# Isolation and Preliminary Characterization of Extracellular Proteases Produced by Strains of *Xylella fastidiosa* from Grapevines

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This study was funded in part by the North Carolina Agricultural Research Service, Raleigh.

We thank D. Neher for assistance with statistical analysis and W. Marvin Williams for photographic assistance. We also thank D. L. Hopkins and C. J. Chang for providing strains of *Xylella fastidiosa* and R. C. Fites, P. B. Lindgren, and R. G. Upchurch for critical review of the manuscript.

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Accepted for publication 16 December 1993.

## ABSTRACT

Fry, S. M., Huang, J.-S., and Milholland, R. D. 1994. Isolation and preliminary characterization of extracellular proteases produced by strains of *Xylella fastidiosa* from grapevines. Phytopathology 84:357-363.

Virulent and weakly virulent grape strains of *Xylella fastidiosa* grew well on PD3 amended with gelatin and produced zones of hydrolysis on this medium, indicating the presence of proteolytic activity. Proteases also were produced in PD3 broth and degraded gelatin, azocasein, and azocoll. Gelatin was the best substrate for the proteases. In addition to using the gelatin assay, native activity and sodium dodecyl sulfate activity gels aided in visualizing and monitoring protease activity. Strain P of *X. fastidiosa* produced at least two proteases, designated P1 and P2, of 54 and 50 kDa, respectively. Protease activity was most abundant in the 31–60% ammonium sulfate fractions. Protease production was positively correlated with bacterial growth in PD3 broth and reached a maximum near the stationary phase of bacterial growth. P2 became more and P1 became less predominant over time in PD3 broth. Protease activity was not diminished by temperatures up to 60 C and was optimal at 50 C and pH 9.0. P1 and P2 were similarly affected by pH and temperature.

*Additional keywords*: Pierce's disease.

*Xylella fastidiosa* (33) is a xylem-limited bacterium that causes Pierce's disease of grapevines (*Vitis* spp.) and symptoms of marginal leaf necrosis on several other hosts (16). The marginal leaf burn or leaf scorch is reported to be due to prolonged water stress as a result of bacterial multiplication in the xylem and the host substances that occlude vessels (10,16,18). It is not known how *X. fastidiosa* moves systemically or even how the bacteria survive in the dilute nutritional environment of the xylem. Hopkins (16) suggested that pathogenicity of *X. fastidiosa* may depend on the ability of the bacteria to move systemically in the plant. Evidence suggested that avirulent strains were able to multiply as well as virulent ones soon after inoculation of grapevines but were not able to move systemically. Bacteria may move through pit membranes to other vessel elements. These membranes are composed of cellulose, hemicellulose, pectin substances, lignin, and protein. Hopkins (15), however, reported that grape strains did not produce pectolytic, proteolytic, or cellulolytic enzymes in cultures. The bacterium did produce a lysophospholipase, but its role in pathogenesis was not investigated. Wells et al (33) reported that strains of *X. fastidiosa* from various hosts did produce an extracellular protease. Although pectolytic and cellulolytic enzymes were not produced in culture, they may be produced in the host (16). Hopkins (16) suggested that bacterial enzymes may be present in low quantities and that the presence of a glycocalyx and the resulting aggregation of bacteria in microcolonies are needed to conserve and concentrate the enzymes on the pit membrane. Hopkins (16) also suggested that perhaps host cell-wall-degrading enzymes might be released in the xylem in response to the presence of the bacteria, or that the pressure created by bacteria in vessels may rupture the pit membranes, allowing the bacteria to move systemically.

Several studies have been undertaken to characterize proteases from phytopathogenic bacteria and to determine their role in disease development (2,4,7,13,14,20,21,23,25–27,29,30,34,35). It has been hypothesized that proteases may be required for nutritional purposes or to degrade protein in the plant cell wall to allow spread of the bacteria or overcome host defenses (7). Tseng and Mount (30) reported that a protease from *Erwinia carotovora* subsp. *carotovora* causes cellular death in intact cucumber tissue and causes cucumber protoplasts to burst, indicating modification of the plasma membrane. Willis et al (34) described a protease from a strain of *E. c. carotovora* that degrades hydroxyproline-rich glycoprotein from potato tubers and tomato plants, although conflicting evidence has been reported (19). Dow et al (7) found that turnip leaf cell walls are effective inducers of protease activity in *Xanthomonas campestris* pv. *campestris* when added to a minimal medium. Studies on the effects of proteases on other host proteins also have been described (2,26).

There is evidence both for and against the role of proteases in disease development; however, the role may vary depending on the particular host-pathogen system. The role of proteases in the *X. fastidiosa*-grapevine system is unknown. The objective of this study was to isolate and characterize the proteases of grape strains of *X. fastidiosa* produced in liquid cultures.

## MATERIALS AND METHODS

**Bacterial strains.** Grape strains of *X. fastidiosa* used in these studies are listed in Table 1. Isolation of strains from infected grapevines in the field were done as previously described (11). Strains were designated as virulent or weakly virulent based on the ability of the strains to multiply in, and cause symptoms on, grapevines.

