Some characteristics of extracellular proteinases from *Coprinus cinereus*

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Spent culture medium contained five electrophoretically distinct proteolytic activities when *Coprinus cinereus* was grown in culture medium which contained protein as sole source of nitrogen. The enzymes were separated by ultrafiltration, gel filtration and ion-exchange chromatography into two groups designated EL and AD fractions. The EL fraction passed through a DE-52 column with eluant, and contained three electrophoretically distinct activities. The AD fraction was desorbed with 0·2 M-NaCl, and contained two electrophoretically distinct activities. Non-proteolytic protein bands were absent from the purified samples. Studies with inhibitors suggested the presence of cysteine, metallo- and serine proteinases within the EL fraction. The EL enzyme activity was stable between pH 6 and pH 11, and up to 30 °C. Activity was optimal at pH 9, with a second peak at pH 6, and at 45°. The AD fraction may contain all four types of proteinases, with metallo- and cystein enzymes being the major species. The AD enzymes were stable over broader ranges of pH (5–11) and temperature (up to 39°) than the EL proteinases, with optimum activities at pH 5 and 9, and at 56–62°. Both groups of enzymes had an apparent molecular weight of about 31 kDa, and isoelectric points of pH 4·9 (AD) and 5·6 (EL).

Key words: *Coprinus cinereus*, Proteinase purification, Extracellular enzymes, *Coprinus* proteinases.

Coprinus cinereus (Schaeff.: Fr.) S. F. Gray is a highly competitive weed in mushroom beds. The form of the available nitrogen sources in compost and similar substrates is still unknown, but protein is probably the most abundant in various forms including proteins/peptides bound to lignin, microbial protein and plant protein. *C. cinereus* has been shown to use protein readily as a source of carbon, nitrogen and/or sulphur (Kalisz, Moore & Wood, 1986). This ability to use protein as a major growth substrate is correlated with production of extracellular proteinase activities. The continued catabolism of protein in the presence of glucose and/or ammonium (Kalisz, Wood & Moore, 1987) shows regulation of production of these enzymes to be free of the catabolite repression systems for proteinase production seen in lower fungi (Cohen, 1980). Thus the regulation of proteinase production in basidiomycetes may differ considerably from that previously described for fungi such as *Aspergillus* and *Neurospora* (Cohen, 1973, 1981; Hanson & Marzluf, 1973; Drucker, 1973, 1975).

Proteinases are a complex group of enzymes varying greatly in their physicochemical and catalytic properties, and playing important roles in the catabolic and regulatory processes of eukaryotes and prokaryotes alike (see reviews by Kalisz, 1988 and North, 1982). Proteolytic enzymes are divided into two major groups, peptidases and proteinases, on the basis of their nature of attack. Peptidases are subdivided further according to whether they act at the C- or N-terminal (carboxy- or aminopeptidases) or on a dipeptide (dipeptidases). Proteinases are classified into four groups by their catalytic mechanism. This classification is determined indirectly through reactivity towards inhibitors of particular amino acid residues in the active site region (Morihara, 1974). The four groups are: (i) serine proteinases, (ii) cysteine proteinases, (iii) aspartic proteinases, and (iv) metalloproteinases.

Extracellular proteinase production by *C. cinereus* is regulated by induction and sulphur derepression. Under conditions of induction, *C. cinereus* proteinase activities are produced in a growth-associated matter, with increases in extracellular activity paralleling increases in biomass. Hence, maximal extracellular proteinase activities occur in the late exponential growth of the organism. During the early stages of growth, when substrate protein in the medium decreases rapidly in concentration, virtually no proteolytic activity can be demonstrated in the medium (Kalisz *et al.*, 1987). This suggests that the enzymes concerned are probably wall-bound during the initial stages of growth. In this paper we report observations on the properties of those enzymes.

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MATERIALS AND METHODS

Organism and culture conditions

Stocks of the *C. cinereus* Birmingham dikaryon (ATCC 42721) were maintained and harvested as described previously (Kalisz *et al.*, 1986). Proteinase secretion was induced on Treschow defined medium (Fermor & Wood, 1981) supplemented with 1·0% (w/v) insoluble casein (BDH no. 44018) and 1·0% (w/v) glucose. Volumes of 25 ml of medium were inoculated with 1·0 ml of a mycelial suspension prepared as described by Kalisz *et al.* (1986). The cultures were incubated at 20° for 15–18 days, and were harvested when maximal proteinase activity was observed.

Crude proteinase preparation

Mycelium was harvested by filtration through cheesecloth, and the medium refiltered under vacuum using Whatman no. 1 filter paper (7 cm diam). The filtrate (usually 1–2 l) was concentrated 100-fold using an Amicon Ultrafilter system, with either PM 10 (for larger volumes) or UM 2 (for volumes less than 2100 ml) ultrafilter membranes at a pressure of 50 p.s.i., at room temperature.

