for each tube), samples (20 μl containing 10 μg of protein) were run at 100 V, 10 mA, for 2 hr.

Qualitative isozyme staining was done immediately after electrophoresis by transferring the gels into 0.01 M citrate buffer, pH 4.8, for 10 min. The gels were incubated for 12 hr at 30 C in a solution containing 1.2% Na-polypectate and 10^{-3} M Na-hydrosulphite dissolved in the same buffer. The gels were washed twice in tap water and stained in a solution of ruthenium red dye (Sigma) (1 mg/ml, dissolved in the buffer and double filtered) for

30 min. Activity was revealed as colorless bands on a pink gel (16). Quantitative measurements of activity from unstained gels was done by slicing the gels into 1-mm segments. Each segment was put in the reaction mixture and products were detected as described above. Isozymes were located on the original gels by their distance from the edge of the gel.

RESULTS

Enzymatic degradation of tomato leaf cuticular wax. No enzymatic activity on wax was observed in any of the reaction mixtures listed in Materials and Methods, and the pathogen did not grow in the medium containing wax.

Properties of cutinases. Enzymes were extracted from diseased plants 24 hr after inoculation. Activity was tested at temperatures ranging from 4–37 C and detected between 12–37 C with an optimum at 22–30 C (6.8–7.2 units). At higher or lower temperatures, a drastic decrease in activity was observed. The effect of pH was tested over the range pH 3–9, activity was detected within pH 4–8, and the optimum was between pH 5 and 6 (7.15 units). The effect of incubation time was tested between 12–100 hr at pH 5.0 at 30 C. After 24 hr, activity reached a maximum. Therefore, the following incubation conditions were adopted for all other experiments: 30 C, pH 5.0, for 24 hr (7 ± 0.2 units).

The products from cutin degradation by enzyme extracts had a free fatty acid nature ($R_f = 0.32$ in TLC identical to the free fatty acid marker). Enzyme activity was detected in extracts of both susceptible and resistant inoculated plants (6.38 and 6.42 units, respectively), but not in uninoculated plants.

Degradation of tomato cuticles was observed in stained preparations. Only in necrotic sites was the cuticle intensely stained (Fig. 1a). Other parts of the cuticle were only slightly stained, similar to the cuticles of healthy plants (Fig. 1b). *P. s.* pv. *tomato* produced very low levels (0.6–1.1 units) of cutinase activity in synthetic BOH medium with galactose and asparagine. However, supplementation with tomato cuticles from either susceptible or resistant cultivars in BOH medium enhanced cutinase production by *P. s.* pv. *tomato* (6.84–7.02 units), whereas other supplements such as sugars and cucumber cuticle preparation had no effect (0.6–1.1 units).

Activities of cutinases in susceptible and resistant plants during disease development. Activities of cutinases was observed mainly during the first 48-hr period of disease development in both cultivars (Fig. 2A and B). At later stages, there was a significant decrease in activity. The decrease was significantly greater in the resistant cultivar than in the susceptible cultivar.

The effect of inoculation level (10^2, 10^5, and 10^9 CFU/ml) on activities of cutinases in susceptible diseased plants were tested during the first 72 hr after inoculation (Fig. 3). At the lowest