*Vitis vinifera* L. 'French Colombard' and 'Carignane' plants were grown in a sand, soil, and peatmoss mix (3:3:1, v/v), fertilized, and maintained in the greenhouse as described (9). Five petioles per plant (three plants per strain) were inoculated.

Log phase PD3 cultures of strains of *X. fastidiosa* were suspended in succinate-citrate-phosphate (SCP) buffer (17; trisodium citrate, 1.0 g; disodium succinate, 1.0 g; $KH_2PO_4$, 1.0 g; and $K_2HPO_4$, 1.5 g; per liter of deionized water, pH 7.0), and the suspension was adjusted to an absorbance of 0.27 at 484 nm. Ten microliters of a bacterial suspension was placed on the petioles, and a pin was used to inoculate each petiole. Inoculated petioles were removed 3 wk after inoculation and assayed to determine bacterial populations in the petioles and leaf veins (9). Symptoms of marginal leaf necrosis were noted on inoculated petioles 3 wk after inoculation and on whole plants about 12 wk after inoculation.

**Protease assay.** A modification of the gelatin assay of Tseng and Mount (30) was used to quantify protease activity. The reaction mixture, consisting of 0.25 ml of 1% gelatin in 0.05 M Tris-HCl (pH 8.0) and 0.25 ml of the protease sample, was placed in microfuge tubes. The mixture was incubated for 4 h at 30 C in a water bath. After the incubation period, 0.75 ml of 20% trichloroacetic acid (TCA) was added, and the contents were thoroughly mixed. The mixture was allowed to stand for at least 15 min at room temperature. The solution was centrifuged at 10,000 rpm for 5 min at room temperature in a microfuge. The supernatant was poured into another microfuge tube, and the solution was centrifuged again. The absorbance of the supernatant was determined at 280 nm. Controls were prepared by incubating the substrate and sample separately and then combining the two for treatment as described above. The controls were used to zero the spectrophotometer before reading the samples. One unit of protease activity was defined as an increase in absorbance of 0.01.

TABLE 1. Grape strains of *Xylella fastidiosa* used in studies of protease production

| Strain[a] | Virulence[b] | Source[c] |
|---|---|---|
| CHC-P21 | V | Fry and Milholland (10) |
| C-P20 | V | Hopkins and Thompson (17) |
| FC-P22 | V | Hopkins and Thompson (17) |
| FC-P36F[d] | V | |
| PD1-P17 | V | Hopkins[e] |
| PD1-P21F | V | |
| PD888-P17 | V | Hopkins |
| PD888-P21F | V | |
| ATCC 35881-P31 | V | ATCC[f] |
| P-P22F | V | Pride grapevine, Horticultural Crops Research Station, Castle Hayne, NC |
| CF-P31 | V | Carlos grapevine, Horticultural Crops Research Station, Castle Hayne, NC |
| PD1a-P144 | WV | Hopkins |
| A-4#3-P215 | WV | Chang[g] |
| Vidal R112V2#3-P196 | WV | Chang |
| ATCC 35879-P169 | WV | ATCC |
| FC-P131 | ND | |
| CHC-P133 | ND | |
| C-P133 | ND | |
| CF-P74 | ND | |
| P-P74 | ND | |
| PD1-P49 | ND | |
| PD15a-P106 | ND | |
| ATCC 35881-P181 | ND | Hopkins |
| A-4#3-P80 | ND | |
| 19#1-P79 | ND | |
| 19#1-P174 | ND | Chang |
| Vidal R112V2#3-P84 | ND | |
| R30#4-P84 | ND | |
| R30#4-P197 | ND | Chang |
| R118V3#7-P84 | ND | |
| R118V3#7-P196 | ND | Chang |
| R25V18 1/300-P34 | ND | |
| R25V18 1/200-P34 | ND | Chang |
| R113V1#7-P34 | ND | Chang |
| R113V1#7-P145 | ND | Chang |
| R30V15-P34 | ND | |
| R112V14 1/200-P34 | ND | Chang |
| R112V14 1/200-P144 | ND | Chang |

[a] The number of times a strain has been subcultured is indicated by P followed by a number. For example, PD4-P24 indicates that strain PD4 has been subcultured 24 times.

[b] V = virulent, WV = weakly virulent, ND = virulence not determined.

[c] Strains that have not been designated a source are those cultures of strains that have been subcultured for extended periods of time compared with cultures of strains (which have been designated a source) that have been subcultured fewer times.

[d] Strain designations followed by an F indicate that the strain was reisolated from artificially inoculated plants in the greenhouse. Other strains were kept on PD3 at 28 C or in glycerol at −80 C.

[e] D. L. Hopkins, University of Florida, Leesburg. Strains were isolated from grapevines in Florida.

[f] American Type Culture Collection, Rockville, MD.

[g] C. J. Chang, University of Georgia, Griffin. Strains were isolated from grapevines in Georgia.

