Protease Production During Pathogenesis of Bacterial Leaf Spot of Alfalfa and by Xanthomonas alfalfae In Vitro

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ABSTRACT

Xanthomonas alfalfae produced an extracellular protease which liberated Congo Red from Congocoll, hydrolyzed gelatin, and clotted milk. The enzyme was most active at pH 8.0. Dialysis and EDTA reduced activity. Co^{++}, Zn^{++}, Mn^{++}, and Fe^{++} increased protease activity, whereas Hg^{++}, Fe^{+++}, Cu^{++}, cyanide, and mercaptoethanol reduced it.

Protease activity was about 20 times greater in extracts from susceptible inoculated plants than in extracts from noninoculated control plants. Broths prepared from susceptible and resistant alfalfa plants supported nearly equal bacterial growth, but protease activity was greater in broth from the susceptible plants. Phytopathology 61:361-365.

Bacterial leaf spot of alfalfa (*Medicago sativa* L.) caused by *Xanthomonas alfalfae* (Riker, Jones, & Davis) Dows. is characterized by postemergence damping-off, marked stunting of seedlings, spotting and blighting of leaves and stems, severe defoliation, and (in severe cases) death of mature plants (25, 27). Histopathological studies (7) revealed that *X. alfalfae* spreads inter- and intracellularly within hypocotyls, cotyledons, leaves, and stems. No evidence of pectolytic enzyme activity was found either in culture media supporting growth of *X. alfalfae* or in extracts from alfalfa plants with severe bacterial leaf spot (24).

In view of the occurrence of protein in the middle lamellae and cell walls (10, 19, 22) of higher plants, proteolytic enzymes could have a role in tissue maceration.

Several microorganisms produce proteases in culture (5, 12), but only a few plant pathogens are known to be proteolytic. In the original description of *X. alfalfae*, Riker et al. (25) reported that it liquefied gelatin and was caseinolytic. Keen et al. (15, 16) reported that *Pseudomonas lachrymans* produced caseinolytic protease in culture and during pathogenesis in cucumber. Increased protease activity was also reported in tobacco tissue infected with *Agrobacterium tumefaciens* (9). Protease activity on gelatin was obtained from several species of *Pseudomonas* (12), and Friedman (8) postulated that virulence of *Erwinia carotovora* could be attributed to increased proteolytic activity. This paper deals with some characteristics of a protease produced by *X. alfalfae* and protease activity in alfalfa during pathogenesis. An abstract of this work was published earlier (23).

Materials and Methods.—Two alfalfa clones were used. They were selected from an observation nursery on the basis of their reaction to *X. alfalfae* under severe natural infection. Clone 22-54 was considered resistant, as symptoms were restricted to a few small lesions on stems and leaves. Clone 22-102 was considered susceptible, as lesions were large, often spreading several cm on stems or until they coalesced; defoliation was severe. Cuttings from these plants were rooted in sand, potted in soil, and maintained in the greenhouse.

Culture media used were Starr's medium (26) supplemented with 0.5 g/liter of yeast extract (Difco) and alfalfa broths. Broths were prepared from healthy plants of the alfalfa clones by homogenization for 2 min in a blender with 10 parts water (w/v). The homogenate was filtered through four layers of cheesecloth, autoclaved for 5 min at 15 psi pressure, and refiltered through cheesecloth. All media were adjusted to pH 6.8 with 0.1 N NaOH or 0.1 N HCl, dispensed in 250-ml Erlenmeyer flasks at 100 ml/flask and sterilized by autoclaving for 20 min at 15 psi pressure.

Each flask was seeded with 1 ml of culture medium (Starr's medium with 48 hr of bacterial growth) and was incubated 48 hr at 22 to 24 C on a rotary shaker. Isolate KX-1 of *X. alfalfae* was used. Alfalfa plants were inoculated with cultures grown 48 hr in Starr's medium and diluted to 0.2 *A* at 620 mμ (ca. 4×10^8 viable cells/ml). Inoculum was sprayed onto plants until runoff with a No. 152 DeVilbiss atomizer. Inoculated plants were kept in a moist chamber at 22 to 25 C for 48 hr, then transferred to a growth chamber at 28 C with 500 ft-c of continuous fluorescent lighting.