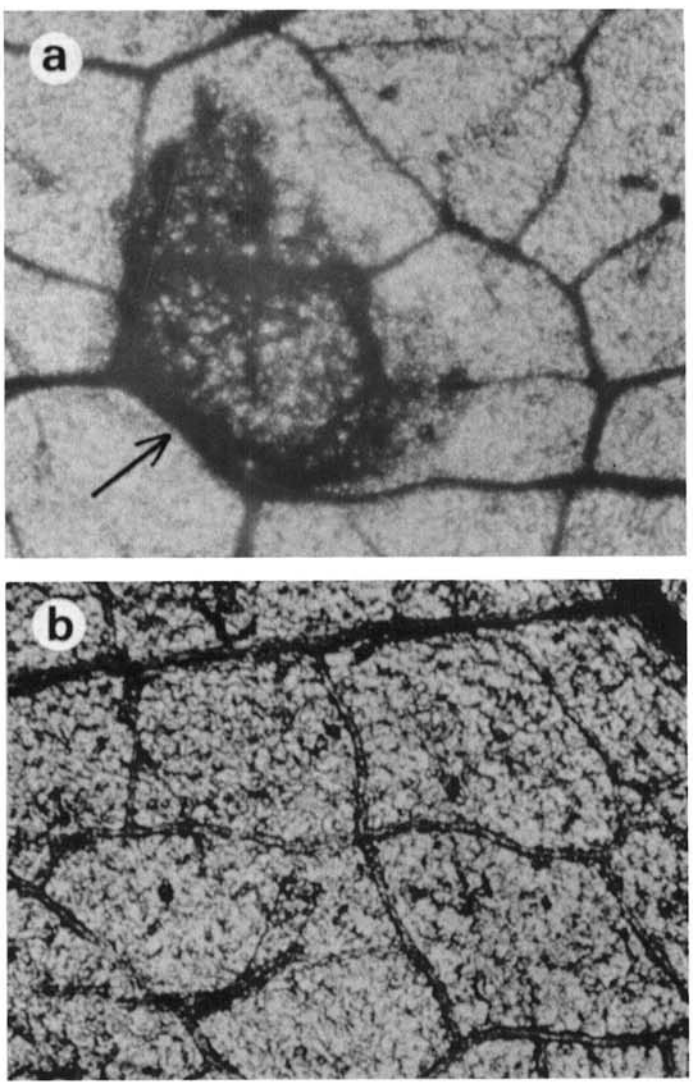

Fig. 1. Tomato cuticle stained with Sudan Black III (×100). **a,** Diseased plant. Arrow shows staining of necrotic area. **b,** Healthy plant.

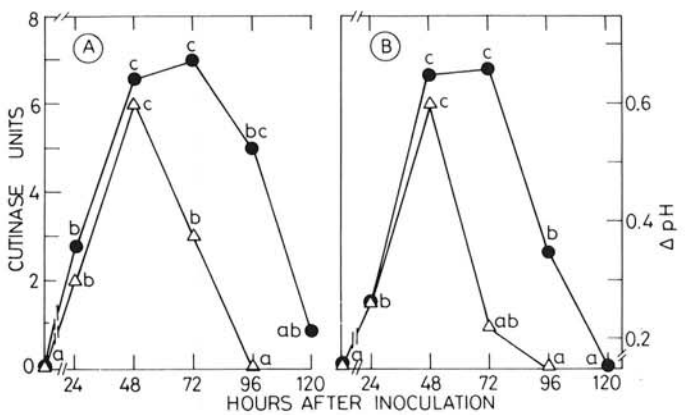

Fig. 2. Cutinase activity in inoculated susceptible and resistant tomato plants. △ = Resistant, ● = susceptible. **A,** Titration method; **B,** decrease in pH method. Points on the graph followed by different letters differ significantly at $P \leq 0.05$.