**Screening strains for protease production.** Strains of *X. fastidiosa* were grown on PD3 (5) plates for 3 days at 28 C. Bacteria were suspended in SCP buffer, and the suspension was adjusted to an absorbance of 0.10 at 484 nm. PD3 amended with 1% gelatin was spotted with 2.0 μl of a bacterial suspension on each of four areas on a plate. Each plate was inoculated with one bacterial strain. After incubation at 28 C for 7 days, plates were flooded with mercuric chloride solution (3), and the diameter of each colony and the diameter of zones of hydrolysis were measured. The ratio of zone of hydrolysis to colony diameter was calculated, and an average was determined for each strain. The experiment was performed once.

**Polyacrylamide gel electrophoresis.** The Bio-Rad Mini-Protean II dual slab gel apparatus was used as recommended by the manufacturer (Bio-Rad Laboratories, Richmond, CA). Protease activity was visualized in native activity gels and sodium dodecyl sulfate (SDS) activity gels. For native activity gels, protease samples were combined (1:4) with sample buffer (0.0625 M Tris-HCl, pH 6.8, 10% glycerol, and 0.001% bromophenol blue) and applied to a native gel consisting of a 5% separating gel with 0.01% gelatin, and a 4% stacking gel. The gel was electrophoresed at 180 V. The gel was incubated in 0.05 M Tris-HCl (pH 8.0) for 5 h at 28–30 C, then stained with 0.1% Coomassie blue in 40% methanol and 10% acetic acid for 15 min, and destained for 15 min. The location of the proteases on the gels was revealed as clear bands where the gelatin had been degraded. Protease samples also were examined on native gels. Samples were combined with native gel sample buffer as described above and applied to a native gel consisting of an 8% separating gel and a 4% stacking gel. The gel was electrophoresed as described above and silver stained (8).

For SDS activity gels (6,22), protease samples were combined (1:4) with SDS sample buffer (sample buffer containing 2% SDS) and incubated 30 min at 37 C. Samples were applied to SDS gels, which consisted of a 9% separating gel and 0.01% gelatin, and a 4% stacking gel. The gel was electrophoresed as described above. The gel was washed in 2.5% Triton X-100 (Bio-Rad) for 3 h on a shaker at room temperature, then incubated in 0.05 M Tris-HCl (pH 8.0) for 3 h at 28–30 C. Gels were stained with Coomassie blue as described.

**Protease production in liquid media.** Strain P was grown on PD3 plates for 3 days at 28 C and suspended in PD3 broth. The absorbance of the suspension was adjusted to 0.10 at 530 nm. Sixteen milliliters of the suspension was inoculated into 800 ml of PD3 broth in 2-L flasks. Flasks were shaken at 150 rpm at 28 C. Twenty-six milliliters of broth was removed from the flasks immediately after addition of bacteria and once every 24 h for 6 days. At each sampling time, the samples were placed in 50-ml plastic centrifuge tubes. One hundred microliters of each sample was serially diluted in SCP buffer and plated onto PD3. Plates were incubated 10 days at 28 C after which time colonies were counted. The remaining broth was centrifuged at 14,500 *g* for 15 min to remove the bacteria. The supernatant was stored at −80 C until use.

The supernatants were lyophilized, resuspended in 2.0 ml of

0.05 M Tris-HCl (pH 8.0), and dialyzed in Spectrapor membranes (Spectrum Medical Industries, Los Angeles, CA; molecular weight cutoff 14,000) at 8 C against 4 L of the same buffer. The buffer was changed two times over a period of 24 h. Samples were centrifuged in 15-ml glass centrifuge tubes at 14,500 *g* for 5 min, and the supernatant fluids were collected. The volume of each sample was adjusted to 6.1 ml with 0.05 M Tris-HCl (pH 8.0). Samples were stored at −80 C until use. The gelatin assay was used to determine protease activity. The Bio-Rad protein assay was used to determine protein content. Bovine serum albumin was used as a standard.

Each experiment included three inoculated flasks. Three replications from each sample date were tested and the average was determined. The average protease activity for each sample date of the three flasks in each experiment was also determined. The experiment was performed three times.

To visualize protease activity over time, protease samples from one flask per experiment were applied to native, native activity, and SDS activity gels as described.

**Isolation of proteases.** Strain P was grown on PD3 plates. Cells were suspended in PD3 broth and then inoculated into PD3 broth as described in the previous experiment. Flasks were placed on a rotary shaker at 150 rpm at 28 C. After 4 days, the liquid cultures were centrifuged for 15 min at 14,600 *g* at 4 C to remove the bacteria. The supernatants were filtered through Whatman no. 1 filter paper to remove clumps of bacteria that came loose upon decanting. A sample of the supernatant was removed and frozen before ammonium sulfate was added. Ammonium sulfate was slowly added to 750 ml of supernatant to make 20% solutions at 0 C. The supernatant was incubated at 0 C for 12 h, and the precipitate was collected by centrifugation. Ammonium sulfate fractions were collected at 10% intervals up to 90% saturation by repeating the aforementioned procedure. Supernatants were filtered through Whatman no. 1 filter paper if pellets came loose during decanting. Filtration was always used with the 0–20, 41–50, and 51–60% fractions and sometimes used with the 31–40 and 61–70% fractions. Pellets were suspended in 20 ml of 0.05 M Tris-HCl (pH 8.0) and stored at −80 C until use. All ammonium sulfate fractions were dialyzed subsequently in Spectrapor membranes (molecular weight cutoff 14,000) at 8 C against 4 L of 0.05 M Tris-HCl buffer (pH 8.0). The buffer was changed two times over a period of 24 h. Dialyzed fractions were centrifuged at 14,500 *g* for 15 min, the supernatant fluids collected, and their volumes adjusted to 25 ml with buffer. The supernatant from the 41–50% fraction was usually filtered again to remove any loose pellet before storage. Samples were stored at −80 C until use. The gelatin assay was used to determine protease activity in each fraction. The Bio-Rad protein assay was used to determine protein content in each fraction.