Proteinase assay

Proteinase was measured colorimetrically by the liberation of a dye from an insoluble substrate, Remazol Brilliant Blue (RBB) hide powder conjugate (Rinderknecht *et al.*, 1968), using the modified method of Fermor & Wood (1981).

Determination of protein

Protein was measured by the method of Bradford (1976), following the procedure of Spector (1978), using Coomassie brilliant blue G reagent (Sigma).

Electrophoresis

Proteinase purity was determined by non-dissociating discontinuous polyacrylamide slab gel electrophoresis (PAGE), following the procedure of Davis (1964), as described by Hames (1981). Zones of proteolytic enzymes on the polyacrylamide electrophoretograms were detected by making contact print zymograms (Westergaard *et al.*, 1980).

Proteinase purification

Gel filtration. The concentrated culture filtrates were applied in 5 ml volumes (= about 50 mg protein) to a Sephadex G-50 column (bed height = 95 cm, diam = 2·5 cm). The column was equilibrated and eluted with 50 mM phosphate buffer, pH 7·0, at a flow rate of 10 ml h^{-1}. Fractions of 2 ml were collected and assayed for activity at pH 4·0, 7·0 and 9·0; those containing proteolytic activity were pooled and concentrated by ultrafiltration.

Ion-exchange chromatography. Samples fractionated by gel filtration were equilibrated with the eluent buffer (10 mM acetate buffer, pH 5·0), and applied on to a DEAE-cellulose (DE-52) (Whatman) column in 2 ml volumes (= 3–4 mg protein). Unbound substances were washed from the column using a volume of the starting buffer. Adsorbed samples were eluted with 0·2 M-NaCl. The eluted and desorbed fractions were collected in batches, concentrated by ultrafiltration, and used for proteinase characterization. Eluted and adsorbed fractions containing proteinases were designated EL and AD fractions, respectively.

Proteinase characterization

PAGE was used to establish the number of electrophoretically distinct extracellular proteinases and to follow changes in protein and proteinase profiles during growth of *C. cinereus*. For the latter, mycelium was harvested on days 4, 6, 9. 11, 13 and 15 and the filtrates concentrated 10-fold by ultrafiltration, then electrophoresed and proteinase activity detected with contact-print zymograms.

Molecular weights of the proteinases were determined by both gel filtration (on Sephadex G-50 Fine and Superfine columns) and sodium dodecyl sulphate (SDS) PAGE which was carried out in a discontinuous buffer system, in 7·5% or 15% gels, using the method of Laemmli (1970). The isoelectric point (pI) of the proteinases was determined by isoelectric focusing, following the method of Winter, Ek & Andersson (1977). Concanavalin A–sepharose chromatography was used to establish the possible presence of carbohydrate moieties on the proteinase monomers.

The influence of pH on proteinase activity was determined by incubating the enzyme with RBB-hide powder at 25° in the presence of 0·1 M buffers of a wide pH range (pH 3·0–12·0). Stability of the EL and AD proteinases in the pH range 3·0–12·0 was determined by incubating each enzyme in 0·1 M buffer of each pH at room temperature for 1 h. Residual activity at pH 9 was subsequently measured. Activity was measured as a percentage of the maximum. The buffers used were: pH 1–3, HCl/KCl; pH 4–6, sodium acetate/acetic acid; pH 7, sodium phosphate; pH 8–12, Tris/NaOH.

Temperature optimum was determined by incubating the proteinases with RBB and buffer at different temperatures. The EL fraction was incubated for 1 h at pH 9·0. The AD fraction was incubated for 30–40 min at pH 5·0 and 9·0. Heat stability was determined by incubating samples of the enzyme with buffer (pH 6·0 for AD; pH 8·0 for EL) for 2 h at a range of temperatures between 0 and 72°. The mixtures were subsequently transferred to the assay medium (pH 5 or 9 for AD; or pH 9 for EL) and incubated at 25°. Activity was defined as a percentage of maximum.

A variety of compounds was used to determine the effects of inhibitors on extracellular proteinase activity. Pepstatin, bestatin, leupeptin and phenylmethylsulphonyl fluoride (PMSF) were dissolved in 100% methanol; 8-hydroxyquinoline and *N*-tosyl-L-lysine chloro-methyl ketone (TLCK) were prepared in 50% methanol; *p*-chloromercuribenzoate (*p*CMB) was dissolved in NaOH in Tris buffer pH 8·0, and the pH readjusted with HCl to pH 10·0 (lowering the pH further resulted in precipitation); soybean trypsin inhibitor, iodoacetamide, iodoacetic acid, mercuric chloride, KCN, 2-