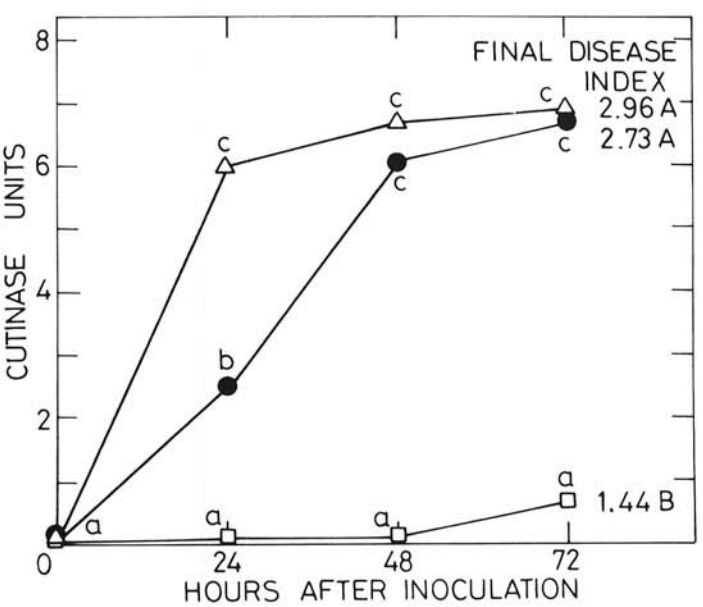

Fig. 3. Effect of inoculum level on cutinase activity and disease severity in inoculated susceptible tomato plants. □ = 10^2 CFU/ml; ● = 10^5 CFU/ml; △ = 10^9 CFU/ml. Points on the graph and the disease index values (separately) followed by different letters differ significantly at $P \leq 0.05$.

inoculation level (10^2 CFU/ml) activities of cutinases were negligible; at higher inoculation levels (both 10^5 and 10^9 CFU/ml) it increased significantly. A significant difference between disease indices was observed 8 days after inoculation between the lowest and the higher inoculation levels.

Proteolytic and cellulolytic activities in culture and in diseased plants. *P. s.* pv. *tomato* in culture is capable of using proteins but not cellulose as a sole carbon source (6). A proteolytic activity of 6.8 enzyme units was obtained in culture medium, whereas 1.6 enzyme units were obtained from plant extracts during the first 48 hr after inoculation. Cellulolytic activity could not be detected in culture or in the infected plant by the assay method used.

Pectic enzymes produced by *P. s.* pv. *tomato*. Highest pectolytic activity in culture supernatant was that of PG (42 units), whereas pectinlyase, pectate-lyase, and pectin-methyl-esterase activities were much lower (0.5 units maximum). In extracts from diseased susceptible plants pectinolytic activity of 7–8 enzyme units was found.

Further characterization of PG was done by running the products of enzymatic activity on TLC plates. The enzyme extracted from diseased plants was tentatively identified as endo-PG because, in addition to one spot with an R_f value similar to the galacturonic acid standard acid ($R_f = 0.46$), four other spots were detected. Their R_f values were 0.38, 0.32, 0.27, and 0.22. These different spots are apparently oligomers of galacturonic acid as deduced by Ayers et al (1) and Smith (28).

Further identification of the pectinase produced in culture as endo-PG was obtained by analyzing the type of the reaction in a viscosimeter. Fig. 4 shows a sharp decrease in viscosity and no increase in liberation of galacturonic acid during the first 180 min of the reaction (21).

PG activity during disease development in susceptible and resistant plants. Enzyme activity in plants was followed daily, after inoculation with 10^5 CFU/ml. It was observed that activity in the plant tissue increased during the first 48 hr after inoculation. At 48 hr, there was a significant difference in activity between resistant and susceptible cultivars. Activity did not increase further in the susceptible cultivar, but activity decreased in the resistant cultivar (Fig. 5).

The effect of inoculum level (10^2, 10^5, and 10^9 CFU/ml) of *P. s.* pv. *tomato* on pectinolytic activity was tested during the first 48 hr of infection. Pectic enzyme activity was low at the low inoculum level (Fig. 6) and increased at the higher inoculum levels (10^5 and 10^9 CFU/ml). The disease index in the plants 8 days later was significantly lower in plants inoculated at low level than in plants inoculated at high levels. Thus, pectic enzyme activity depended on bacterial multiplication in the tissue which increased with the concentration of the inoculum applied (4).