To visualize the protease activity in the fractions, protease samples were applied to native activity and SDS activity gels as described above.

For the characterization studies described below, a 31–60% ammonium sulfate fraction was utilized. The sample was dialyzed extensively against 0.05 M Tris-HCl (pH 8.0) before use.

**Thermal stability.** Two milliliters of protease sample were placed in borosilicate disposable culture tubes (13 × 100 mm), and the tubes were covered with Parafilm. Tubes were incubated at 4, 45, 50, 55, 60, 65, or 70 C for 15 min. There was one tube per treatment. After incubation, tubes were placed in an ice water bath for 5 min. The gelatin assay was used to test for residual protease activity. Four samples (replications) were removed from each tube and tested for protease activity. The experiment was performed three times. Protease samples were applied to native, native activity, and SDS activity gels as described previously.

**Optimum temperature.** The gelatin assay was used to determine the effect of temperature on enzyme activity in the protease sample. Reaction mixtures were incubated at 8, 25, 30, 35, 40, 45, 50, 55, 60, or 65 C for 4 h, and treated as previously described. There were four replications per treatment. The experiment was performed three times.

**Optimum pH.** The protease sample was dialyzed at 8 C against 4 L of deionized water. The water was changed one time over a period of 16 h. The sample was used in the assay immediately after dialysis. One percent gelatin solutions were prepared in each of the following buffers: 0.1 M acetic acid-sodium acetate buffer (pH 5), 0.1 M monobasic sodium phosphate-dibasic sodium phosphate buffer (pH 6 and 7), 0.1 M Tris-HCl buffer (pH 8 and 9), and 0.1 M glycine-sodium glycinate buffer (pH 10 and 10.7). The gelatin assay was performed as described above. There were four replications per treatment. The experiment was performed three times.

To determine if the proteases were affected differently by pH, protease samples were applied to native activity and SDS activity gels as described previously. Gels were incubated in the above buffers with ionic strength of 0.05 M at 28–30 C for 5 h and stained with Coomassie blue as described previously.

**Molecular weight determination.** A nondenatured protein molecular weight marker kit (Sigma) was used to estimate the molecular weight of the proteases. Standards and protease samples were applied to 7, 8, 9, and 10% native separating gels. Standards were α-lactalbumin (14.2 kDa), carbonic anhydrase (29 kDa), chicken egg albumin (45 kDa), and bovine serum albumin (monomer, 66 kDa; dimer, 132 kDa). Standards and protease samples were applied to the left half of gels and protease samples only were applied to the right half of gels. After electrophoresis, the gels were cut in half. The left half of each gel was silver stained, and the right half was placed on a petri dish containing a thin layer of 1.5% Bacto-agar, 8% gelatin, and 0.01% thimerosal (Sigma). The plate was incubated for about 10 h at 28–30 C and then flooded with mercuric chloride solution. The $R_f$ of each standard and protease was calculated, and the molecular weight of the proteases determined using a standard curve generated according to the manufacturer's instructions.

## RESULTS AND DISCUSSION

Preliminary studies revealed that grape strains of *X. fastidiosa* did not grow on skim milk or 1% Bacto-agar amended with 1% gelatin but did grow on milk agar and nutrient agar amended with 1% skim milk (8). Zones of hydrolysis were not observed on any of the media. Strains grew well on nutrient agar and PD3, each amended with 0.4% gelatin, and zones of hydrolysis were observed 2 and 1 wk, respectively, after inoculation. Studies also revealed that the proteases degraded gelatin and azocasein and slightly degraded azocoll in assay solutions. Gelatin was the best substrate for determining protease activity (8). Hopkins (15)

