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Purification and Characterization of an Extracellular Acid Proteinase from the Ectomycorrhizal Fungus *Hebeloma crustuliniforme*

HONG ZHU,[1]†* DA-CHENG GUO,[2] AND BRUCE P. DANCIK[1]

Department of Forest Science[1] and Department of Plant Science,[2] University of Alberta, Edmonton, Alberta, Canada T6G 2H1

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***Hebeloma crustuliniforme* produced an extracellular acid proteinase in a liquid medium containing bovine serum albumin as the sole nitrogen source. The proteinase was purified 26-fold with 20% activity recovery and was shown to have a molecular weight of 37,800 (as indicated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and an isoelectric point of 4.8 ± 0.2. The enzyme was most active at 50°C and pH 2.5 against bovine serum albumin and was stable in the absence of substrates at temperatures up to 45°C and pHs between 2.0 and 5.0. Pepstatin A, diazoacetyl-DL-norleucine methylester, metallic ions Fe^{2+} and Fe^{3+}, and phenolic acids severely inhibited the enzyme activity, while antipain, leupeptin, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, and trypsin inhibitor inhibited the activity moderately. The proteinase hydrolyzed bovine serum albumin and cytochrome *c* rapidly compared with casein and azocasein but failed to hydrolyze any of the low-molecular-weight peptide derivatives tested.**

Hebeloma crustuliniforme (Bull. ex St. Am.) Quél. is an ectomycorrhizal fungus associated with a number of tree species in boreal and arctic regions. This fungus, like ectomycorrhizal symbionts in general, benefits trees by increasing their root surface area for absorbing greater levels of nutrients through sheath formation and hyphal emanation to the surrounding soil. Studies have shown that *H. crustuliniforme* utilizes a broad range of nitrogen sources, including ammonium, nitrate (13), amino acids (2), and proteins, and facilitates a rapid transfer of the assimilated protein nitrogen to its tree host (1). The access to protein nitrogen accorded to trees via ectomycorrhizal fungi is of considerable importance because in boreal and arctic forests low temperatures and acidic soil inhibit mineralization and lead to an accumulation of organic matter.

The utilization of exogenous proteins by fungi requires the enzymatic degradation of proteins to peptides and amino acids before cellular uptake. The enzymes involved in this action are mainly extracellular proteinases. A considerable number of fungal species from each of the major taxa are known to produce extracellular proteinases (8). The majority of these have low specificities with regard to the proteins which they degrade and have biochemical properties consistent with aspartic, metallo-, serine, or cysteine proteinases (19). Although there is much information about fungal extracellular proteinases, little is known about the physiological functions and biochemical properties of the proteinases from mycorrhizal fungi. Available data suggest that the ericoid mycorrhizal fungus *Hymenoscyphus ericae* secretes acid proteinases (3), the ectomycorrhizal fungus *Cenococcum geophilum* produces alkaline proteinases (6), and *H. crustuliniforme* produces an acid proteinase (1) under the conditions of induction. Proteolytic activity has also been detected in mineral nutrient medium in which several other ectomycorrhizal fungi grew for 3 weeks (21). These observations, however, were based on biomass growth in protein medium or total proteolytic activity in crude culture filtrate without pure enzyme preparations. Therefore, the function of the extracellular proteinases of mycorrhizal fungi in protein utilization or in other biological processes (e.g., the infection of roots) is hindered. In order to elucidate the precise roles of proteinases produced by this group of fungi, we have conducted a series of studies on the biochemical properties and physiological regulation of extracellular proteinases from ectomycorrhizal fungi. This article reports the purification and characterization of a proteinase produced in vitro by *H. crustuliniforme*.

MATERIALS AND METHODS

Culture and growth conditions. *H. crustuliniforme* UAMH 5640 used throughout this study was obtained from the University of Alberta Microfungus Collection and Herbarium, Edmonton, Alberta, Canada. The strain was collected from a lodgepole pine forest in the Clearwater forest region of Alberta in 1986. A dicaryon mycelial culture isolated from its basidiocarp had been maintained on modified Melin-Norkrans agar (15) with regular transfers at 3-month intervals.

Mycelium plugs 6 mm in diameter cut from margins of 15-day-old colonies on modified Melin-Norkrans agar were transferred to plates (15 by 100 mm) containing 25 ml of liquid medium. The medium consisted of 10 g of D-glucose, 0.76 g of KH_2PO_4, 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of $CaCl_2$, 0.025 g of NaCl, 100 μg of thiamine hydrochloride, 5 μg of biotin, 0.34 g of bovine serum albumin (BSA), and 1 ml of micronutrient mixture (18) in 1 liter of distilled water. The pH of the medium was adjusted to 5.6 with 0.1 M HCl and 0.1 M NaOH. The inoculated plates were incubated at 24°C for up to 30 days.