mercaptoethanol, dithiothreitol and ethylenediamine tetra-acetic acid (EDTA) were dissolved in distilled water. The proteinases were pre-incubated with each inhibitor at either pH 6·0 or 8·0 for 1 or 2 h at 25°. The samples were subsequently added to the assay medium and incubated at 25°. Enzymes pre-incubated at pH 6·0 were assayed at pH 5·0 (AD) or pH 6·0 (EL). Proteinases pre-incubated at pH 8·0 were assayed at pH 9·0 (both AD and EL). Controls with distilled water and methanol were treated in the same way as the test samples. To establish degree of inhibition by EDTA and subsequent re-activation by divalent metal ions, proteinases were pre-incubated for 2 h with a final concentration of 2 or 10 mM-EDTA at pH 6 (AD and EL) or pH 8 (AD) at room temperature. Subsequently, 0·7 ml samples were transferred to 0·35 ml of a solution of 1·0, 2·0, 5·0 or 10·0 mM (final concentration) divalent ions (calcium, manganese, magnesium, zinc or ferrous ions) or distilled water, incubated at room temperature for 2 h, then assayed for activity.

RESULTS

PAGE profiles of crude and purified samples

Crude extracts were separated by PAGE into five proteolytic bands active at pH 9, three active at pH 7, and one active at pH 4 (Fig. 1). These activities were designated proteinase I–V, according to their electrophoretic mobility, with proteinase I having the lowest mobility. The relative mobilities of the proteinases are presented in Fig. 1. About 15 protein bands were visible in electrophoretograms of crude culture filtrates (data not shown); these were reduced to the five proteinase bands by the purification procedures described.

Changes in the proteinase profiles of 10-fold concentrated crude filtrates of *C. cinereus* are presented in Fig. 2. Proteinase I increased in activity (in terms of clearing intensity on zymograms) during the growth period. Proteinase III was observed on day 15 only. Proteinase II and IV were not observed, probably due to relatively low concentrations of the filtrates. Three proteolytic activities were observed with relative mobilities corresponding to proteinase V. The relative mobilities on 7·5% polyacrylamide gels of the three proteinases were 0·50, 0·53 and 0·55 (designated proteinase V_a, V_b and V_c, respectively). The three proteolytic bands increased in intensity over the first 11 days. By day 13 most of the activity corresponding to proteinase V_a and V_c was lost. At the same time, proteinase V_b increased in intensity (Fig. 2).

Fig. 1. Diagrammatic illustration of the protein and proteinase spectra of crude culture filtrates from 18-d cultures of *Coprinus cinereus* following ultrafiltration and electrophoretic separation on 7·5% acrylamide gels. Proteinase activity was determined with contact-print zymograms.

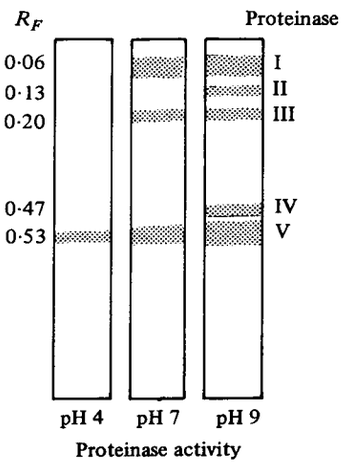
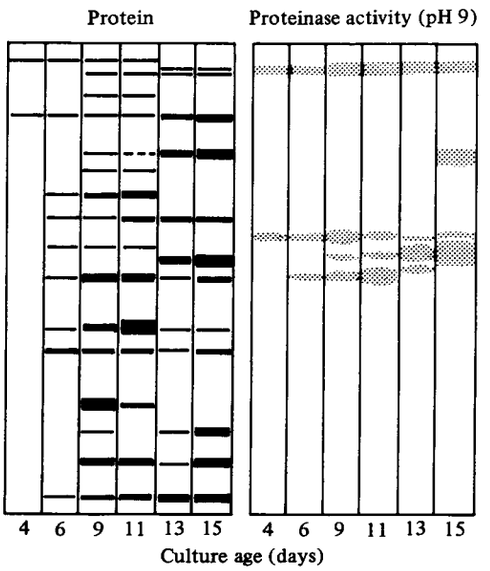


Fig. 2. Diagrammatic illustration of the protein and proteinase spectra of crude culture filtrates following ultrafiltration and electrophoretic separation on 7·5% acrylamide gels; medium fluids were harvested from cultures of *Coprinus cinereus* grown for various times, as indicated.



Proteinase purification

Two proteolytic peaks active at pH 9 (designated G-50 peaks 1 and 2) were separated by Sephadex G-50 filtration (Fig. 3). Both peaks contained all five proteolytic activities but only peak 2 was purified to any significant extent. The G-50 peak 1 may have contained aggregates of the proteinases as the molecular weights of its components were identical to those of peak 2 when measured under dissociating conditions. G-50 peak 2 was further purified by DEAE-cellulose chromatography. Three of the five proteinases (I, II and III) passed through the DE-52 column with the eluent and were designated the EL fraction. The other two enzymes (IV and V) were desorbed from the column with 0·2 M-NaCl and designated the AD fraction.