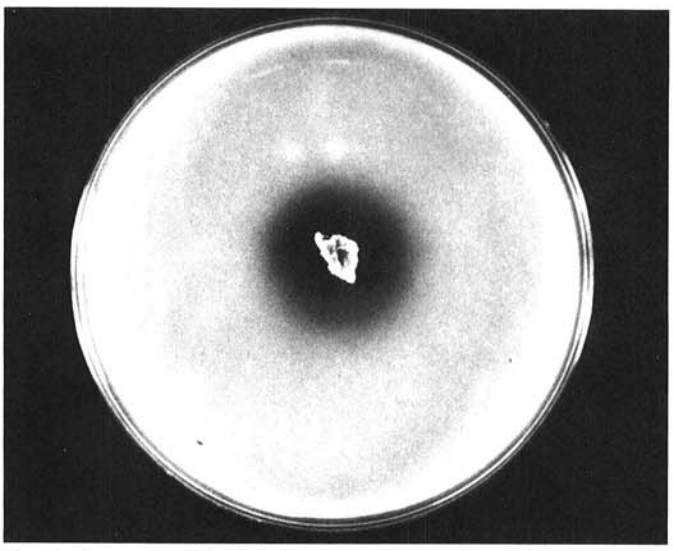

**Fig. 1.** Clear zone of hydrolysis surrounding a colony of a grape strain of *Xylella fastidiosa* on PD3 amended with 1% gelatin. After incubation at 28 C for about 7 days, the plate was flooded with mercuric chloride solution to make the zone visible.

did not detect protease activity by grape strains of *X. fastidiosa* in PD3 medium by the hide powder azure method. Hide powder consists of collagen and is similar to azocoll, which was not the best substrate for the proteases of *X. fastidiosa*. Wells et al (33), however, reported that strains of *X. fastidiosa* produce a gelatinase in PW medium that is detected by the gelatin-charcoal test. Similar to our results with *X. fastidiosa*, other phytopathogenic bacteria have been shown to degrade a range of proteins (1,4,20,21,23,26, 28,30).

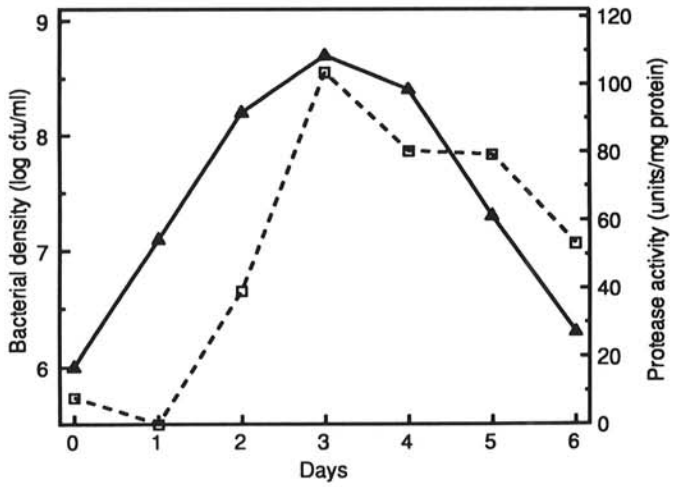


**Fig. 2.** Protease production (dashed line) and bacterial growth (solid line) in PD3 broth cultures of *Xylella fastidiosa* strain P as determined by the gelatin assay and dilution plating, respectively. Cultures were shaken at 150 rpm at 28 C. Data are from one representative experiment.

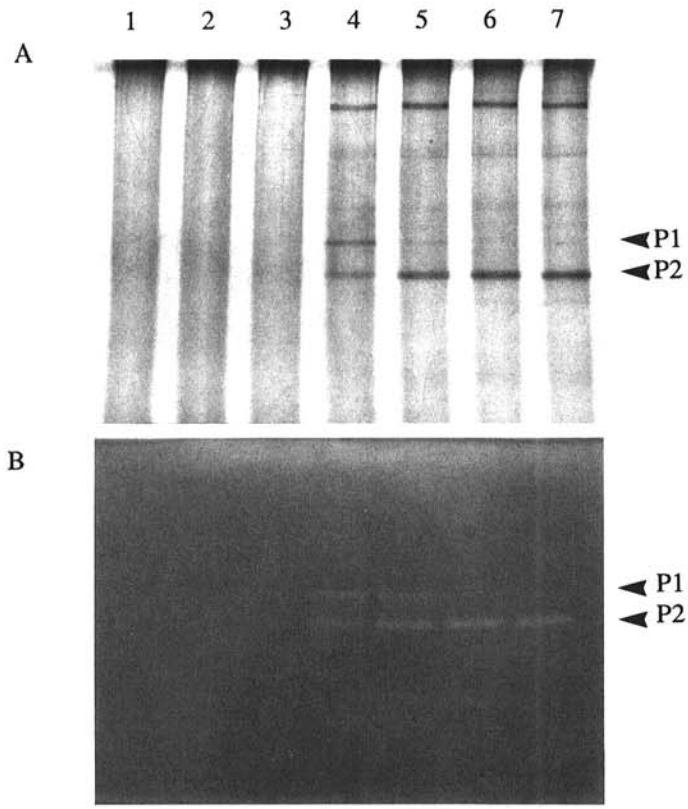


**Fig. 3. A,** The appearance of two proteases, designated P1 and P2, (indicated by arrows) on a silver-stained native gel in samples of liquid cultures from 0 to 6 days (lanes 1–7, respectively) after inoculation of the flasks with *Xylella fastidiosa* strain P. **B,** Protease activity (light bands) visualized on a sodium dodecyl sulfate activity gel. Protein content was 0.3 $\mu$g per lane.