For enzyme preparations, infected shoots were harvested 4 (early) and 8 (advanced stage of disease development) days after inoculation. They were homogenized in a blender for 2 min with 2 parts (w/v) of water. A few drops of Antifoam (Antifoam A, Dow Corning Corp., Midland, Mich.) were used to prevent foaming. The homogenate was strained through four layers of cheesecloth and then centrifuged for 30 min at 6,780 *g*. The supernatants were dialyzed at 4 C for 20 hr against distilled deionized water, and assayed or stored at 4 C. Toluene or 1:1,000 Merthiolate (Lilly)

in water was added as a preservative. These were termed "plant extracts".

Enzyme preparations were made from culture media in the following manner. After the 48-hr incubation period at 22 to 24 C, the culture media were centrifuged at 2 C and 27,000 *g* for 40 min to remove bacterial cells. The supernatants were dialyzed and assayed or stored as described above. These were termed "culture supernatants". Plant and culture supernatants were assayed for protease activity within 1 day after preparation.

Protease activity was assayed by four techniques, each employing a different substrate. The standard casein method of Kunitz (18) was used most often. A 1% casein (Hammersten quality, Nutritional Biochemicals Corp., Cleveland, Ohio) solution was prepared in 0.1 M Tris[tris (hydroxymethyl) amino methane]-HCl buffer (pH 8.5) (11), heat-denatured at 100 C for 15 min in a water bath, cooled, and used as substrate. One ml of enzyme preparation was added to 1.0 ml of substrate, mixed thoroughly, and incubated in a water bath at 35 C. Three ml of 10% trichloroacetic acid (TCA) in water (w/v) were added to the reactants after 40 min of incubation, and the contents mixed thoroughly and allowed to stand 30 min to allow the protein to precipitate. The supernatants containing TCA-soluble peptides were separated from precipitates by centrifugation for 15 min at 6,780 *g*. Absorbancy of the supernatant was measured at 280 mμ with a Beckman DB spectrophotometer against a blank prepared at 0 time. The data were expressed as arbitrary protease units where 1 unit was defined as that amt of enzyme that caused an increase of 0.02 *A*.

The second assay for protease activity was described by Nelson et al. (21), who used Congo Red hide powder (Congocoll, Calbiochem, Los Angeles, Calif.) as the substrate. The substrate was prepared in 0.1 M Tris-HCl buffer (pH 8.5) at 5 mg/ml. Eight ml of culture supernatant were mixed with 40 ml of substrate and incubated at 35 C. Five-ml portions were withdrawn periodically and filtered through lens paper. Change of absorbancy due to the release of Congo Red was determined by using a B & L Spectronic-20 spectrophotometer at 495 mμ against a blank. The blank was prepared as stated above, except that the culture supernatant was autoclaved 20 min at 15 psi pressure before adding to the substrate.

Protease activity was assayed viscosimetrically, using 10% gelatin (Difco) (w/v) in water as the substrate (20). Substrate and culture supernatant were adjusted to pH 8.5 with 0.1 N NaOH and maintained at 40 C. Three ml of each were transferred to an Ostwald-Fenske viscosity pipette (size 300) maintained at 40 C in a water bath. The reactants were mixed thoroughly, and efflux times were determined immediately (0 time) and periodically thereafter. Decrease in efflux times during incubation was attributed to protease activity. As controls, dialyzed and nondialyzed culture supernatants were autoclaved 15 min at 15 psi pressure before assaying.

Protease activity of *X. alfalfae* also was assayed by determining the time required for clotting of milk (1, 6). Dried nonfat milk ('Lucerne', Safeway Stores, Inc., Durand, Wisc.) was suspended in 0.1 M phosphate buffer (pH 5.6) (11) or 0.1 M Tris-HCl buffer (pH 8.0) (11) to 20% (w/v). Four ml of this "reconstituted milk" were mixed with 2 ml of culture supernatant. Merthiolate was added at 2 mg/100 ml to prevent microbial activity. The reaction mixture was incubated at 35 C in a water bath, and the time required for clotting was recorded. Results are expressed as the reciprocal of the clotting time.

The effect of casein concn in the reaction mixture on protease activity was determined by assaying with substrates containing 0.5, 1, 2, and 3% casein in 0.1 M Tris-HCl buffer (pH 8.5).