The purification steps and yields of the *Coprinus* proteinases are summarized in Table 1. Proteinases of the EL and AD fractions could not be further separated by CM-cellulose ion-exchange chromatography, affinity chromatography or chromatofocusing.

Fig. 3. Protein separation by gel filtration. A sample of protein concentrated by ultrafiltration was applied to a Sephadex G-50 column. Proteinase activity was determined at pH 9 with Remazol Brilliant Blue hide powder conjugate as substrate.

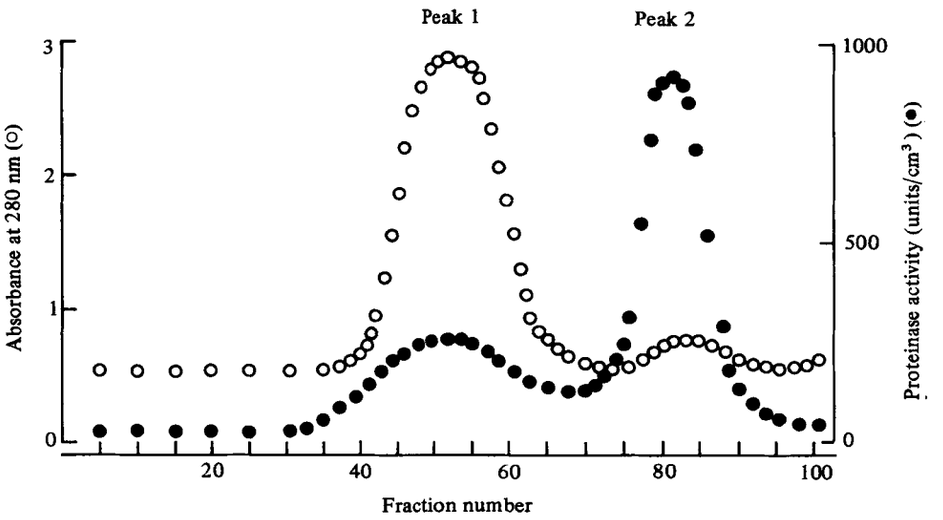
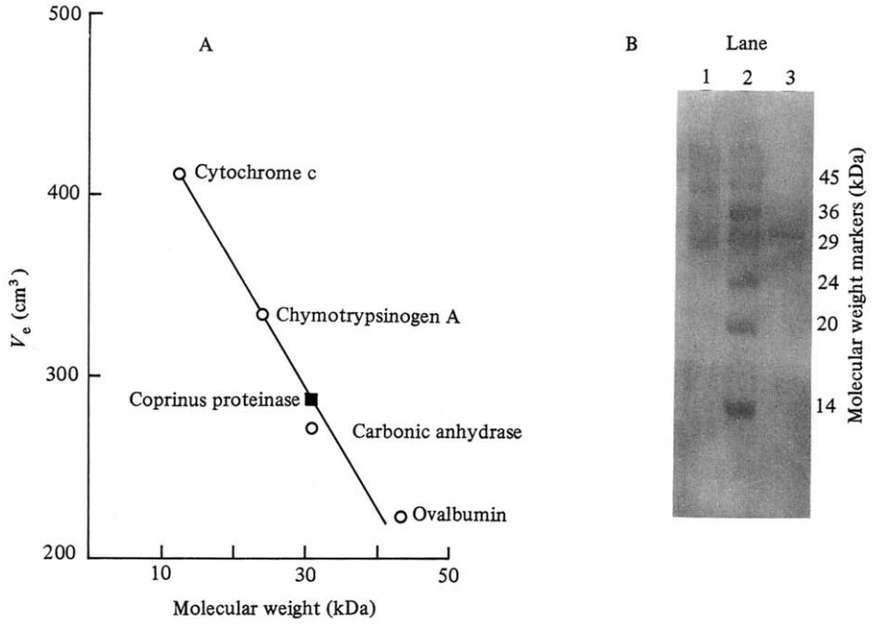


Table 1. Summary of the purification steps and yields of *Coprinus* extracellular proteinases

Fraction	(mg) Total protein	Proteinase (units/h)	Specific activity (units/mg)	Purification factor	Yield (%)
(*a*) pH 9 proteinase activity					
Crude filtrate	524·7	626230	1193·5	1·00	100
Ultrafiltrate 1	467·6	575355	1230·4	1·03	91·9
Sephadex G-50	69·1	536058	7757·7	6·50	85·6
Ultrafiltrate 2	48·5	478040	9856·5	8·26	76·3
DE 52 and ultra-filtration					
EL fraction	4·5	43701	9711·3	8·14	7·0
AD fraction	0·8	9629	12036·4	10·08	1·5
(*b*) pH 7 proteinase activity					
Crude filtrate	524·7	353210	673·2	1·00	100
Ultrafiltrate	467·6	317889	679·8	1·01	90·0
Sephadex G-50	69·1	287874	4164·8	6·19	81·5
Ultrafiltrate 2	48·5	247572	5104·6	7·58	70·1
DE 52 and ultra-filtration					
EL fraction	4·5	35039	7786·5	11·57	9·9
AD fraction	0·8	2760	3443·0	5·11	0·8
(*c*) pH 4 proteinase activity					
Crude filtrate	524·7	529684	1009·5	1·00	100
Ultrafiltrate 1	467·6	495509	1059·7	1·05	93·5
Sephadex G-50	69·1	290394	4202·5	4·16	54·8
Ultrafiltrate 2	48·5	239370	4935·5	4·89	45·2
DE 52 and ultra-filtration					
EL fraction	4·5	0	0·0	0·0	0·0
AD fraction	0·8	2777	3471·1	3·44	0·5

