Purification of a Protease Secreted by Colletotrichum lindemuthianum

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ABSTRACT

Colletotrichum lindemuthianum secretes a proteolytic enzyme into culture medium when grown on plant cell walls or on an artificial medium of salts supplemented with collagen as the nitrogen source and glucose as the carbon source. Protease activity has been assayed using a collagen-red dye compound (Azocoll) as substrate. Maximal extracellular protease activity on the glucose-collagen medium occurs after 7 days growth. The protease has been purified from extracellular proteins by $(NH_4)_2SO_4$ fractionation, ion-exchange chromatography on sulphoethyl Sephadex, and by passage through a polyacrylamide sizing column. The protease has a molecular weight of 25,000 as determined by gel filtration, exhibits optimum activity at pH 8.6, and is stable for several months at 0 C. This is the first reported proteolytic enzyme from a plant pathogen that has been extensively purified and characterized.

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Additional key words: pathogenic fungi, extracellular enzymes.

The plant cell wall is a complex structure composed of cellulose, hemicellulose, pectic substances, and proteinaceous compounds (1). Plant pathogens have been shown to secrete enzymes capable of degrading the polysaccharides of plant cell walls (3). It therefore seems possible that plant pathogens would also secrete enzymes capable of degrading the protein present in the plant cell wall (13). Early investigators, using a trichloroacetic acid precipitation assay, demonstrated the secretion of proteolytic enzymes by several plant pathogenic organisms (9, 10, 11, 16). The authenticity of these early findings was questioned when Keen et al. (8) discovered that the assay used in these early studies could lead to erroneous conclusions because the assay measures not only protease activity but also the non-enzymatic solubilization of protein. Recently, Mussell & Strouse (15) reported that *Verticillium albo-atrum* secretes a proteolytic enzyme, but these workers did not describe the assay used nor did they attempt purification of the protease.

The present report describes the purification and some properties of a proteolytic enzyme secreted by *Colletotrichum lindemuthianum.*

MATERIALS AND METHODS.—*Culture.*—The α-strain of *Colletotrichum lindemuthianum* was cultured as reported (4). The protease secreted by this fungus was found to be induced by growing the organism in shake cultures on minimal salts medium lacking KNO_3 and supplemented with glucose and collagen. Maximum extracellular protease activity was found when the medium contained 5% glucose and 0.5% collagen. Collagen was chosen as the sole nitrogen source because it is a structural protein rich in the amino acid L-hydroxyproline and therefore has some similarity to the structural protein present in plant cell walls (13). A standard culture contained 1 liter of medium in a 2.8-liter Fernbach flask. The cultures were inoculated with 10^6 spores and were incubated at 23 C on a shaker rotating at 60-70 rpm.

Assays.—Protease activity was assayed using Azocoll (Calbiochem) as substrate. Azocoll is powdered collagen to which a red dye is attached. As the peptide linkages of the collagen are hydrolyzed, the bound dye is solubilized. A typical reaction contained 1 ml of enzyme solution and 2 ml of 0.5% Azocoll suspended in 100 mM glycine-NaOH buffer at pH 8.6. A standard reaction was incubated for 60 min at 37 C with constant stirring to maintain the substrate in suspension. The reaction was terminated by filtering through GF/A glass fiber filter paper to remove undegraded substrate. The concentration of solubilized red dye was determined by measuring the optical density at 520 nm with a Gilford Model spectrophotometer. Enzyme activity is expressed as arbitrary units of substrate hydrolyzed. One unit of protease activity has been equated to the solubilization of that amount of red dye which results in an absorbance change of 1.0 at 520 nm under the assay conditions described above. To assure linearity, enzyme solutions were diluted before assay so that each reaction contained no more than 0.8 units of protease activity. Control reactions were carried out as described above except that the enzyme preparation was preheated to 70 C for 5 min and the absorbance of controls was subtracted from enzyme reaction mixtures.