Purification of proteinase. After 25 days of incubation, culture medium from about 65 plates was pooled and passed first through a no. 4 filter paper (Whatman, Inc.) and then through a micropore filter (Millipore Corp.; pore size, 0.45 μm) to remove mycelium. CM-Sephadex C-50 ion-exchanger (1 g) equilibrated with 0.01 M citric phosphate buffer (pH 3.5) was added to approximately 1 liter of the filtrate. The mixture was gently stirred for 2 h at 4°C, and the

* Corresponding author.
† Present address: Seabright Corporation Ltd., c/o Memorial University of Newfoundland, St. John's, Newfoundland, Canada A1C 5S7.

exchanger was removed by a glass filter. After being washed with excess equilibrating buffer, the exchanger was suspended in 120 ml of the same buffer containing 0.3 M NaCl and stirred for 1 h to elute enzyme activity. The eluate was collected by filtration and dialyzed (molecular weight cutoff, 3,500) exhaustively against 0.01 M citric phosphate buffer (pH 3.5). The dialysate was applied to a column (1 by 10 cm) packed with CM-Sephadex C-50. After being washed with starting buffer (0.01 M citric phosphate buffer, pH 3.5), the enzyme was eluted with a linear gradient of NaCl from 0 to 0.3 M at a flow rate of 25 ml/h. Fractions (1.2 ml each) were collected and measured for protein content and proteolytic activity. Fractions showing activity were pooled, dialyzed against 0.01 M citric phosphate buffer (pH 5), and concentrated against polyethylene glycol 20000 with Spectro membrane tubing (molecular weight cutoff, 3,500). The concentrated sample (about 3 ml) was then applied to a Sephadex G-75 column (2 by 25 cm) which was equilibrated with 0.01 M citric phosphate buffer (pH 5). The enzyme was eluted with the same buffer at a flow rate of 15 ml/h, and fractions (1.5 ml each) were collected and analyzed. Fractions showing proteolytic activity were pooled, dialyzed against 0.01 M citric phosphate buffer (pH 3), and stored at −18°C.

Enzyme assay. Routine proteinase assays were performed according to the fluorimetric method described by Twining (24) with 0.5% fluorescein isothiocyanate-labeled BSA (FITC-BSA) and casein (FITC-casein) as substrates. A reaction mixture containing 10 μl of enzyme solution, 20 μl of substrate, and 30 μl of 0.2 M citric phosphate buffer (pH 3 for FITC-BSA and pH 5 for FITC-casein) in a covered 1.5-ml micro-test tube was incubated at 37°C for 3 h. The reaction was stopped by the addition of 120 μl of 5% trichloroacetic acid, and the reaction mixture was allowed to stand at room temperature for 1 h and sit overnight at 4°C. Precipitated protein was sedimented by centrifugation for 5 min in a microcentrifuge. A 100-μl sample of the supernatant was diluted to 3 ml with 0.5 M Tris hydrochloride buffer (pH 8.5) and measured by using a spectrofluorimeter at an excitation wavelength of 485 nm and an emission wavelength of 525 nm. Proteinase activity was expressed in relative fluorescence units; one unit was arbitrarily defined as the amount of enzyme which produced a reading of 100 under the conditions described above.

Substrate specificity and exopeptidase assay. Substrate specificity was tested by using BSA, cytochrome *c*, casein, and azocasein. A reaction mixture consisting of 0.1 ml of enzyme solution and 0.5 ml of 1% substrate in 0.2 M citric phosphate buffer (pH 3 and 5) was incubated at 37°C for 1 h, and the reaction was stopped by the addition of 0.6 ml of 10% trichloroacetic acid. After centrifugation, the A_{366} was measured for azocasein, the A_{400} was measured for cytochrome *c*, and the A_{280} was measured for BSA and casein. One proteolytic unit was defined as the amount of enzyme which produced a reading of 0.1 under the study conditions.

The aminopeptidase assay was based on the method described by Balasubramanian and Manocha (4). The *p*-nitroanidides, benzoyl-L-arginine-β-naphthylamide, benzoyl-L-tyrosine ethyl ester, benzoyl-L-arginine-*p*-nitroanilide, and *p*-tosyl-L-arginine methyl ester, were dissolved in dimethyl sulfoxide and made up to a final concentration of 1 mM with 0.1 M sodium acetate buffer (pH 3 and 5). An assay mixture consisting of 0.5 ml of substrate and 0.1 ml of enzyme solution was incubated for 1 h at 37°C. The reaction was stopped by the addition of 0.9 ml of 1 M Na_2CO_3. The *p*-nitroaniline formed was measured at 420 nm.