Proteinase activity was determined at pH 4, 7 and 9 using Remazol Brilliant Blue hide powder conjugate as substrate.

Fig. 4. A, Determination of the molecular weight of *Coprinus cinereus* proteinase using gel filtration with Sephacryl S-200 Superfine columns. B, Determination of the molecular weight of the EL proteinase fraction (lane 3) by SDS–PAGE electrophoresis using a 15% polyacrylamide gel. Lanes 1 and 2 contain, respectively, high- and low-molecular-weight marker proteins.



Proteinase characterization

Five proteolytic activities were observed at pH 9·0 on non-dissociating gel electrophoretograms (about 20 g protein/well). The EL and AD fractions used for partial characterization consisted of three (proteinases I, II and III) and two (proteinases IV and V) proteolytic activities, respectively. The two fractions contained no protein band detectable by silver staining other than those corresponding to the proteinases. Hence, both fractions were free of non-proteolytic proteins.

Molecular weight. The molecular weight of proteinases in both the EL and AD fractions was estimated at approximately 31 kDa. An apparent molecular weight of 30·75 kDa was estimated using Sephadex G-50; Sephacryl S-200 yielded a molecular weight of 31·2 kDa, and a molecular weight of 30·7 kDa was estimated by SDS–PAGE (Fig. 4). The proteinases were shown by their behaviour on concanavalin A-sepharose chromatography as likely to be unglycosylated.

Isoelectric point. The isoelectric point of the crude and G-50-separated enzymes was estimated at pH 4·9 and 5·6 by isoelectric focusing.

pH optimum and stability. The crude sample exhibited optimal activity from pH 3·0 to 11·0. Outside this range, activity was lost rapidly. The EL and AD fractions exhibited a narrower pH range of optimum activity. Optimum pH of both fractions was 9·0. However, activity of the AD fraction decreased rapidly outside pH 9. Activity of the EL fraction was optimal in the range pH 8–11.

A second peak of activity was also observed at pH 6 with the EL fraction and pH 5 with the AD fraction. Using 8-hydroxyquinoline the AD proteinase activities at pH 5 and pH 9 were shown to result from two different types of enzyme (Fig. 5).

Both the EL and AD fractions were stable over a broad pH range. The EL fraction was stable between pH 6 and 11, with optimum at pH 6. The AD fraction exhibited maximum stability between pH 5 and 11, with an optimum at pH 8.

Temperature optimum and stability. Activities of the EL and AD fractions at different temperatures are outlined in Fig. 6. EL fraction activity increased rapidly from 30° to a

Fig. 5. Influence of pH on activity of the EL (open squares) and AD (circles) proteinase fractions. Proteinase activity was measured at pH 9. Also shown is the effect of treating the AD fraction with 8-hydroxyquinoline (closed circles).

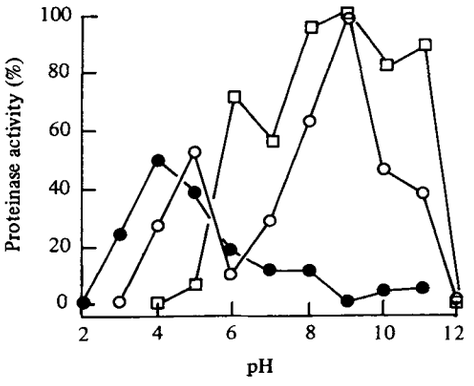


Fig. 6. Effect of temperature on the activity of EL (open squares) and AD (circles) proteinase fractions. The assays were carried out at pH 9 (open symbols) or pH 5 (closed symbols) at the indicated temperatures without pre-incubation.