The activity of the protease was also measured using 10 mM benzoyl L-arginine ethyl ester, a trypsin substrate. Reactions were incubated in 46 mM sodium phosphate buffer at pH 7.6 and the reaction rate monitored at 253 nm (18).

Protein concentrations were determined by the method of Lowry et al. (14). Protein concentrations were calculated relative to a standard prepared from crystallized bovine plasma albumin (Armour Pharmaceutical).

Endopolygalacturonase was assayed by the method of English et al. (5).

All purification steps were performed at 4 C. The

standard buffer used in purification was 10 mM sodium phosphate, pH 6.3. This solution is referred to in this manuscript as 'Buffer'. The protease is more stable and binds better to ion-exchange resins in Buffer than under the conditions used in the protease assay, 100 mM glycine-NaOH, pH 8.6.

RESULTS.–Experiments were carried out to maximize protease yield by varying the carbon source and its concentration. Pectin, collagen, hydroxyproline, and glucose were tested as carbon sources. Media containing 5% glucose and 0.5% collagen increased protease secretion several fold over media containing 0.5% collagen, 0.5% collagen plus 0.25% hydroxyproline, or 0.5% collagen plus 1% pectin. The data in Figure 1 shows the effect of varying the glucose concentration on the level of protease secretion when the collagen concentration is held constant at 0.5%. A glucose concentration of 5% stimulates maximal protease activity in the extracellular medium after 7 days of incubation (Fig. 1). The optimum time to harvest for maximal enzyme activity is of brief duration as the protease activity quickly rises and then rapidly declines (Fig. 1).

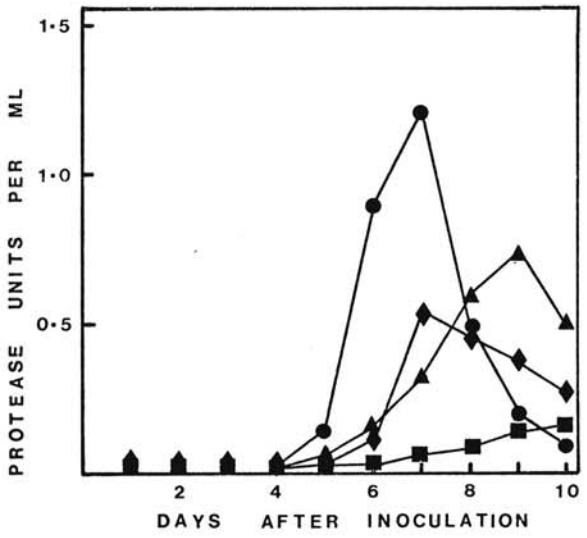

Fig. 1. The hydrolysis of Azocoll by an enzyme in the medium of *Colletotrichum lindemuthianum* cultures was determined as a function of culture age and as a function of culture medium glucose concentration. Each of a series of 100 ml cultures containing either 1% (▲), 5% (●), 10% (♦), or 20% (■) glucose was inoculated with 1×10^5 spores and the cultures grown as described in MATERIALS AND METHODS.

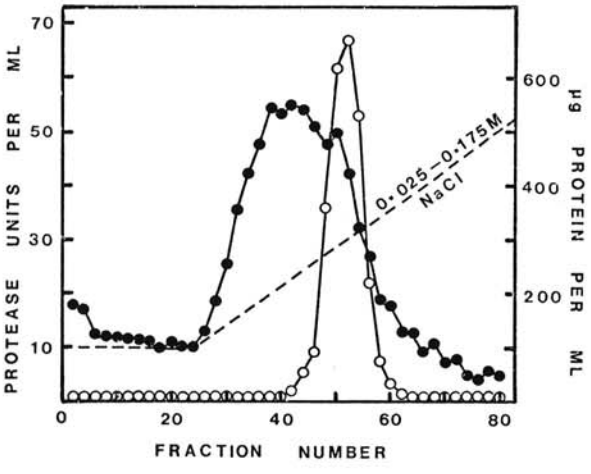

Fig. 2. Purification of protease by ion-exchange chromatography on sulphoethyl-Sephadex. The protease (○), protein (●), and salt gradient (dashed line) are plotted versus fraction number. This step in the purification procedure follows $(NH_4)_2SO_4$ fractionation.