Carboxypeptidase activity was determined by using the method of Ichishima (11) with carbobenzoxy-L-glutanyl-L-tyrosine (CBZ-L-glutanyl-L-tyrosine) and CBZ-L-leucine-*p*-nitrophenyl ester. The substrates were dissolved in dimethyl sulfoxide and made up to a final concentration of 1 mM with 0.1 M sodium acetate buffer (pH 3 and 5). A 0.5-ml portion of substrate was incubated with 0.1 ml of enzyme solution at 37°C for 1 h. The reaction was stopped by the addition of 0.2 ml of 0.3 M NaOH. After incubation for a further 30 min, 0.2 ml of 2.5% acetic acid, 2 ml of 0.05 M sodium citrate (pH 5), and 1 ml of ninhydrin solution (2% wt/vol) were added. The mixture was heated at 100°C for 15 min to develop the color, which was measured at 570 nm.

Protein determination. Protein content was determined by the method of Lowry et al. (14) with BSA as a standard. The concentration of BSA in the culture medium during the fungal growth was determined by the Bradford method (7). Bradford reagent was insensitive to the extracellular proteins produced by the fungus under the conditions of this study.

Electrophoresis. Native polyacrylamide gel electrophoresis (PAGE) was carried out by using the method of Reisfeld et al. (22) to assess the number of proteinases in the culture filtrate. The gels used were 10% polyacrylamide in acetic acid-KOH buffer (pH 4.3) and were run with the same buffer (pH 3.8) at 4°C for 2.5 h with the enzyme migrating towards the cathode. The proteinase was located in the gel by application of an X-ray film directly on the gel for 3 h at 40°C. Once washed, the film showed the presence of proteinase activity where the gelatin had been hydrolyzed.

Sodium dodecyl sulfate (SDS)-PAGE was performed in 12% polyacrylamide gels in Tris hydrochloride buffer (pH 8.9) by using the method of Laemmli (12). Low-molecular-weight standards (14,000 to 93,000; Bio-Rad Laboratories) were run in parallel and used for the molecular weight determination. The gels were stained for protein with 0.2% Coomassie blue R-250 in methanol-acetic acid-water (3:2:11) at room temperature for 30 min and destained in methanol-acetic acid-water (3:1:11).

Polyacrylamide gels for isoelectric focusing were prepared according to the method of O'Farrell (20) with carrier ampholytes (pH 3.5 to 10). The pH was determined in the gel after electrofocusing either by using an electrode or by dividing the gels into small slices and macerating them in distilled water. The positions of proteins in the gels were determined by staining with Coomassie blue R-250.

Effects of pH and temperature on proteinase activity. The optimal pH for proteinase activity was measured over a range of 1.5 to 9 by using KCl-HCl buffer (pH 1.5 to 3), citric phosphate buffer (pH 2.8 to 7), and Tris hydrochloride buffer (pH 7 to 9) under standard assay conditions. The effect of pH on enzyme stability was determined by using the same buffer systems in the pH range of 1.5 to 9. Equal volumes of enzyme solution and buffer were incubated for up to 3 h at 37°C. The pH of the mixture was then adjusted to 3 or 5 with 0.5 M citric phosphate buffer before the addition of substrate for the enzyme assay.

The temperature optimum was determined by performing the standard assay within a range of 24 to 70°C. Thermal stability was determined by holding the enzyme solution at various temperatures for 0 to 1 h. Remaining activity was measured under standard assay conditions.

Inhibition of proteinase activity. Purified enzyme was treated with the following proteinase inhibitors: antipain, leupeptin, pepstatin A (dissolved in dimethyl sulfoxide), soybean trypsin inhibitor, phenylmethylsulfonyl fluoride (PMSF; dissolved in dimethyl sulfoxide), *N*-α-*p*-tosyl-L-

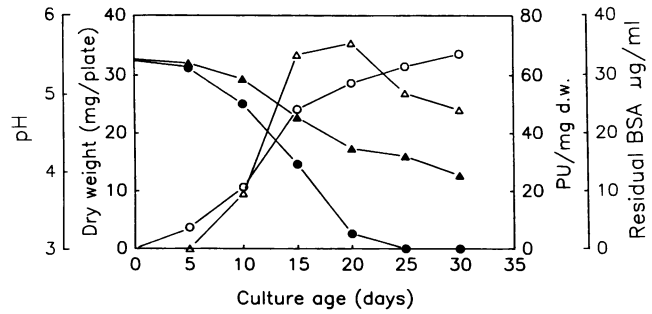

FIG. 1. Proteolytic activity (△), biomass growth (○), final pH (▲), and residual BSA (●) in the medium of *H. crustuliniforme* as a function of culture age. PU, Proteolytic units; d.w., dry weight.

lysine chloromethyl ketone (TLCK), diazoacetyl-DL-norleucine methyl ester (DAN), and EDTA. The enzyme was preincubated with each of the inhibitors at various concentrations for 30 min at 37°C, and the remaining enzyme activity was measured relative to an uninhibited control.

