PROTEOLYTIC ENZYMES PRODUCED BY PHYTOPATHOGENS IN VITRO*

TSUNG-CHE TSENG and SHIH-LO LEE**

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A role for enzymes of pathogen origin in pathogenesis of infected plant tissues was suggested by the early studies of de Bary (1886). Studied by Jones (1909) and Brown (1915, 1917) during the early part of this century clearly indicated that pectic enzymes were involved tissue degradation in soft rot diseases caused by bacteria and fungi. At the present, the involvement of enzymes of pathogen origin in pathological processes is an accepted fact. A great deal of information concerning to the role in pathogenesis of pectinolytic enzymes produced by microorganisms is available (Bateman and Millar 1966), but very little work has been done on either *in vitro* or *in vivo* production and role of proteolytic enzyme by plant pathogenic organisms. The purpose of this investigation was to determine the ability of selected phytopathogens to produce proteolytic enzymes.

The studies were made with three isolates of *Rhizoctonia solani* Kühn (R_2, RB, and RB_2), two isolates of *Erwinia carotovora* (Jones) Holland (EC_{14} and EC_{32}), and one isolate each of *Botrytis cinerea* Person (BC), *Fusarium solani* (Mart.) Appel Wr. f. sp. *phaseoli* (Burk.) Snyd. and Hans. (FB). All cultures were grown on potato-dextrose agar (PDA) at 23 C and transfers were made at one month intervals. Production of proteolytic enzymes by the above organisms was examined by growing isolates on an autoclaved bean (*Phaseolus vulgaris* var. 'Red Kidney') hypocotyls. Healthy bean hypocotyls were obtained from plants grown in steamed soil in growth chambers at 29–30 C for 7 days. The medium for proteolytic enzyme productions was prepared by autoclaving 50 g of bean hypocotyls with 20 ml of distilled water in 1 liter Erlenmeyer flasks for 30 min at 121 C. Sterilized medium was seeded with a week-old cultures of a given organism and incubated at 28±2C for 10 days.

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** Assistant Research Fellow and Research Assistsnt of the Institute of Botany, Academia Sinica, Nankang, Taipei, Taiwan, China.

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Cultures grown on the bean hypocotyl medium were harvested by grinding the flask contents with 50 ml of distilled water in a Virtis '45' homogenizer for one minute at high speed. The extracts were filtered through 4 layers of cheesecloth to remove solid material and then centrifuged at 20,000 g for 15 min at 5 C. The supernatant fluids were lyophilized and stored at -20°C in deep freezer until used.

Proteolytic activity was estimated by 'cup plate' method described by Doery *et al.* (1965). The substrate of gelatin (South America Minerals & Merchandis Corp.) was prepared by blending 1.2 g of the gelatin with 100 ml of 0.1 M citrate or phosphate buffer which contained 0.01% Thiomersalate (Eli Lilly Co.) in a Virtis '45' homogenizer at full speed for 10 min. The homogenizer vessel was kept in an ice bath to prevent overheating. 'Cup plates' were prepared by adding 50 ml of 2.4% gelatin in 0.02% Thiomersalate to 50 ml of 4% melted agar in 0.1 M of citrate or phosphate buffer. Then the pH was checked with a pH meter and adjusted to the exact desired pH value with 0.1 N of NaOH or HCl. This mixture was poured into 9 cm (dia.) Petri dishes to give a 5 mm layer of substrate which was allowed to solidify at room temperature. Wells were made in the solified plates with a No. 5 cork borer (10 mm dia.); the bottoms of the wells were sealed by adding 2 drops of liquid agar. The lyophilized culture extracts were reconstituted in distilled water and added to the wells of 'cup plates' at the rate of 0.2 ml/ well. 'Cup plates' were incubated at 30 C for 24 hr and then the clear zone of substrate hydrolysis was measured by treatment with 20% trichloroacetic acid (TCA). Proteolytic activity was indicated by development of a clear zone around the wells containing active enzyme; enzyme activity was expressed as the area hydrolysis per mg of protein and autoclaved enzymes were served as controls. The protein content of the enzyme preparation was determined according to the procedure of Lowry *et al.* (1951). Crystalline bovine serum albumin was used as a protein standard.