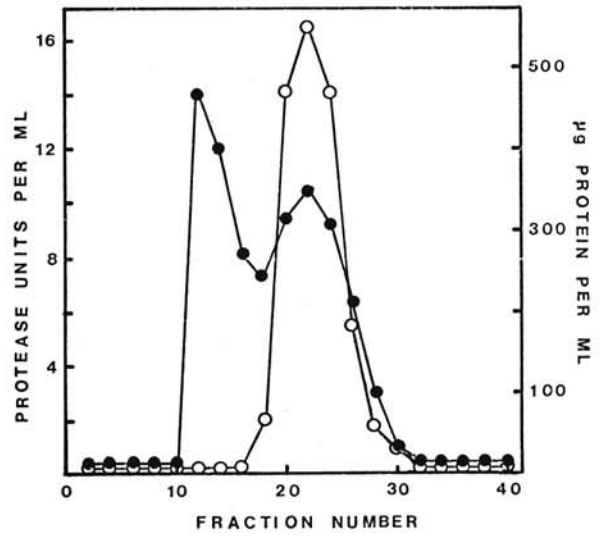

Fig. 3. Gel filtration (Bio-Gel P-150) of the protease (○) to separate this enzyme from larger molecular weight proteins (●). This step in the purification follows elution of the enzyme from sulphoethyl-Sephadex. Fractions 18-30 were combined and used as purified protease.

Similar growth experiments in which the collagen concentration was varied while the glucose concentration was held constant at 5% indicated that maximal enzyme activity occurs when the collagen concentration is between 0.5 and 2.0%. Therefore, the growth medium was supplemented with 0.5% collagen.

When 'Pinto' bean cell walls, prepared by the method of English et al. (4), were used as the carbon source for *C. lindemuthianum* growth, and the activities in the culture medium of both the protease and endopolygalacturonase (5) were assayed with time, it was observed that maximal protease activity occurred approximately 24 hr after maximal endopolygalacturonase activity. However, the culture age at which the activity of these enzymes in the culture medium is at a maximum appears to be variable.

Purification of the protease.–Cultures were

TABLE 1. Summary purification of *Colletotrichum lindemuthianum* protease

Fraction	Activity (units/ml)[a]	Volume (ml)	Total activity (units)[a]	Yield (%)	Protein (μg/ml)	Specific activity (units/μg)[a]	Purification (relative)
Crude	1.5	1,800	2,700	100	3,430	0.00044	1.0
$(NH_4)_2SO_4$	2.5	1,000	2,500	92	892	0.00028	6.4
SE-Sephadex	11.7	58	680	25	158	0.074	168.0
Bio-Gel P-150	12.6	41	517	19	33	0.38	864.0

[a] One unit of enzyme activity equals a change in absorbance of 1.0 at 520 nm/ml per hr at 37 C when the substrate is 2 ml of 0.5% Azocoll and the reaction is carried out in 3 ml of 100 mM glycine-NaOH, pH 8.6.

