Characteristics of intracellular peptidase and proteinase activities from the mycelium of a cord-forming wood decay fungus, *Serpula lacrymans*

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Serpula lacrymans is a basidiomycete cord-forming wood decay fungus which reallocates nitrogen within an extensive perennial mycelial system in response to spatial discontinuities in external nutrient supply. Intracellular stored protein is mobilised by conversion to amino acids at nutrient-poor sites within a mycelium or when a whole mycelium is starved. Intracellular peptidase and proteinase activities of the mycelium were investigated with the aim of identifying proteases specifically activated in response to a nitrogen demand. Mycelium for enzyme extraction grown as surface mats in static liquid culture was homogenised, and the extract used in assays for proteinase and peptidase with various synthetic peptide substrates conjugated to 4-nitroaniline. Activities against different substrates were characterised with respect to pH, inhibitor sensitivity, requirements for divalent metal ions, isoelectric point, and by changes in activities in starved mycelium. Four different activities were found, comprising two peptidases one of which had metalloprotease characteristics, a serine-type proteinase, and a proteinase active at pH 2.5 which was not affected by any of the inhibitors tried. Both the latter were most active in starved mycelium. Isoelectric focusing showed peaks with activities corresponding to the serine-type proteinase and one of the manganese-activated peptidases. Possible roles for these enzymes in nitrogen reallocation during mycelial foraging are discussed.

INTRODUCTION

Mycelium of saprotrophic and ectomycorrhizal cord forming fungi accumulates, immobilises and redistributes significant amounts of nitrogen within their habitats, thereby affecting the process of wood decomposition (Dighton 1997, Boddy & Watkinson 1995). Nitrogen accumulated from wood (Watkinson, Davison & Bramah 1981) and from the environment is stored in the mycelium as protein, which is hydrolysed when mycelium is starved of nitrogen (Venables & Watkinson 1989a), and transported through the mycelial network (Watkinson 1984), probably in the form of amino acids, to support new growth and colonisation of woody resources. Fungi forming such distributive networks include the woodland decomposer *Phanerochaete velutina*, the ectomycorrhizal *Suillus bovinus*, and the timber dry rot fungus *Serpula lacrymans*, reviewed respectively by Boddy (1993), Leake & Read (1997), and Watkinson (1999). In mycelial foraging, biomass is preferentially allocated to cord development in those parts of the hyphal network which encounter and colonise fresh food sources. Other parts of the mycelium which do not connect to fresh resources are autolysed and resorbed, a process which presumably involves local activation of intracellular hydrolases. The signal transduction pathway for the resource reallocation response following encounter with a localised fresh food source is not known. This investigation of intracellular proteases was undertaken in a search for the specific proteinases activated when non-bridging mycelium regresses. Ultimately, purification of enzymes could lead to development of a cellular reporter for an autolysis-inducing signal. Wadekar, North & Watkinson (1995) characterised four distinct proteinase activities of *S. lacrymans* by using SDS–PAGE to characterise bands in terms of inhibitor sensitivity and pH optima, and hydrolysis of azocasein to quantify net proteolytic activity in mycelial extracts. The purpose of using a range of synthetic substrates in this investigation was to differentiate the different protease activities present in mycelium, and to quantify and further characterise them, as a basis for investigating their physiological roles.

† Deceased.

MATERIALS AND METHODS

Organism and culture

Culture

Serpula lacrymans, culture 12C from the Forest Products Laboratory (Building Research Establishment, Garston, Watford), was subcultured and maintained on 2% malt agar (20 g l^{-1} malt extract, 15 g l^{-1} Oxoid no. 3 agar) at 22 °C.

Growth of mycelium and preparation of extracts for assays

Conical flasks, capacity 250 ml or 50 ml, containing respectively 50 or 20 ml of modified Czapek–Dox medium (g l^{-1}: glucose 2; Oxoid bactopeptone 1; KH_2PO_4, 1; KCl, 1; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.01), inoculated with 10 mm agar plugs cut submarginally from a 10-d-old agar culture and floated on the surface of the medium, were incubated for three to five weeks at 22 °C. Except where otherwise stated, mycelium was separated from the medium by vacuum filtration on nylon mesh, washed with 100 mM ice-cold KH_2PO_4/Na_2HPO_4 buffer pH 6.5, and fragmented by grinding in a mortar with a further 5–10 ml ice-cold buffer. The resulting mycelial fragments were homogenised with a Polytron homogeniser run at half maximum speed. The homogenate was centrifuged at 35 000 g for 20 min at 0 °, and the supernatant kept ice cold until used in the assay.