The effects of pH on protease activity were determined by assaying for enzyme activity in reaction mixtures of 1% casein in 0.1 M Tris-HCl buffers at pH 7 to 10. Due to isoelectric precipitation of casein, pH values below 7 were not included.

The stability of protease in storage at 4 and 22 to 24 C was determined. Portions of culture supernatants were removed periodically, and their protease activity was determined.

The effect of certain chemical compounds that have activated or inhibited protease activity was determined on *X. alfalfae* protease by using these compounds in the reaction mixture at 0.0025 M final concn. The reaction mixture contained 0.5 ml (0.01 M) activator or inhibitor, 0.5 ml dialyzed culture supernatant, and 1.0 ml of 1% (w/v) casein in 0.1 M Tris-HCl buffer (pH 8.5). Protease activity was determined by the standard casein method (18).

RESULTS.—*Xanthomonas alfalfae* produced protease in Starr's medium which was proteolytic to casein, Congocoll, and gelatin (Fig. 1). Protease production was closely related to bacterial growth (Fig. 1-A). *Xanthomonas alfalfae* produced more growth and more protease in Starr's medium than in the alfalfa broths (Table 1), but protease production relative to growth was greater in the broths. Broths from susceptible and resistant plants produced nearly equal bacterial growth, but protease activity, like protease units and milk clot-

TABLE 1. Bacterial growth and proteolysis of casein and clotting of milk by protease produced by *Xanthomonas alfalfae* in Starr's medium and broths from alfalfa plants

Medium	Growth at 48 hr as *A* at 620 mμ	Protease units[a]	Clotting units[b]	
			pH 5.6	pH 8.0
Starr's medium	2.34	14.0	10.00	16.60
Alfalfa broth from:				
Resistant clone (22-54)	0.68	9.6	3.80	4.60
Susceptible clone (22-102)	0.67	11.5	10.00	25.00

[a] One unit is arbitrarily defined as the amount of enzyme that releases trichloroacetic acid-solube peptides from casein to increase 0.02 *A* at 280 mμ.

[b] The reciprocal of the time in hr required for clotting milk. (×100)