Virulent and weakly virulent grape strains of *X. fastidiosa* (Table 1) grew well on PD3 amended with 1% gelatin. All strains tested produced zones of hydrolysis (Fig. 1). Because the borders of the zones were diffuse, it was difficult to obtain precise measurements. The diffuse borders may be an indication of the small amount of protease that is produced by *X. fastidiosa*, because purified commercial proteases produced zones of hydrolysis with defined borders. There did not appear to be distinct differences in the ability of strains to produce protease according to this method. Since the weakly virulent strains were derived by long-term subculturing of virulent strains, the strains may be affected in characteristics other than protease production. Fuji and Uematsu (12) found that virulence in *Xanthomonas campestris* pv. *oryzae* did not correlate with production of protease when strains became less virulent as a result of subculturing over a long time.

Preliminary studies involving growth of strains of *X. fastidiosa* in PD3 broth, PD3 broth amended with 0.4% gelatin, nutrient broth, nutrient broth amended with 0.4% gelatin, salt solution (8; trisodium citrate, 1.0 g; disodium succinate, 1.0 g; $KH_2PO_4$, 1.0 g; $K_2HPO_4$, 1.5 g; and $MgSO_2 \cdot 7H_2O$, 1.0 g; per liter of deionized water), and salt solution amended with 0.4% gelatin revealed that growth was poor in nutrient broth, nutrient broth amended with gelatin, and salt solution. Growth was slow in salt solution amended with gelatin but was good in PD3 and PD3 amended with gelatin (8). PD3 broth was chosen because the bacteria grew well and produced protease without the presence of gelatin.

Both bacterial density and protease activity (Fig. 2) of strain P increased to a maximum at about 3 days, then rapidly declined during culture in PD3 broth. A similar correlation between bacterial growth and protease production was observed in cultures of *Erwinia chrysanthemi* (1).

The two proteases, designated P1 and P2, were not observed on native gels (Fig. 3A) after 1 to 2 days of bacterial growth. On the third day, P1 and P2 were present in higher quantities, but P1 was more prevalent than P2. On the fourth day, P2 was more prevalent than P1 and was predominant up to the sixth day, whereas P1 decreased. The protease activity as revealed on the native activity (not shown) and SDS activity gels (Fig. 3B) showed the same pattern in the presence and intensity of the protease activity of P1 and P2 over time as that on the native gel.

Information on phytopathogenic bacteria that produce multiple proteases in which the production of the proteases have been monitored over time in cultures is lacking. It is unknown at this time whether the proteases of *X. fastidiosa* are distinct or whether there is actually one protease that is being partially degraded

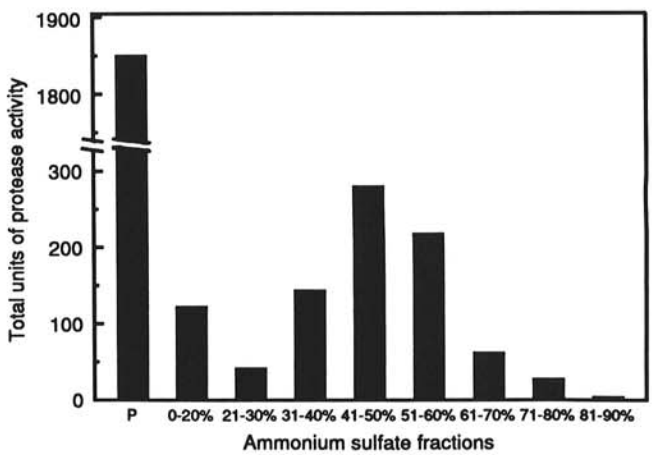


**Fig. 4.** Protease activity in ammonium sulfate fractions of the bacterial cell-free supernatant as determined by the gelatin assay. P represents the bacterial cell-free supernatant. Data represent means from six purifications. Standard deviations are not shown due to the variation in protease activity of the bacterial cell-free supernatant.

due to autocatalytic activity or another protease that was not detected in this study. Other phytopathogenic bacteria that produce more than one protease include *E. chrysanthemi* (31,32), *Xanthomonas campestris* pv. *campestris* (27), and *X. c. malvacearum* (14).

About 50% of the total activity of the bacterial-cell-free supernatant was recovered in the sum of the ammonium sulfate fractions (Fig. 4). Protease activities were most abundant (approximately 35% of the total) in the 31–60% ammonium sulfate fractions. Based on specific activity, ammonium sulfate precipitation of the 31–60% fractions resulted in an average purification of 1.6-fold. Native and SDS activity gels were used to visualize protease activity in the ammonium sulfate fractions. Based on the results using the native activity gels, P1 and P2 were most prevalent in the 41–50% fraction and were usually present in similar quantities (*data not shown*). The proteases also were present in similar quantities to each other in the 31–40% fraction. P2 was predominant in the 51–60% fraction. Based on the results using SDS activity gels, P1 was predominant in the 31–40% fraction, P1 and P2 were present in similar quantities in the 41–50% fraction, and P2 was predominant in the 51–60% fraction (Fig. 5). Attempts to separate the two proteases using gel filtration and ion-exchange high-performance liquid chromatography were unsuccessful.