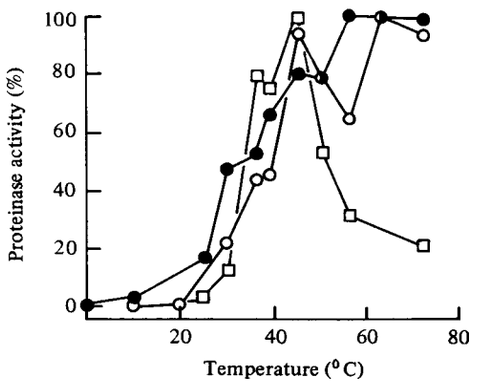
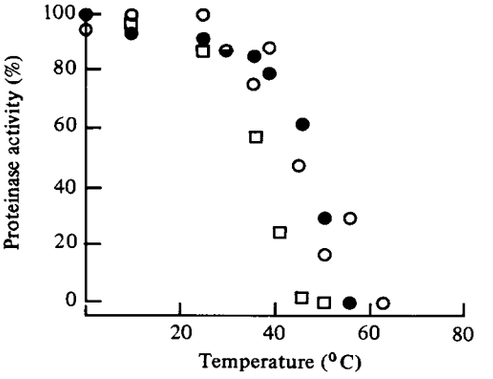


Fig. 7. Effect of temperature on the stability of EL (open squares) and AD (circles) proteinase fractions. Enzyme fractions were incubated at the indicated temperatures for 2 h prior to assay at 25° at either pH 9 (open symbols) or pH 5 (closed symbols).



maximum at 45°, with rapid loss of activity at temperatures above 45°. Activity of the AD fraction increased linearly from 25° to a maximum at 56° at pH 5, and 62° at pH 9.

The AD fraction was stable over a broader temperature range than the EL proteinase (Fig. 7). The EL fraction was rapidly inactivated above 30°, with a complete loss of activity above 45°. Little loss in activity of the AD fraction occurred at temperatures up to 39°. Complete inactivation of this fraction at pH 5 and pH 9 was effected by temperatures above 56° and 62°, respectively.

Effect of inhibitors. The effects of inhibitors on the activity of the EL and AD fractions are summarized in Table 2. Both enzyme fractions were completely inhibited at acid and alkaline pHs by inhibitors of different types of proteinase. This indicates the presence of more than one proteinase enzyme within each fraction.

The proteolytic activity of the EL fraction at pH 6 was completely inhibited by cysteine proteinase inhibitors, pCMB, mercuric chloride and iodoacetic acid, as well as the serine inhibitor, PMSF (which also inhibits some cysteine proteinases). Activity at pH 9 was also completely inhibited by pCMB and mercuric chloride, but was unaffected by iodoacetic acid and PMSF. More than 50% inhibition of the EL fraction was also caused at pH 9 by the soybean trypsin inhibitor and at pH 6 by TLCK and EDTA. Both acid and alkaline activities of the EL fraction were stimulated up to 30% by the reducing agents 2-mercaptoethanol, dithiothreitol and KCN. The pH 6

Table 2. Effect of inhibitors on proteinase activity of EL and AD fractions

		Inhibition (as % of control)			
		EL fraction		AD fraction	
	Pre-incubation pH	6	8	6	8
Inhibitor	Assay pH	6	9	5	9
Trypsin inhibitor		27	65	8	29
PMSF		100	0	27	12
Leupeptin		−15	33	5	17
TLCK		58	9	4	14
pCMB		100	100	76	100
Iodoacetamide		22	30	6	32
Iodoacetic acid		100	0	100	37
Mercuric chloride		100	100	100	100
Potassium cyanide		−83	−22	100	100
2-Mercaptoethanol		−32	0	0	30
Dithiothreitol		−23	−35	11	73
Pepstatin		40	3	0	0
Bestatin		0	6	0	0
EDTA		56	−95	100	100
8-Hydroxyquinoline		−21	40	32	100

Both EL and AD fractions were pre-incubated for 2 h at pH 6 and 8 and were assayed for activity at pH 6 and 9 (EL fraction) or pH 5 and 9 (AD fraction), as described in Materials and Methods. Negative signs indicate activation of proteinase relative to the control.

Table 3. Reactivation of EDTA-inhibited proteinase by divalent cations: restoration of activity (%)

Cation identity	Pretreatment.. Cation concentration (mM)	10 mM-EDTA 2	5	10	2 mM-EDTA 2
	(*a*) EL fraction activity assayed at pH 6				
Ca^{2+}		3	22	21	28
Mn^{2+}		1	0	33	31
Mg^{2+}		13	0	16	40
Zn^{2+}		4	21	−32	−26
Fe^{2}		−46	−100	−100	−77
	(*b*) AD fraction activity assayed at pH 5				
Ca^{2+}		0	29	19	21
Mn^{2+}		0	0	6	23
Mg^{2+}		0	0	0	0
Zn^{2+}		0	31	36	27
Fe^{2+}		0	6	7	12
	(*c*) AD fraction activity assayed at pH 9				
Ca^{2+}		0	28	49	25
Mn^{2+}		0	17	8	8
Mg^{2+}		0	5	7	2
Zn^{2+}		0	ND	7	10
Fe^{2+}		0	7	0	0

The EL and AD fractions were inhibited by EDTA at 2 mM and 10 mM; subsequent incubation with a divalent cation often restored activity, which is shown here as a percentage reactivation of proteinase activity relative to the EDTA-inhibited preparation. Negative values represent further inhibition by the cation. ND, not determined.

activity was also stimulated by leupeptin and 8-hydroxyquinoline, with EDTA stimulating the pH 9 activity.