TABLE 1. Purification of the extracellular proteinase from *H. crustuliniforme*

Procedure	Vol (ml)	Protein		Proteinase		
		Concn (mg/ml)	% Recovery	Total activity (units)	Sp act (units/mg of protein)	% Recovery
Culture	1,400	0.29	100	147,000	362	100
Batch C-50	110	0.70	19	107,590	1,397	73
C-50	35	0.44	3.8	62,540	4,061	43
G-75	10	0.31	0.7	28,920	9,329	20

Various metallic ions, phenolic acids, and reducing reagents were also tested for their effects on proteinase activity under the conditions described above.

Chemicals. FITC-BSA, FITC-casein (type III), BSA (fraction V), casein (purified powder), azocasein, cytochrome *c* (type V), proteinase inhibitors, *p*-nitroanidides, CBZ-L-glutanyl-L-tyrosine, and CBZ-L-leucine-*p*-nitrophenyl ester were obtained from Sigma Chemical Co. CM-Sephadex C-50 and Sephadex G-75 (superfine) were obtained from Pharma-

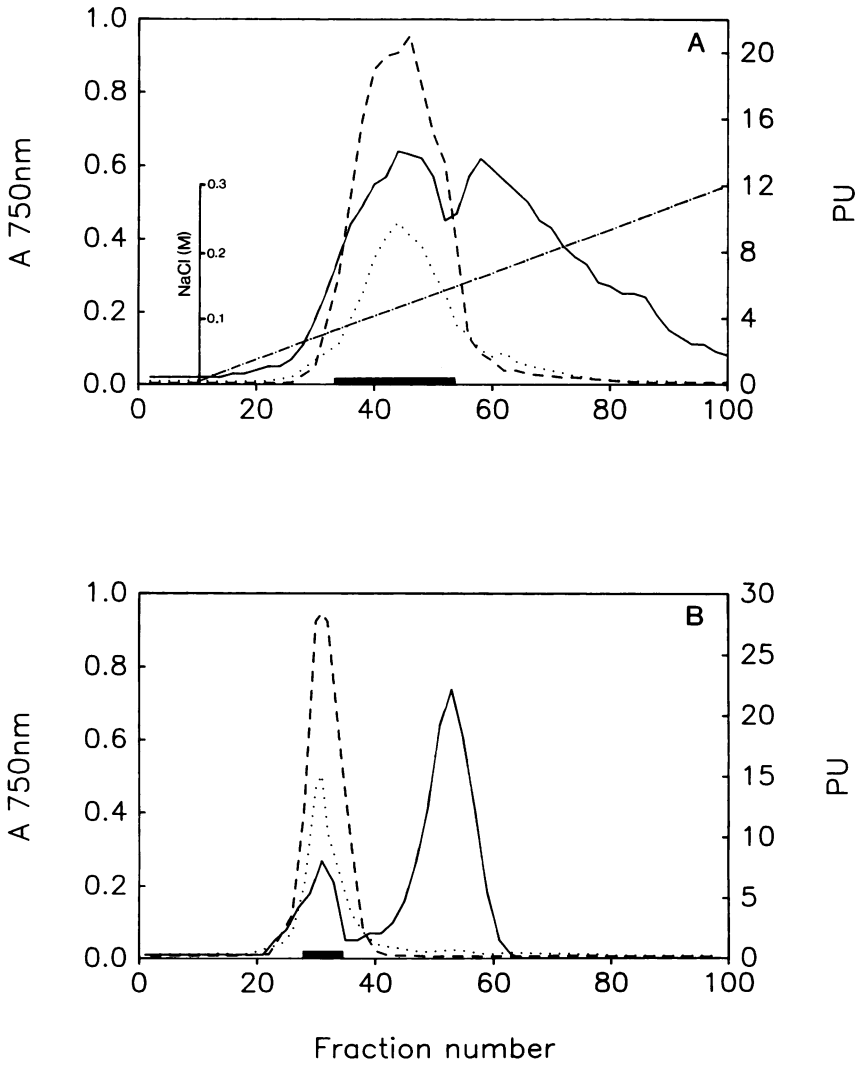

FIG. 2. Elution profile of protein (——) and proteinase activity against FITC-BSA (– – –) and FITC-casein (·····). (A) CM-Sephadex C-50 ion-exchange chromatography with a linear gradient of 0 to 0.3 M NaCl (— · — · —). (B) Sephadex G-75 gel filtration, PU, Proteolytic units.