For analysing the effects of starvation, the peptone medium listed above was carefully removed by pipette, without damaging the floating mycelial mat, and replaced by salts-only medium in which the glucose and bactopeptone were omitted. Cultures were initially grown for 3 weeks in 50 ml flasks each containing 25 ml peptone medium. Experimental cultures were selected to be at a similar stage of growth, with mycelial mats just reaching the sides of the flask. The duration of the starvation period was varied by exchanging full medium for salts-only medium in two duplicate sets of three flasks at 4, 3, 2 and 1 d before the determination of enzyme activities, with two sets kept on full medium as a control. Mycelial mats were harvested in pooled sets of three from each set of replicate flasks, rapidly blotted on cellulose tissue and transferred to liquid nitrogen, then ground with a pestle. Fragments were suspended in 5 ml ice-cold 100 mM Tris/HCl buffer (pH 8.5). The mixture was homogenised and centrifuged as above. The supernatants from pooled mycelium harvested at each sampling period were assayed for activity with four substrates, and for protein by the assay of Bradford (1976).

Proteinase assays

Initial screen for suitable substrates

To assess the range of activities present various peptide substrates conjugated with *p*-nitroanilide were used. For 3 mM stock solutions, peptidase and proteinase substrates were dissolved in dimethyl formamide. Culture filtrate samples (50 µl) were added to microtitre plate wells each containing approx. 200 µl of stock solutions of synthetic peptidase or proteinase substrates diluted to 0.15 mM in three buffers to cover three pH values: 0.1 M sodium acetate/acetic acid (pH 5.0), 0.1 M sodium phosphate/phosphoric acid (pH 7.0) and 0.1 M sodium carbonate/HCl (pH 9.5). Further assays of some substrates were carried out over a wider range with buffers including 0.1 M glycine/HCl (pH 2.5). All synthetic substrates contained *p*-nitroanilide (pNA) coupled to the α-carboxylic group of a single amino acid or short peptide (two to four amino acid residues in length). Some of the substrates were blocked on the NH_2-terminus by either succinyl (Suc) or benzoyl (Bz) groups to prevent aminopeptidase action, but others were not blocked. The plates were agitated to mix, and covered with clingfilm for 1 hour's incubation at 35°, after which enzyme activity was assessed as light absorbance at 405 nm wavelength, using an ELISA plate reader.

Enzyme assays

Activities against 0.15 mM peptide substrates were measured by addition of 50 or 100 µl culture filtrate to the substrate in 950 or 900 µl of 100 mM buffer at 35 °. Initial rate of increase in A_{405} was measured continuously for 10 min in a recording dual-beam spectrophotometer. Activity is expressed as moles per minute per ml of mycelial extract, using the molar absorbance coefficient of 10 500 M^{-1} cm^{-1} (Beynon & Bond 1994). All subsequent experiments were done at the optimum pH. Chemicals used in assays were Analar grade or equivalent.

pH ranges

The effect of pH on activity was determined using mycelial extract over a pH range initially from 2.5 to 12 with each of four substrates at 0.15 mM. Buffers, at 100 mM, were: sodium phosphate/citric acid, pH values 2.5–5.5; sodium phosphate/phosphoric acid, pH values 5.5–8.0; Tris/HCl, pH values 7.5 and 8.5; glycine/HCl, pH values from 8.5–10.5 and sodium carbonate/NaOH, pH values from 9.5–11.5. Stock substrate solution (50 µl) was mixed with 900 µl buffer, followed by 50 µl of mycelial extract, and the enzyme activity was measured at 35 ° immediately after mixing. In some experiments 100 µl of extract was used in the assays, and the buffer volume was adjusted accordingly. Any cuvettes giving non-linear rates were discarded. All the pH/activity curves were carried out at least twice, using separate extracts. The curves shown in Fig. 1 are representative of the curves confirmed by replicate experiments. All subsequent experiments, below, were performed at the optimum pH for the enzyme activities, i.e. the buffer for assays of Arg-pNA hydrolysis and Leu-pNA hydrolysis was 100 mM Glycine/HCl, pH 8.5; for Suc-Ala-Ala-Pro-Phe-pNA hydrolysis, 100 mM Na_2HPO_4/phosphoric acid or 100 mM MES, pH 6.5; and for Bz-Phe-Val-Arg-pNA hydrolysis, 100 mM Na_2PO_4/citric acid pH 2.5.