The thermal stability of the proteases was high. Residual protease activity changed nominally as the enzyme solution was incubated at intervals between 45 and 60 C (Fig. 6). However, activity decreased substantially upon incubation at 65 and 70C. The native gel (Fig. 7A) also revealed that the intensities of the P1 and P2 bands were diminished after treatment at 65 C and not detectable after treatment at 70 C. SDS activity gels (Fig. 7B) and native activity gels (not shown) revealed that both P1 and P2 were affected similarly by incubation at various temperatures. Zones of hydrolysis were similar up to 60 C. At 65 C, the zones of hydrolysis for P1 and P2 were reduced in size, whereas at 70 C zones of hydrolysis were not evident. It is important to note the effect of heat on the other protein bands (Fig. 7A). The three high molecular weight proteins in greatest abundance continued to be retained even at 70 C. The three protein bands that migrated just above P1 decreased in intensity at 55 C but were still present at 65 C. The protein band below P2 decreased in intensity at 55 C and was no longer evident at 60 C. Thus, heat denaturation was not used as a step in purifying the proteases since high temperatures would not have preferentially removed

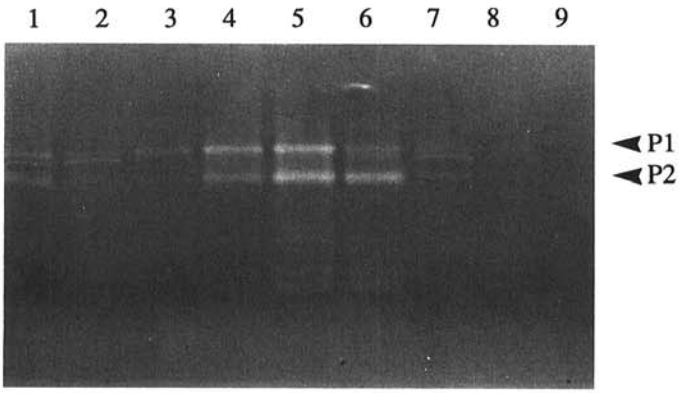


**Fig. 5.** Protease activity of P1 and P2 (indicated by arrows) in ammonium sulfate fractions as visualized on a sodium dodecyl sulfate activity gel as clear zones. Lanes 1–9 correspond to the x-axis in Fig. 4. Sample volume was 12 $\mu$l per lane.

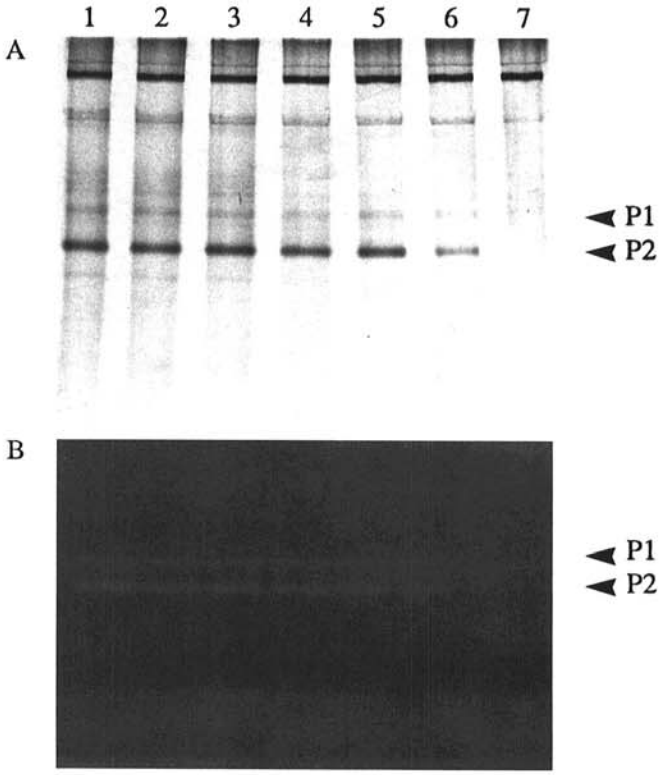


**Fig. 7.** Effect of temperature on enzyme stability as visualized on a **A,** native gel and **B,** sodium dodecyl sulfate activity gel. Temperatures tested were 4, 45, 50, 55, 60, 65, and 70 C (lanes 1–7, respectively). Sample volume was 17.5 $\mu$l per lane.