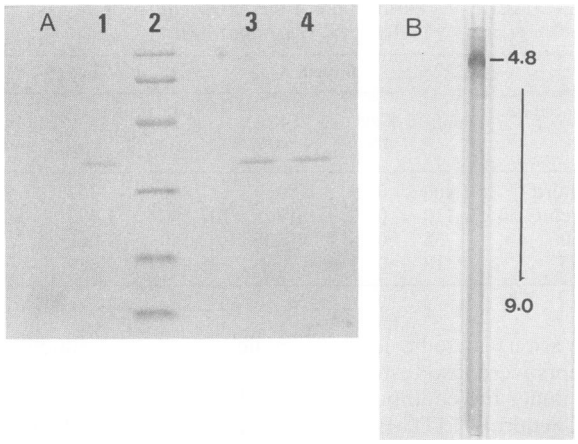

FIG. 3. (A) SDS-PAGE (12% polyacrylamide) of purified proteinase after Sephadex G-75 gel filtration and staining of the gel with Coomassie blue R-250. Lane 2 contains low-molecular-weight standards of 92,200 (phosphorylase *b*), 66,200 (BSA) , 45,000 (ovalbumin), 31,000 (carbonic anhydrase), 21,500 (soybean trypsin inhibitor), and 14,400 molecular weight (lysozyme), in descending order. Lanes 1, 3, and 4 are 5, 8, and 10 μg of purified protein, respectively. (B) Isoelectric focusing of purified protein (13 μg) after Sephadex G-75 gel filtration and staining of the gel with Coomassie blue R-250. The pH of the gel is indicated at the right; the position of the purified protein is at pH 4.8 ± 0.2.

cia LKB. SDS, polyacrylamide, and biacrylamide were obtained from BDH. Ampholines and low-molecular-weight standards were obtained from Bio-Rad. All other chemicals and reagents were of the highest purity commercially available.

RESULTS

Enzyme production. Biomass growth and extracellular proteinase production by *H. crustuliniforme* were monitored for 30 days in the medium containing BSA as the sole nitrogen source (Fig. 1). Acid proteinase activity appeared in culture filtrate after 5 days of incubation and increased rapidly to the maximum at day 20. A slow decline of proteinase activity was then followed at a linear rate of about 2 units/day on a dry weight (milligram) basis. Specific detection of the number of acid proteinases in the culture filtrate on PAGE showed a single band of dissolved gelatin. No proteolytic activity in the alkaline pH range was detected in the culture filtrate during the growth period. Biomass growth was closely associated with an increase of enzyme activity and decreases of pH and BSA concentration in the medium through the logarithmic growth phase to early stationary phase.

Enzyme purification. A summary of the proteinase purification is presented in Table 1. Batch adsorption with CM-Sephadex was used as the first step to remove mineral salts from the culture filtrate. About 70% of the total activity was recovered by using this procedure. Assays of the filtrate after batch adsorption showed that the remaining activity failed to adsorb the exchanger, probably as a result of an ionic strength interference of the culture medium. When the batch adsorption sample was applied to CM-Sephadex C-50 chromatography, most of the enzyme activity was eluted as a single peak in the range of 0.1 to 0.2 M NaCl (Fig. 2A). Pooled fractions under the activity peak showed several faint protein bands on SDS-PAGE. Sephadex G-75 gel filtration of the ion-exchange sample gave two protein peaks, and only the first-eluted peak contained proteolytic activity (Fig. 2B). The enzyme was obtained with a high purity after gel filtration, since a single band appeared on SDS-PAGE (Fig. 3A) and on isoelectric focusing gels with a pH range from 4.0 to 9.0 (Fig. 3B). The enzyme apparently had a molecular weight of 37,800 and an isoelectric point of 4.8 ± 0.2. The purification scheme recovered about 20% of the total activity and resulted in a 26-fold purification of the proteinase compared with the crude culture filtrate.