harvested after 6 to 7 days growth by filtering the culture fluid through GF/A glass fiber filter paper. The mycelium and that portion of the collagen remaining insoluble were discarded and the filtrate was termed "crude" protease. The filtrate (2 liters) was cooled, adjusted to 30% of saturation with $(NH_4)_2SO_4$, and stirred for 30 min. The resulting suspension was centrifuged at 13,300 *g* for 10 min and the pellet was discarded. Further ammonium sulfate fractionation of the supernatant solution yielded the proteins insoluble in 30-50% $(NH_4)_2SO_4$. This fraction was dissolved in 1 liter of Buffer and dialyzed for 36 hr against Buffer. The nondialyzable material was passed through a sulphoethyl-Sephadex column (2.5 x 25 cm) that had been equilibrated with Buffer. The protease was thus bound to the column. The column was then washed with 300 ml of Buffer, and then with 300 ml of 25 mM NaCl in Buffer. Up to this point, all the material passing through the sulphoethyl-Sephadex column was discarded. Finally, the column was subjected to gradient elution by mixing in linear fashion 200 ml of Buffer containing 25 mM NaCl and 200 ml of Buffer containing 400 mM NaCl. The protease and protein concentrations in each of 2.8 ml fractions collected were assayed (Fig. 2). Fractions 46-60, which contained protease activity, were combined and dialyzed against Buffer for 36 hr. To concentrate the protease, the non-dialyzable material was lyophilized and the residue taken up in 2 ml of Buffer. Concentrated protease was then chromatographed on a Bio-Gel P-150 column (1.7 X 110 cm, void volume = 43 ml). This sizing column was prepared as described by Sachs & Painter (17). Such a "bead column" greatly shortened fractionation time and resulted in well defined peaks. Fractions (4.1 ml) were collected and assayed for both protein and protease (Fig. 3). The protease eluted from the column at 1.83 void volumes. This elution volume indicates that the protease has a molecular weight of about 25,000 provided that the enzyme behaves as a normal globular protein. Fractions 18-30, which contained the protease activity, were combined, dialyzed against Buffer, concentrated by freeze-drying, taken up in 2 ml of Buffer, and frozen. This preparation was termed "purified protease". Further efforts at greater purification were attempted on Sephadex G-100 columns, but the yield was sharply reduced (<1%).

The data of Table 1 summarizes the purification of the proteolytic enzyme secreted by *C. lindemuthianum*. The enzyme has been purified 864-fold from those proteins present in the extracellular medium of *C. lindemuthianum* cultures. After four purification steps, the yield was 19%.

Characterization of the purified protease.—The protease is completely denatured by heating at 70 C for 5 min.

The results presented in Fig. 4 demonstrate that optimal activity of the enzyme in glycine-NaOH buffer is about pH 8.6. The protease demonstrates no activity at or below pH 6.0.

Various known inhibitors of proteolytic enzymes were used in an attempt to inhibit this protease. Trypsin inhibitor was ineffective at reducing protease activity. Recently, Green & Ryan (6) demonstrated

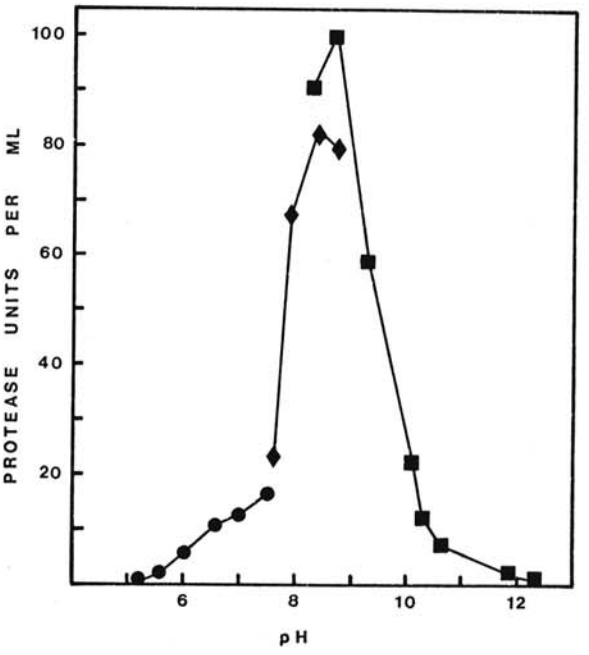

Fig. 4. Effect of hydrogen ion concentration on the hydrolysis of Azocoll by purified protease. The reactions contained 0.8 units of enzyme and were incubated for 60 min at 37 C. The buffers were 100 mM sodium acetate (●), 100 mM sodium phosphate (♦), and 100 mM glycine-NaOH (■).

a protease inhibitor in extracts of mechanically wounded potato or tomato plant leaves. Extracts from mechanically wounded bean plants do not inhibit the *C. lindemuthianum* protease. Similarly, the inhibitor of the C. lindemuthianum endopolygalacturonase (2) had no effect on protease activity.