Isozyme of PG in bacteria, and in healthy and diseased plants. A single isozyme with a high specific activity was detected in the bacterial culture supernatant (Fig. 7B). A similar isozyme was present (with a lower specific activity) in the diseased plant. In addition, another PG isozyme was produced in the diseased plant (Fig. 7A). No PG isozyme could be detected in the uninoculated plants by this method (Fig. 7C).

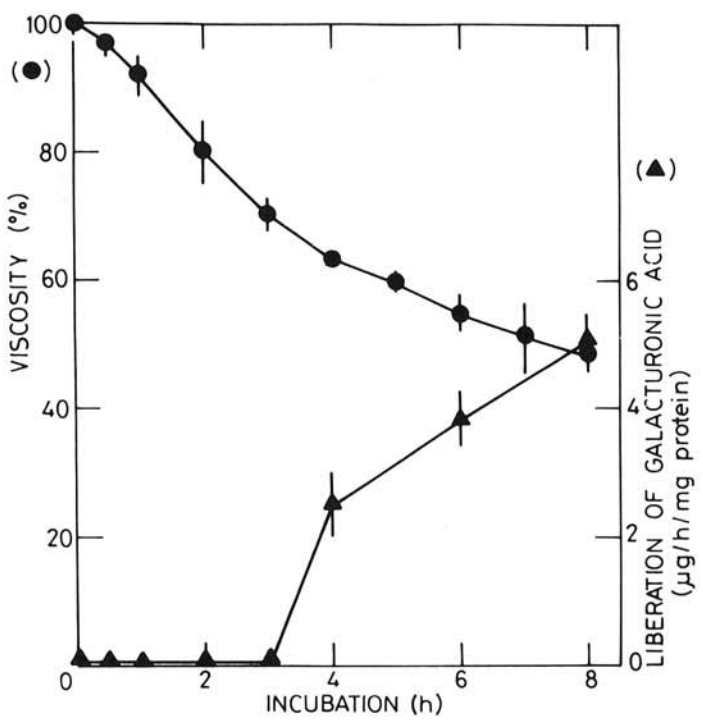

Fig. 4. Changes in viscosity and liberation of galacturonic acid by polygalacturonase after different incubation times.

DISCUSSION

The mechanisms involved in penetration, establishment, and proliferation of phytopathogenic bacteria in their host are not fully understood. Possibly, enzymes active in degradation of the plant cuticle layer are induced. These enzymes either degrade the whole epidermal wall or certain of its constituents such as wax, cutin, cellulose, pectin, and protein. Similar to observations of many other bacterial diseases (8,32) enzymes that degrade wax could not be detected in this system. On the other hand, the involvement of enzymes that hydrolyze cutin has been reported (2,29). In the present study, the existence of cutinases in the phytopathogenic bacterium *P. s.* pv. *tomato* has been demonstrated. The biochemical properties of cutinases from this bacterium were similar to those of the cutinase obtained from some fungi (29). However, their specific activity was lower when compared to cutinase obtained from *Botrytis* species (26). It was concluded that cutinases from *P. s.* pv. *tomato* may degrade side chains of the cutin polymer (full degradation of the leaf cutin layer was not observed) because the intensive staining in necrotic tissue may have been caused by cutinase liberation of fatty acid groups with higher affinity to the stain. However, suberin produced in response to infection could also take up Sudan III. Other studies have suggested that the degradation of side chains may weaken the cell wall cutin and aid penetration of the pathogens (26,29).

It is suggested that the enzymes that we studied participate in the primary stages of disease development, because their activity was mainly detected during the first 48 hr after inoculation. The high enzymatic activity could have resulted from multiplication of the pathogen inside the stomata which have a thin waxless cutin layer (11). Thus, microcolonies of *P. s.* pv. *tomato* multiplying in the stomatal chamber (7) may concentrate enough cutinase to cause successful penetration in the substomatal chamber wall.