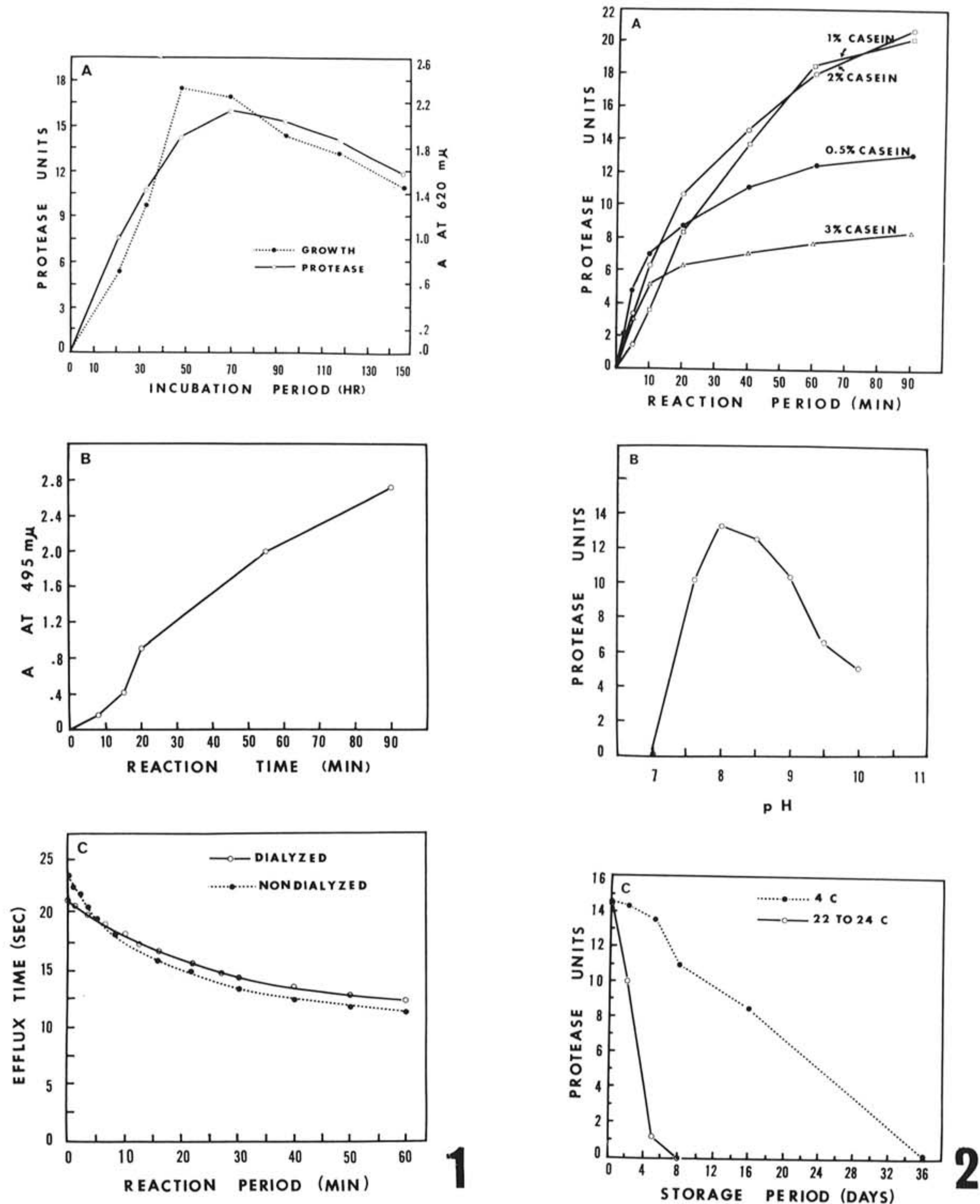

Fig. 1-2. A) Growth of *Xanthomonas alfalfae* (as absorbance) and protease production in Starr's medium. One protease unit = 0.02 $\triangle A$ at 280 mμ using the standard casein method. **B)** Increase in absorbance of reaction mixture with Congocoll. **C)** Protease activity in culture supernatants assayed viscosimetrically against gelatin. **2)** Effect of **A)** casein concn; **B)** pH of the reaction mixture on the activity of *X. alfalfae* protease; **C)** its stability in culture supernatant during storage.

ting ability, was much greater in broths from susceptible plants. Clotting of milk was always followed by clearing; both occurred earlier at pH 8.0 than at pH 5.6. Autoclaved culture supernatant controls yielded no protease activity in any of the assays.

More TCA-soluble peptides were liberated from 1 and 2% than from 0.5 or 3% casein solutions (Fig. 2-A). Protease activity was greatest between pH 7.5 and 8.5, and decreased rapidly outside this range (Fig. 2-B). Protease in culture supernatant was not stable in storage (Fig. 2-C); activity was lost by 8 days at 22 to 24 C and by 36 days at 4 C.

Dialysis during preparation of culture supernatant reduced protease activity about 26% (Table 2). Protease activity was further reduced by EDTA, $HgCl_2$, $FeCl_3$, NaCN, $CuSO_4$, and mercaptoethanol. But $CaCl_2$, $CoCl_2$, $ZnSO_4$, $MnCl_2$, and $FeSO_4$ not only restored the activity loss due to dialysis but in some cases stimulated it. Highest activity occurred with $FeSO_4$.

Protease activity increased during bacterial leaf spot development (Fig. 3). At 8 days after inoculation, protease activity in susceptible plants was more than 2-fold that in the resistant ones and about 20-fold that in the noninoculated control plants. Resistant and susceptible plants had small, water-soaked areas on the leaves and stems at 4 days after inoculation. In susceptible plants, these lesions continued to enlarge in size, while in resistant plants the lesions increased very little in size after 4 days.

Due to the abnormally large inoculum load we used, disease severity was greater on the resistant clone than we observed in the field. On the other hand, disease severity on the susceptible clone was not nearly as great 8 days after inoculation as we noted on naturally infected plants.