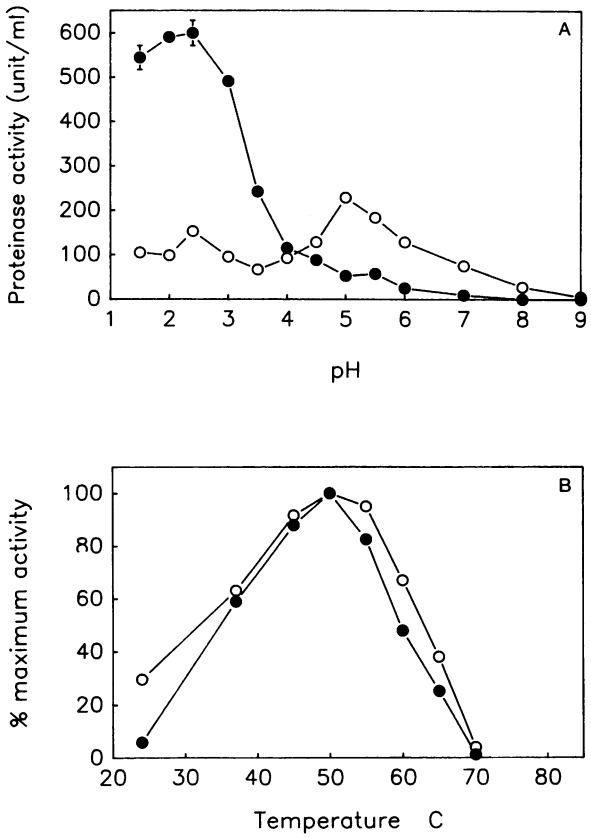

FIG. 4. Effects of pH (A) and temperature (B) on the proteinase activity from *H. crustuliniforme* as measured by using the substrates FITC-BSA (●) and FITC-casein (○) under standard conditions.

Effects of pH and temperature on proteinase activity. The optimal pH for proteinase activity was 2.5 against FITC-BSA and 5 against FITC-casein (Fig. 4A) under standard assay conditions. When assayed with unlabeled substrates, the optimal activity was at pH 2.5 against BSA and cytochrome *c* and at pH 3 against casein and azocasein (data not shown). The optimal temperature for the proteinase activity was 50°C, and about 90% of the maximal activity was retained at 45 or 55°C (Fig. 4B).

The enzyme was stable over a broad range of pHs, as demonstrated by the fact that more than 85% of the maximal activity remained following 3 h of preincubation in buffers with pHs between 2 and 5 (Fig. 5A). The enzyme was stable at temperatures below 40°C but was inactivated at higher temperatures (Fig. 5B). Less than 15% of the maximal activity remained within 10 min of inactivation at 60°C, although about 50% of the activity was retained at 55°C for 40 min.

Activity towards proteins and peptides. The proteinase was active towards a range of proteins; it hydrolyzed BSA,

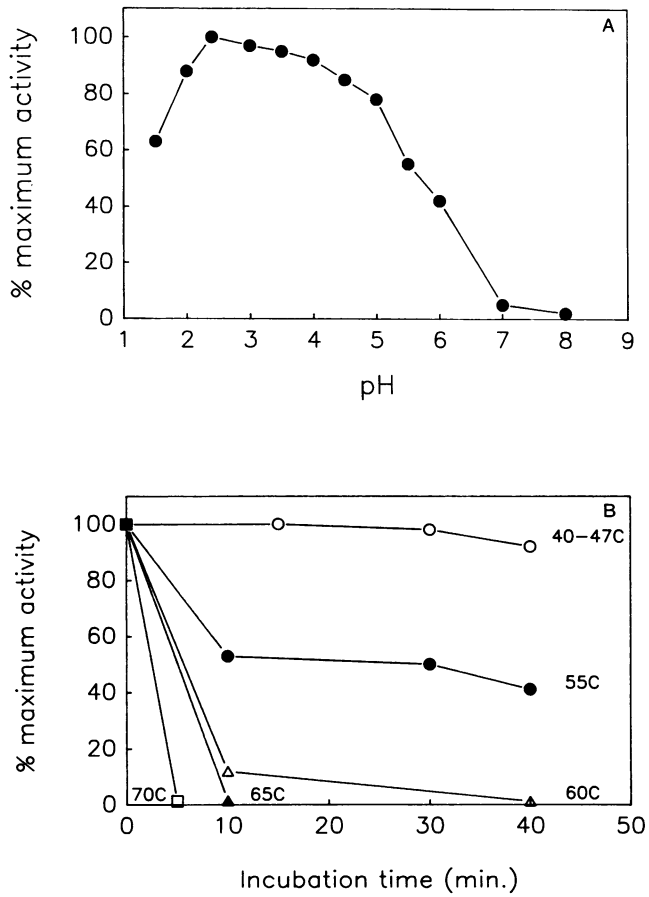

FIG. 5. Effects of pH and temperature on stability of the purified proteinase from *H. crustuliniforme* as measured by using the substrate FITC-BSA. Proteinase was preincubated with buffers with various pH values for 3 h at 37°C (A) or at various temperatures (B), and remaining activity was assayed under standard conditions.

casein, azocasein, cytochrome *c* (Table 2), and gelatin as indicated by dissolving X-ray film (data not shown). The activity against cytochrome *c* and BSA was greater than that against casein and azocasein. However, the purified enzyme had no detectable activity towards any of the peptide derivatives tested (Table 2).