At later stages (more than 48 hr after inoculation), cutinase activity was arrested in spite of the occurrence of secondary infections (7). Possibly, inhibition of the activity was caused by an increase in the pH level of the tissue as a result of ammonia accumulation (5), since high pH levels are known to inhibit cutinase activity (26,29). An interesting point is that similar activities were

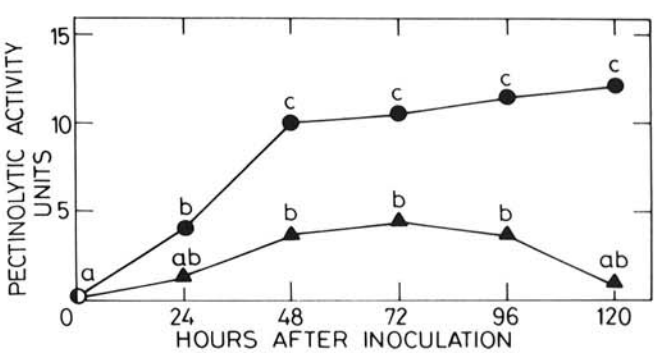

Fig. 5. Pectinolytic activity in inoculated susceptible and resistant tomato plants. ● = Susceptible; ▲= resistant. Points on the graph followed by different letters differ significantly at $P \leq 0.05$.

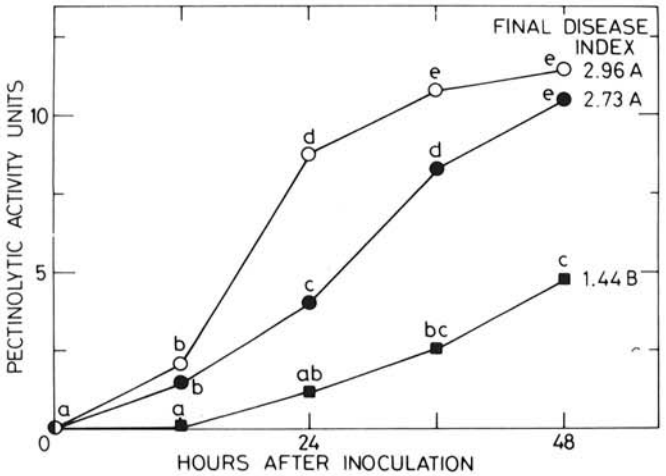

Fig. 6. Effect of inoculum level on pectinolytic activity in inoculated susceptible tomato plants. ■ = 10^2 CFU/ml, ● = 10^5 CFU/ml; O = 10^9 CFU/ml. Points on the graph or disease index values (separately) followed by different letters differ significantly at $P \leq 0.05$.

observed in a resistant and a susceptible tomato cultivar corresponding to the primary multiplication of the pathogen in these plant types (7). Therefore, resistance to the pathogen seems not to be related to the inhibition of cutinase.

The cutinase activities found in this work may also explain the phenomenon observed in previous work (4) with the same pathogen. Spraying of tomato leaves with diluted organic solvents to dissolve only the wax layer resulted in severe disease development after artificial inoculation. Perhaps the exposure of the cutin layer (which is usually protected by the wax layer) to enzymatic degradation caused an increase in disease development.

The importance of cellulase in pathogenesis is controversial, because of its low specific activity and its repression in the presence of glucose in plant tissue. The finding that *P. s.* pv. *tomato* has no cellulolitic activity is in accord with reports on many other phytopathogenic bacteria (32).