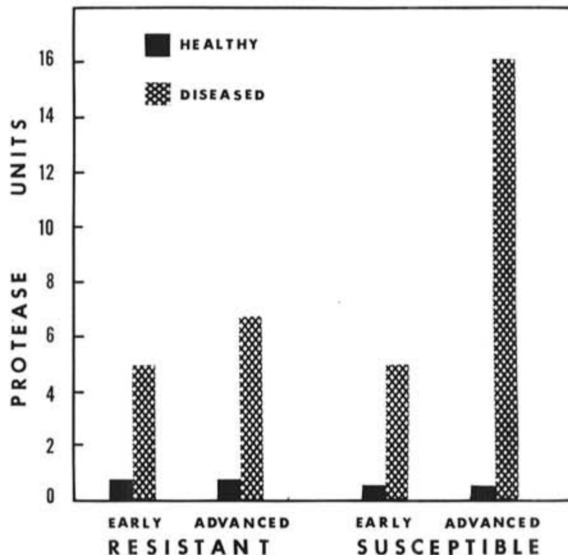

Fig. 3. Protease activity in extracts from healthy and diseased, resistant (22-54), and susceptible (22-102) alfalfa clones. Infected material was harvested at 4 (early) and 8 (advanced stage of disease development) days after inoculation with *Xanthomonas alfalfae*.

TABLE 2. Effect of various chemical compounds on the activity of *Xanthomonas alfalfae* protease[a]

Chemical compound	Protease units
EDTA	3.80
$CaCl_2$	7.65
$CoCl_2$	8.50
$ZnSO_4$	8.90
$MnCl_2$	9.90
$FeSO_4$	12.55
$HgCl_2$	4.10
$FeCl_3$	4.35
$CuSO_4$	4.90
NaCN	4.90
Mercaptoethanol	5.10
Water (control)	6.25
Water + nondialyzed enzyme preparation[b]	8.40

[a] Reaction mixture contained 1.0 ml of 1% (w/v) casein in 0.1 M Tris-HCl buffer (pH 8.5), 0.5 ml of dialyzed culture supernatant and 0.5 ml of a 0.01 M solution of the specified chemical compound.

[b] Same as culture supernatant except that the enzyme preparation was not dialyzed.

DISCUSSION.—*Xanthomonas alfalfae* protease was active on all proteins tested. It liberated TCA-soluble peptides when incubated with casein, released Congo Red from Congocoll, liquefied gelatin, and clotted milk. Riker et al. (25) used some of these activities as criteria for characterizing *X. alfalfae*.

Xanthomonas alfalfae protease was most active at pH 8 to 8.5. Similar results were reported for other microbial proteases (12).

The inhibition of proteolytic activity by dialysis and EDTA suggests that the *X. alfalfae* protease may require a metal ion activator. The requirement of a specific metallic cofactor appears unlikely, since several compounds restored the lost activity. Among these, Fe^{++} and Mn^{++} were more effective than Zn^{++}, Co^{++}, and Ca^{++}. Inactivation of the protease by EDTA and reactivation by Co^{++}, Zn^{++}, Mn^{++}, and Ca^{++} also was reported by others (2, 3, 12). Fe^{+++} and Cu^{++} ions reduced the activity of *X. alfalfae* protease. Fe^{+++} also inhibited the protease activity of *Phymatotrichum omnivorum* (3) but not *Pseudomonas lachrymans* (14). The reduction of *X. alfalfae* protease activity by NaCN and mercaptoethanol may be due to the chelating activity of these compounds (2, 4).

Keen et al. (14) found that results of an earlier study (15) on the activity of *P. lachrymans* protease were confounded by a lipomucopolysaccharide fraction from the culture filtrates and bacterial cells which produced TCA-soluble materials in casein and some other proteins. In our studies, we found no evidence of this material which withstood autoclaving and dialysis but was not active against Congocoll or gelatin and did not clot milk.

Increased protease activity has been associated with other bacterial (9, 16) and fungal (17, 28) diseases of plants. Friedman (8) reported that virulent strains of *Erwinia carotovora* yielded more protease than did

avirulent strains. Hancock & Millar (13) found that *Ascochyta imperfecta, Colletotrichum trifolii,* and *Stemphylium botryosum* produced caseinolytic protease on autoclaved healthy alfalfa shoots. But, protease activity increased during disease development only in the plants infected with *C. trifolii* or *S. botryosum.* They (13) also found small amounts of protease in healthy alfalfa plants.

We believe that *X. alfalfae* protease could play a role during pathogenesis, particularly in intercellular movement, as the bacterium did not produce any pectolytic enzymes (24).

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