Effects of inhibitors and various compounds on proteinase activity. The enzyme was preincubated with a variety of inhibitors and reducing reagents and then assayed for the remaining activity with FITC-BSA and FITC-casein (Table 3). When treated with proteinase inhibitors, enzyme activity was inhibited severely by the acid proteinase inhibitors pepstatin A and DAN plus copper ions and moderately by the serine and cysteine proteinase inhibitors antipain, leupeptin, and trypsin inhibitor. DAN was not inhibitory in the absence of copper ions or when the reaction was carried out in buffers containing copper chelator ions such as citrate or acetate. Both PMSF and TLCK inhibited less than 30% of the activity at a concentration of 5 mM and about 10% at 1 mM. The metalloproteinase inhibitor EDTA had no effect on enzyme activity. When treated with reagents, the enzyme activity was inhibited by glycerol and SDS but not by dithiothreitol or mercaptoethanol. Urea enhanced enzyme activity twofold at 6 M.

The effects of a number of metallic ions and phenolic acids on the enzyme activity were tested (Table 4). Ca^{2+}, Cu^{2+}, Mg^{2+}, and Zn^{2+} brought about a 15% decrease of activity, while Al^{3+}, Fe^{3+}, and Fe^{2+} had more inhibitory effects. The phenolic compounds tannic acid and gallic acid severely inhibited the enzyme activity.

TABLE 2. Activity of the purified proteinase from *H. crustuliniforme* on various substrates

Substrate	pH	Proteinase activity (units/mg of protein)[a]
Proteins		
Cytochrome *c*	3.0	556
	5.0	29.3
BSA	3.0	140
	5.0	33.6
Azocasein	3.0	100.2
	5.0	62
Casein	3.0	73.4
	5.0	53.4
Peptide derivatives[b]		
BANA	3.0	ND
	5.0	ND
BTEE	3.0	ND
	5.0	ND
L-BAPNA	3.0	ND
	5.0	ND
TAME	3.0	ND
	5.0	ND
CBZ-L-glutamyl-L-tyrosine	3.0	ND
	5.0	ND
CBZ-L-leucine-*p*-nitrophenyl ester	3.0	ND
	5.0	ND

[a] ND, Not detectable.
[b] BANA, Benzoyl-L-arginine-β-naphthylamide; BTEE, benzoyl-L-tyrosine ethyl ester; L-BAPNA, benzoyl-L-arginine-*p*-nitroanilide; TAME, *p*-tosyl-L-arginine methyl ester.

DISCUSSION

H. crustuliniforme produced an extracellular proteinase during growth in liquid protein medium. Purification of the crude enzyme from culture filtrate yielded a preparation that seems to be homogeneous upon electrophoretic analysis. Among the four types of proteinases described by North (19), the purified enzyme can be classified as an acid (aspartic) proteinase because of its optimal pH and inhibition by pepstatin A and DAN. Both pepstatin A and DAN are specific inhibitors of acid proteinases with little or no effect on other types of proteinases, although DAN is reactive with certain cysteine proteinases (5). Antipain, leupeptin, TLCK, and trypsin inhibitor, which are known to be nonspecific inhibitors of serine and cysteine proteinases, partially inhibited the enzyme activity, but the specific activators dithiothreitol and EDTA along with the inhibitor PMSF showed no significant effects. These biochemical characteristics suggest that the purified enzyme is not likely to be a serine or cysteine proteinase. The fact that chelating agent EDTA did not inhibit the enzyme activity excludes the possibility that the enzyme is a metalloproteinase. Urea enhanced the enzyme activity twofold even at concentrations of 2 to 6 M. The reason for this enhancement effect is unknown. It may be that this enzyme was more resistant to urea denaturation and more efficient at hydrolyzing unfolded proteins. The physical properties of the purified enzyme are also similar to those of typical acid proteinases. Its apparent molecular weight (37,800) and isoelectric point (4.8) are consistent with

TABLE 3. Effects of inhibitors and reagents on the purified proteinase of *H. crustuliniforme*

Compound	Concn (mM)	% Relative activity on:	
		FITC-BSA	FITC-casein
Inhibitors			
Antipain	3	60	24
	0.3	100	72
Leupeptin	3	64	40
	0.3	85	89
Pepstatin A	2.5	0	0
	0.25	0	0
TLCK	5	78	74
	1	100	100
DAN	5	95	89
	1	98	99
DAN + Cu^{2+}[a]	5	10	12
	1	60	47
PMSF	5	82	71
	1	94	90
EDTA	10	91	100
	1	100	100
Trypsin inhibitor (soybean)	0.2%	47	87
	0.02%	97	100
Reagents			
Mercaptoethanol	50	107	111
	10	100	103
Dithiothreitol	10	108	118
	1	100	100
SDS	3.5	0	23
	0.35	30	67
Glycerol	10	75	93
	1	93	99
Urea	6,000	210	208
	500	123	112
	50	106	108

[a] The assay was carried out in KOH-HCl buffer.