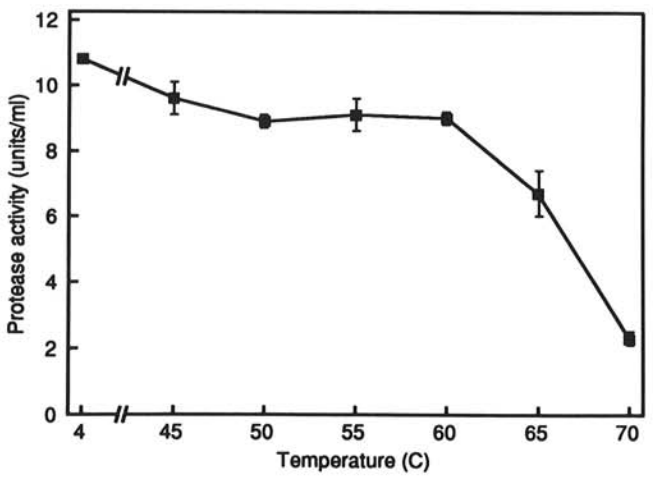


**Fig. 6.** Effect of temperature on enzyme stability. The residual activity was determined using the gelatin assay. Bars represent standard deviation. Data are from one representative experiment.

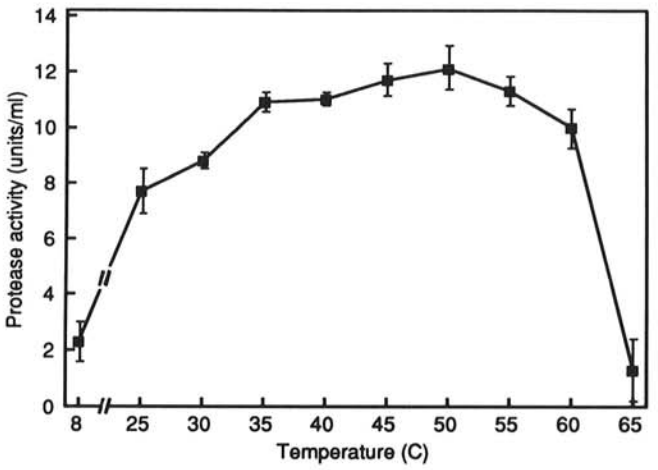


**Fig. 8.** Effect of the reaction temperature of the gelatin assay on protease activity. Bars represent standard deviation. Data are from one representative experiment.

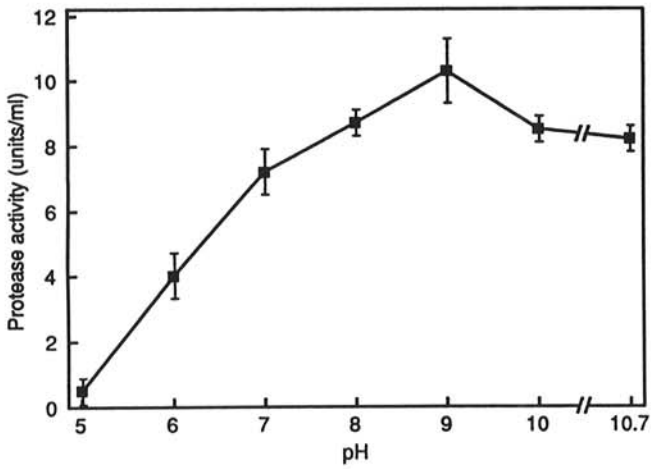


**Fig. 9.** Effect of pH on protease activity. Protease samples were combined with 1% gelatin solutions prepared in each of the following buffers: 0.1 M acetic acid-sodium acetate buffer (pH 5), 0.1 M monobasic sodium phosphate-dibasic sodium phosphate buffer (pH 6 and 7), 0.1 M Tris-HCl buffer (pH 8 and 9), and 0.1 M glycine-sodium glycinate buffer (pH 10 and 10.7). Bars represent standard deviation. Data are from one representative experiment.

the more abundant proteins. Protease activity of *X. c. zinniae* (26) was also resistant to temperatures up to 65 C.

Protease activity was low at 8 C, at which point the reaction mixture solidified due to the gelatin substrate (Fig. 8). The protease was otherwise active across a wide range of temperatures (25–60 C). Protease activity increased to a maximum around 50C, then there was a dramatic decrease in activity from 60 to 65C. Protease activity of *X. c. zinniae* (26) was also optimal at 50C.

P1 and P2 showed optimal activities at about pH 9.0 (Fig. 9). Native activity and SDS activity gels revealed that the two proteases were similarly affected by pH. Protease activity from *Xanthomonas alfalfa* (21) and *X. c. campestris* (7) was optimal near pH 8.0, whereas protease activity from *E. c. carotovora* (24) and *X. c. oryzae* (12) was optimal near pH 7.0 and 9.3, respectively. Protease activity from *X. c. zinniae* (26) was active over a broad range (pH 4.6–10.8).

When molecular weight markers for native gels were used, P1 and P2 were 54 and 50 kDa, respectively. P1 and P2 appear to be similar in size to proteases from *E. chrysanthemi* strains HP3 (31), HP1 (32), and EC16 (4) and from *E. c. carotovora* strain EC14 (24), which range from 50 to 55 kDa.

Further study is required to characterize the proteases of *X. fastidiosa* and their role in disease development or virulence of the bacterium. The production and role of a lysophospholipase and a phospholipase (8) by this bacterium also need to be investigated further. It will be a challenge to determine the ultimate role of these factors in the biology of *X. fastidiosa*, since this bacterium is limited to the xylem.

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