Activity of the AD fraction was not enhanced by any of the reagents used. Complete inhibition was effected by EDTA, KCN, mercuric chloride and pCMB at both pH 5 and pH 9. The fraction was also inhibited 100% at pH 5 by iodoacetate and at pH 9 by 8-hydroxyquinoline. Other reagents inhibited between 15% and 70% of the AD proteinase activity at either pH (Table 2).

Reactivation of EDTA-inhibited proteinases. The effects of the divalent cations on EDTA-treated proteinases are summarized in Table 3. The EL fraction activity was inhibited 60% by 10 mM-EDTA and 20% by 2 mM-EDTA at pH 6. The fraction was reactivated by 20–40% by equimolar calcium, manganese and magnesium cations. Calcium ions (5 mM) also effected 20% reactivation of the 10 mM-EDTA-inhibited proteinase activity of this fraction. Equimolar solutions of zinc and ferrous cations further inhibited the enzyme activity.

The AD fraction was inhibited completely by 2 and 10 mM-EDTA at both pH 5 and pH 9. Reactivation of 20–30% was effected at pH 5 by equimolar solutions of calcium, manganese and zinc cations and by 5 mM calcium and zinc solutions. Ferrous and magnesium ions had little or no effect on the reactivation of the AD fraction at pH 5. Calcium restored 25–50% of pH 9 activity. Less than 10% reactivation was effected by the other cations.

DISCUSSION

Coprinus cinereus has been shown to secrete at least five electrophoretically distinct proteolytic enzymes active at pH 9. Proteinase I and V were the first enzymes to be produced into culture filtrates of *Coprinus*, and were present throughout the growth period. Three proteinases of very similar electrophoretic mobilities to proteinase V were present in the filtrates of logarithmic phase cultures. The three activities appeared to merge into a single activity, corresponding to proteinase V, on transition from logarithmic to stationary growth. Proteinase III activity was observed on day 15 only, and proteinase II and IV were detected only after 100-fold concentration of the culture filtrate.

The crude enzymes were stable over a very broad pH range (3–11) and lost little activity when repeatedly frozen and thawed. The partially purified proteinases were stable over a narrower pH range and were inactivated fairly rapidly on freezing.

The effects of inhibitors imply that the EL and AD fractions each contain more than one type of proteinase. The major enzyme of the EL fraction appears to be a cysteine proteinase. This fraction was completely inhibited by thiol reagents and stimulated by reducing agents. The pH 6 and 9 activities are most probably due to different cysteine proteinases. Only a few fungi, such as *Microsporum* sp. (Roberts & Doetsch, 1967), *Aspergillus oryzae* (Kandu & Manna, 1975) and *Sporotrichum pulverulentum* (Eriksson & Pettersson, 1982), have been reported to produce extracellular cysteine proteinases. The cysteine proteinase of *Coprinus* active of pH 6 exhibited many characteristics similar to the cysteine proteinases of other fungi. These include an acidic optimum pH, a molecular weight of about 31 kDa and an isoelectric point of 5·6.

Partial inhibition of pH 6 or pH 9 activities of the EL fraction by EDTA and 8-hydroxyquinoline respectively implies the presence of one and perhaps two different metalloproteinases. The EL fraction also appears to contain a

serine proteinase. The EDTA-inhibited proteinases were partially reactivated by calcium, magnesium and manganese salts. Zinc, which is essential for the activity of many metalloproteinases, inhibited the enzyme further. A similar phenomenon has been observed with bacterial proteinases (Li & Yousten, 1975) and probably reflects the presence of adequate levels of the cation in the medium, the added excess being inhibitory.

The AD fraction, which was completely inhibited by chelating agents and by thiol reagents and heavy metals, appears to contain metallo- and cysteine proteinases. However, most of the other inhibitors caused partial inhibition of the AD proteinase activity. The EDTA-inhibited proteinases were partially reactivated by calcium and zinc salts, as well as manganese and ferrous ions.

Molecular weights of the EL and AD proteinases were estimated at approx. 31 kDa; thus, although five different proteinases were detected by non-dissociating gel electrophoresis, they all appear to be monomers of about the same molecular weight. Experiments showing the lack of binding of these enzymes to concanavalin A-sepharose suggest that the peptide chains are unlikely to be glycosylated.