TABLE 4. Effects of metallic ions and phenolic acids on the activity of the purified proteinase from *H. crustuliniforme*

Compound	Concn (mM)	% Relative activity on:	
		FITC-BSA	FICT-casein
$NH_4H_2PO_4$	50	100	87
KNO_3	50	98	88
$FeCl_3 \cdot 6H_2O$	4	6	87
	1	20	88
$FeSO_4 \cdot 7H_2O$	4	12	75
	1	20	100
$CuCl_2 \cdot 2H_2O$	4	83	70
	1	86	88
$ZnCl_2$	4	97	96
	1	100	108
$Al_2(SO_4)_3 \cdot 16H_2O$	4	61	31
	1	96	100
$MgSO_4$	10	76	100
	3	99	118
$CaCl_2$	15	84	91
	5	96	100
Gallic acid	1	11	18
	0.3	71	26
Tannic acid	1	2	5
	0.3	3	9

the data obtained for acid proteinases produced by other fungi (19).

The acid proteinase of *H. crustuliniforme* is able to hydrolyze a broad range of native proteins such as BSA, casein, gelatin, and cytochrome *c*. However, casein and azocasein were poorly hydrolyzed by the enzyme in comparison with BSA and cytochrome *c*, probably because of the insolubility of the former pair at lower pH values. The optimal pH for enzyme activity also seems to be dependent upon the substrate, as shown by the discrepancy of pH optima between FITC-casein and FITC-BSA. The failure to hydrolyze low-molecular-weight peptide derivatives is common among fungal acid proteinases that have little or no activity on small substrates (19); an example is *Choanephora cucurbitarum* proteinase (4).

H. crustuliniforme proteinase was remarkably stable under conditions of low pH and high temperature. This result is comparable with the majority of fungal acid proteinases, which are generally stable at pHs between 2 and 6 and lose about 50% of the total activity after 10 to 15 min at 60 to 65°C (16).

The enzyme was sensitive to several metallic ions tested. Inhibition by Fe^{2+} and Fe^{3+} ions draws parallels with the acid proteinases of other fungi (4, 10, 23). It could be due to ionic interaction between these ions and carboxylic groups of the enzyme active sites (25). Partial inhibition (15 to 70%) by Ca^{2+}, Cu^{2+}, Mg^{2+}, and Al^{3+}, in that order, was found when the enzyme was treated with these ions at higher concentrations. None of the metallic ions tested led to a significant enhancement of proteinase activity. In this respect, the *H. crustuliniforme* enzyme resembles the acid proteinases of *Mucor pusillus* (23). The phenolic compounds, tannic acid and gallic acid, severely inhibited enzyme activity. Tannic acid is known for its ability to form insoluble complexes with proteins, and the tannin-protein interaction has been involved with proteolytic enzymes and their substrates (17). It is possible that tannic acid reduces enzyme activity by binding to the enzyme active sites or substrate or both, since this compound has a wide range of affinities for different proteins (9). The action of gallic acid on the enzyme could be similar to that described for tannic acid, and if it is so, its affinity for the enzyme or FITC-labeled substrates seems to be weaker than that of tannic acid.

Many species of fungi produce extracellular proteinases under suitable culture conditions, and these enzymes are generally involved with the degradation of proteins as a source of nutrients (8). A possible role of extracellular proteinase during fungal pathogenesis or morphogenesis has also been discussed (8, 10, 27). It is evident from this study that the extracellular acid proteinase of *H. crustuliniforme* has an important role in protein nitrogen utilization, as indicated by the close associations among enzyme activity, biomass growth, and substrate assimilation (Fig. 1). Consequently, tree seedlings inoculated with this fungus are able to utilize protein nitrogen, as demonstrated by Abuzinadah and Read (1). In natural soil, microbes are often deficient in energy for the synthesis of extracellular enzymes, and competition for available protein nitrogen among them would be expected. Under these conditions, mycorrhizal fungi have an advantage over saprotrophic microbes because they have a continuous carbon supply from tree hosts. It is possible that the extracellular enzyme produced by *H. crustuliniforme* has other functions in addition to its role in nutrition. Our preliminary studies on the physiological regulation of the enzyme have shown that the enzyme production was not repressed by simple forms of nitrogen and carbon such as ammonium and glucose. Since root cell walls contain a great amount of protein (26), the proteinase could assist the fungus

in infecting the host root and developing a tissue network among root cells.

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