The role of pectic enzymes in the invasion process is much less controversial. We found that pectic enzymes play a role in the primary stages of disease development. Activity in the tissue was considered to be pectinolytic activity, since no highly purified pectic enzymes were used, and various cofactors or substrates obtained from the tissue might have accompanied the source of the enzyme used. However, the results indicated that the main enzyme involved in the process might be endo-PG, which is an important enzyme in many diseases because it causes rapid maceration of plant tissue. These indications are based on the following facts: Endo-PG was the main pectic enzyme produced in culture by *P. s.* pv. *tomato* because the reaction mixture and the enzyme source did not contain calcium which is a prerequisite for pectin and pectate lyase activity and viscosity decreased sharply without concomitant increase in galacturonic acid (21). Isozymes of PG of bacterial origin (with high specific activity) were found in diseased plants and in pathogen culture supernatant, and the pathogenic relationship induced a new isozyme which enhanced pectinolytic activity in the tissue. Assessment of the relative contribution of pectinolytic enzymes to the primary stages of disease development shows that activity is relatively low compared to that of soft-rot *Erwinia* sp. (19) but relatively high compared to other pseudomonads (10,20,23). Moreover, it may be speculated that the specific activity is higher at the specific sites of infection, because when the whole leaf is homogenized to extract the enzyme, the activity is diluted by the large proportion of healthy tissue. Further evidence that supports this point of view is that PG activity was much higher after gel electrophoresis to purify the enzyme.

Proteins are found in close relationship with pectin and cellulose in cell walls and middle lamellae. In spite of widely distributed protease activity in bacteria, the relationship of bacterial protease to pathogenicity or plant tissue invasion is not known. In bacterial

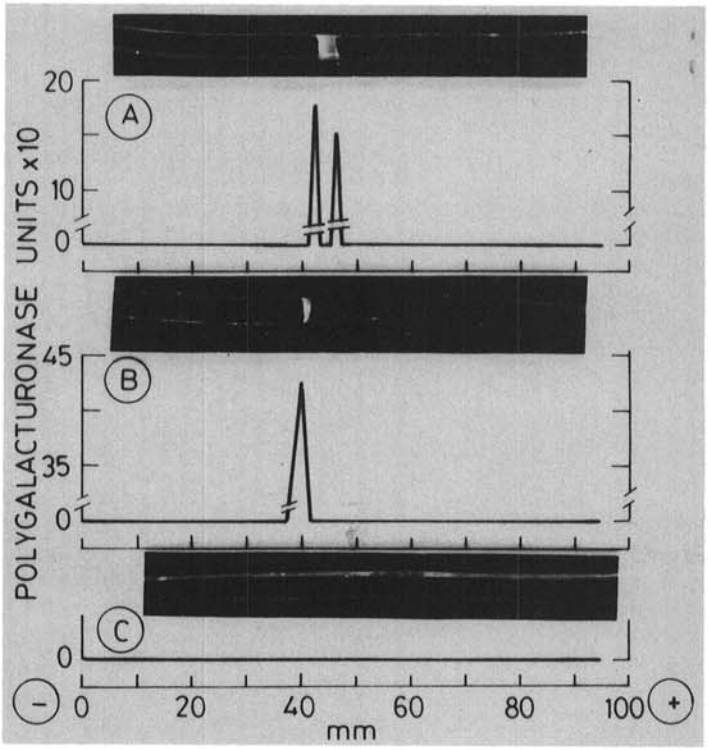

Fig. 7. Isozymes of polygalacturonase: **A,** diseased tomato plants; **B,** *Pseudomonas syringae* pv. *tomato*; and **C,** healthy plants.

speck of tomato, very low proteolytic activity was found during the early stages of disease development; thus, it seems to have only minor importance.

Some basic questions remain unresolved: do the enzymes produced either by the pathogen or by the host-pathogen interaction help in successful penetration of the host or are the enzymes induced as the consequence of a chain of events that occur soon after *P. s.* pv. *tomato* penetration and establishment in the tissue and do they play only a secondary role in the disease syndrome?

By analyzing all the data collected so far on the primary stages of disease development, it can be suggested that degradative enzymatic activity may play a role in initiating infection. However, it seems that although this mechanism is probably not the main one, it may increase the probabilities of successful infection.

For demonstration of direct involvement of degradative enzymes in infection, purified enzymes and enzymatic mutants of the pathogen should be used.

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