The purified *C. lindemuthianum* protease was compared to trypsin in its ability to degrade both Azocoll and benzoyl L-arginine ethyl ester. On a specific activity basis, trypsin was 10-fold better than the *C. lindemuthianum* protease at degrading Azocoll and 125-fold more effective at degrading benzoyl L-arginine ethyl ester.

The ability of the protease to degrade other enzymes produced by *C. lindemuthianum* was also examined. When α-galactosidase or α-arabinosidase were pre-incubated with 2 units of protease activity for 1 hr at 30 C and then assayed for their ability to hydrolyze their respective *p*-nitrophenyl glycosides, their activity was unimpaired. Similar experiments demonstrated that the protease had no effect on the activity of the *C. lindemuthianum* endopolygalacturonase. Protease pre-incubated with the 'Red Kidney' bean protein which inhibits the *C. lindemuthianum* endopolygalacturonase (2) did not affect the ability of the inhibitor to inactivate the endopolygalacturonase.

DISCUSSION.—The protease secreted by *C. lindemuthianum* has been purified 864-fold. This is a major purification considering that the protease, as an extracellular protein, is free, even in the most impure preparations, of the normal levels of many of the cytoplasmic proteins. The degree of purification is even more impressive when one considers that the level of this enzyme in the extracellular medium has been increased by growth of the fungus on specific inducing substrates. The purified protease has resisted attempts at further fractionation. The instability of the purified preparation may be due to autodegradation or to rapid denaturation of the protein.

The *C. lindemuthianum* protease degrades both Azocoll and benzoyl L-arginine ethyl ester, but has no effect on the activities of three polysaccharide-degrading enzymes secreted by *C. lindemuthianum*. Thus, this protease appears able to degrade proteins with extended chains, the structure characteristic of collagen. However, the protease appears to be unable to degrade native globular enzymes.

Other degradative enzymes, such as α-galactosidase, β-xylosidase, cellulase, β-glucosidase, α-arabinosidase, and endopolygalacturonase from *C. lindemuthianum* (4, 5), demonstrate good activity at pH 5.2. As is evident from the data of Fig. 4, the pH optimum of the protease is 8.6 with no activity at pH values below 6.0. It is not understood why the protease has a more basic pH optimum than the other degradative enzymes secreted by this pathogen. Mussell & Strouse (15) reported that a protease secreted by *Verticillium albo-atrum* also has a basic pH optimum.

The possible role of this enzyme in pathogenesis is uncertain, but it could serve one of several purposes. When *C. lindemuthianum* (4) or *Fusarium oxysporum* f. sp. *lycopersici* (7) are grown on isolated plant cell walls, endopolygalacturonases are the first detectable enzymes secreted. As was demonstrated above, the protease is secreted soon after the endopolygalacturonase in the *C. lindemuthianum* system. Therefore, one possible use of a protease to the plant pathogen might be to aid the process of infection by selectively degrading the hydroxyproline-rich structural protein present in plant cell walls (13). However, initial observations indicate that the protease is not highly effective in degrading the hydroxyproline-rich protein of sycamore or tomato plant cell walls, even when the walls have been pre-treated with endopolygalacturonase. Acid-stripping, to remove the protecting tetra-arabinosides (12, 13), renders the hydroxyproline-containing wall protein only slightly susceptible to degradation by the *C. lindemuthianum* secreted protease (*unpublished results*). This would indicate that the major function of the protease is not cell wall degradation. Another possible use for such a protease might be to hydrolyze the proteins released after death of the host cells, thereby supplying the pathogen with a source of amino acids. Similarly, the protease might be used while the organism is growing saprophytically in the soil. This use for the enzyme seems plausible because soils are frequently basic, a condition conducive to effective action by this protease.

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