The lyophilized culture extracts of all tested phytopathogens exhibited proteolytic activity in the 'cup plate' assay. When the various enzyme sources were assayed over the pH range from 3.0 through 8.5 at 0.5 unit intervals, it was observed that the pH optimum was various for the different enzyme sources, and also no proteolytic activity was found in extremely acid range (Table 1). The optima for the proteolytic enzymes of the three isolates of *R. solani*, *B. cinerea* and *F. solani* exhibited the enzyme activities over the pH range 5.0 to 8.5 whereas the enzyme(s) from *E. carotovora* was active from 5.5 to 8.5. The *R. solani* enzymes generally were more active at neutrality but enzymes from *B. cinerea* were most active at pH 8.5.

This study revealed that the ability to produce proteolytic enzymes by

Table 1. *Effect of pH on the proteolytic activity of lyophilized extracts of phytopathogens*[a]

Pathogens	Activity (mm^2)[b]/mg protein											
	pH											
	3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5
Botrytis cinerea	0.0	0.0	0.0	0.0	60.0	85.3	124.4	178.1	136.0	87.4	129.0	308.7
Fusarium solani f. sp. *phaseoli*	0.0	0.0	0.0	0.0	171.1	155.8	168.5	205.8	195.3	188.5	180.1	151.1
Rhizoctoni soldni (R_2)	0.0	0.0	0.0	0.0	91.3	106.9	115.8	167.1	174.7	109.4	146.8	103.2
Rhizoctoni solani (RB)	0.0	0.0	0.0	0.0	115.1	158.0	178.2	214.8	214.8	125.8	172.1	182.4
Rhizoctoni solani (RB_2)	0.0	0.0	0.0	0.0	137.2	168.5	195.4	247.0	295.6	206.2	236.5	206.2
Erwinia carotovora (EC_{14})	0.0	0.0	0.0	0.0	0.0	110.1	118.5	157.5	211.1	169.3	176.4	193.4
Erwinia carotovora (EC_{32})	0.0	0.0	0.0	0.0	0.0	41.1	86.1	78.0	99.6	96.2	104.9	121.1

a All cultures were grown on an autoclaved bean hypocotyl medium for 10 days at 28±2 C.

b Assays were made using the 'cup plates' which contained 1.2% gelatin, 0,01% Thiomersalate, 2% agar, and 0,1 M buffer (citrate, pH 3.0-7.0 or phosphate, pH 7.5-8,5). Enzyme activity was measured as the area of the clear zones around 10 mm diameter wells, containing the reconstituted enzyme at the rate of 0.2 ml/well, made in a 5 mm thick later of substrate after 24 hr incubation at 30 C.

phytopathogens is probably widespread. Also, the quantity and types of proteolytic enzymes produced by different pathogens differ considerably. Since the selected phytopathogenic organisms produced considerable amount of these enzymes thus they should serve as good sources of proteolytic enzymes for future studies.

It is suprising that a paucity of information exists in plant pathological literature concerning proteolytic enzymes which may have a potential importance in plant pathogenesis and would appear to have in disrupting host metabolism and initiating disease processes. Kúc and Williams (1962), working on the rots of apple fruit caused by *Botryosphaeria ribis* Gross. & Dug., *Glomerella cingulata* (Ston.) Spauld. & Schrenk, *Physalospora obtusa* (Schw.) Cke., and a *Physalospora* sp., detected endopeptidase activity in extracts of the lesions produced by these pathogens. The enzyme was proposed to be involved in the degradation of the protein associated with the apple fruit middle lamella. Van Etten and Bateman (1965) have indicated that *Rhizoctoni solani* produced an inducible extracellular protease *in vitro,* the proteolytic activity was detected in healthy and in *R. solani*-infected red kidney bean tissues, but the activity was much greater from the disease tissue. The role of the proteolytic activity was not established. Keen's studies (1966) on *Pseudomonas lachrymans* revealed the participation of a proteolytic enzyme in a disease process. In his study, partially purified *P. lachrymans* protease, trypsin or pronase injected into cucumber leaves caused a vein-limited water soaking which was similar to the symptoms of the angular leaf spot diseases. Klement and Goodman (1967) have postulated that the hypersensitive reaction induced by bacteria results from a drastic change in permeability of host cell membranes. They have suggested that such changes may result from the action of proteases and the disruption of S-S bonds in the protein components of membranes. The role of proteolytic enzymes in plant pathogenesis is still abscure. A current need is a supply of the purified enzymes from the tested organisms that can be used for more detailed studies of the enzyme in relation to their hosts during the plant pathogenesis.

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