The AD fraction was stable over broad pH and temperature ranges; being stable between pH 5 and 11, and up to 39° and exhibiting optimum activity at pH 9 and at pH 5. The pH 5 and 9 activities were maximal at 56° and 62° respectively. The EL fraction was stable over a lesser range of pH (6–11) and temperature (up to 30°) than the AD proteinase. Optimum activity was at pH 9, with a second peak of activity at pH 6 and at 45°. Differences in the temperature stabilities and optima of the two groups of proteinases may reflect possible differences in the role of the enzymes in the natural habitats for *Coprinus*. The more thermostable proteinases are perhaps used to degrade proteins in composts, herbivore dung and similar environments at temperatures at which other competitors are unable to grow or utilize the medium nutrients. The enzyme, though relatively unstable at higher temperatures, could provide the organism with the required amino acids and peptides before other micro-organisms are able to adapt to these conditions.

REFERENCES

BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein dye binding. *Analytical Biochemistry* **72**, 248–254.

COHEN, B. L. (1973). Regulation of intra- and extracellular neutral and alkaline proteases in *Aspergillus nidulans*. *Journal of General Microbiology* **79**, 311–320.

COHEN, B. L. (1980). Transport and utilization of proteins by fungi. In *Microorganisms and Nitrogen Sources* (ed. J. W. Payne), pp. 411–430. Chichester: Wiley.

COHEN, B. L. (1981). Regulation of protease production in *Aspergillus*. *Transactions of the British Mycological Society* **76**, 447–450.

DAVIS, B. J. (1964). Disc electrophoresis. II. Method and application to human serum proteins. *Annals of the New York Academy of Sciences* **121**, 404–427.

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DRUCKER, H. (1973). Regulation of exocellular proteases in *Neurospora crassa*: role of *Neurospora* proteases in induction. *Journal of Bacteriology* **116**, 593–599.

DRUCKER, H. (1975). Regulation of exocellular proteases in *Neurospora crassa*: metabolic requirements of the process. *Journal of Bacteriology* **122**, 1117–1125.

ERIKSSON, K.-E. & PETTERSSON, B. (1982). Purification and partial characterisation of two acid proteases from the white rot fungus *Sporotrichum pulverulentum*. *European Journal of Biochemistry* **124**, 635–642.

FERMOR, T. R. & WOOD, D. A. (1981). Degradation of bacteria by *Agaricus bisporus* and other fungi. *Journal of General Microbiology* **126**, 377–387.

HAMES, B. D. (1981). An introduction to polyacrylamide gel electrophoresis. In *Gel Electrophoresis of Proteins: a Practical Approach* (ed. B. D. Hames & D. Rickwood), pp. 1–91. London: IRL Press.

HANSON, M. A. & MARZLUF, G. A. (1973). Regulation of a sulphur-controlled protease in *Neurospora crassa*. *Journal of Bacteriology* **116**, 785–789.

KALISZ, H. M. (1988). Microbial proteinases. *Advanced in Biochemical Engineering* **36**, (In the Press.)

KALISZ, H. M., MOORE, D. & WOOD, D. A. (1986). Protein utilization by basidiomycete fungi. *Transactions of the British Mycological Society* **86**, 519–525.

KALISZ, H. M., WOOD, D. A. & MOORE, D. (1987). Production, regulation and release of extracellular proteinase activity in basidiomycete fungi. *Transactions of the British Mycological Society* **88**, 221–227.

KANDU, A. K. & MANNA, S. (1975). Purification and characterisation of extracellular proteinases of *Aspergillus oryzae*. *Applied Microbiology* **30**, 507–513.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.

LI, E. & YOUSTEN, A. A. (1975). Metalloprotease from *Bacillus thuringiensis*. *Applied Microbiology* **30**, 354–361.

MERRIL, C. R., GOLDMAN, D., SEDMAN, S. A. & EBERT, M. H. (1981). Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science* **211**, 1437–1438.

MORIHARA, K. (1974). Comparative specificity of microbial proteinases. *Advances in Enzymology* **41**, 179–243.

NORTH, M. J. (1982). Comparative biochemistry of the proteinases of eucaryotic microorganisms. *Microbiological Reviews* **46**, 308–340.

RINDERKNECHT, H., GEOKAS, M. C., SILVERMAN, P. & HAVERBACK, B. J. (1968). A new sensitive method for the determination of proteolytic activity. *Clinica Chimica Acta* **21**, 197–203.

ROBERTS, F. F. & DOETSCH, R. N. (1967). Purification of a highly active protease from a *Microsporum* species. *Antonie van Leeuwenhoek* **33**, 145–152.

SPECTOR, T. (1978). Refinement of the Coomassie blue method of protein quantitation. *Analytical Biochemistry* **86**, 142–146.

WESTERGAARD, J. L., HACKBARTH, C., TREUHAFT, M. W. & ROBERTS, R. C. (1980). Detection of proteinases in electrophoretograms of complex mixtures. *Journal of Immunological Methods* **34**, 167–175.

WINTER, A., EK, K. & ANDERSSON, U.-B. (1977). Analytical electrofocussing in thin layers of polyacrylamide gels. LKB Application Note no. 250.