Inhibitor sensitivities

Stock inhibitor solution (10 or 20 µl), diluted to give the appropriate concentration (see Table 2), was mixed with 900 µl of 100 mM buffer. The buffer and pH were chosen for optimum activity with the substrate. Mycelial extract (50 µl) was added, and then buffer, to give a total volume of 1 ml. Except where otherwise stated, inhibition was assayed immediately after adding the extract. When the inhibitor stock solution was in a solvent, the activity was compared to an assay containing the solvent at the same concentration.

Requirement for divalent cations

Mycelium from three pooled mycelial mats grown in 250 ml flasks was extracted in approximately 5 ml 0.1 M MES (2-[N-

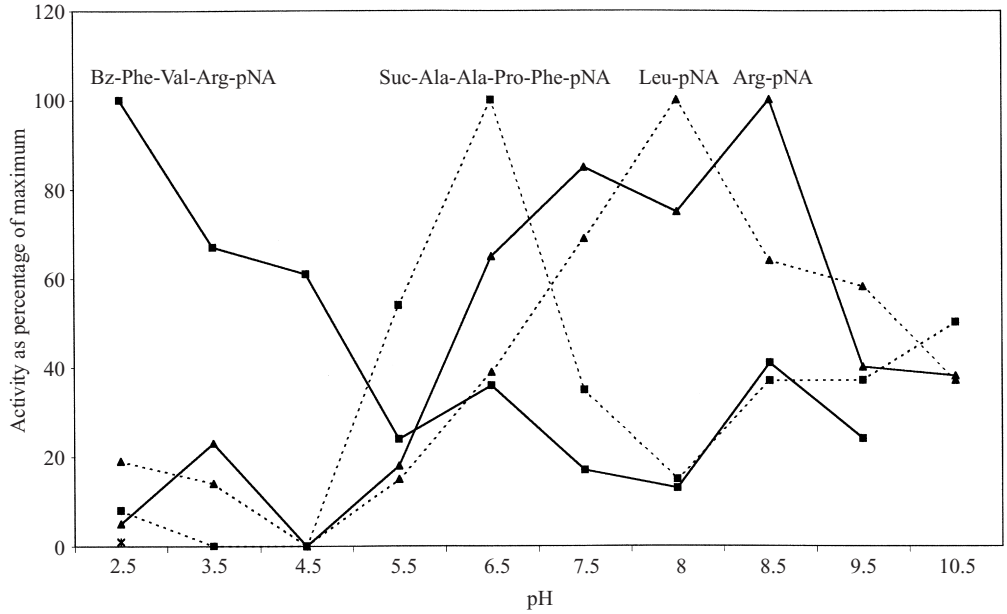

Fig. 1. Effect of pH on proteinase and peptidase activities. Symbols: Arg-pNA solid triangles, solid line; Bz-Phe-Val-Arg-pNA solid squares, solid line; Leu-pNA solid triangles, dotted line; Suc-Ala-Ala-Pro-Phe-pNA solid squares, dotted line. Activity is expressed as a percentage of maximal activity. Maximal activities in nmoles min^{-1} ml $extract^{-1}$ for each substrate were: Arg-pNA, 4.0; Bz-Phe-Val-Arg-pNA, 8.3; Leu-pNA, 5.9 and Suc-Ala-Ala-Pro-Phe-pNA, 5.2. Each experiment was carried out at least twice, and the curves shown are representative of the results obtained in every experiment. The buffers used were as described under Methods. At the pH values where two buffer ranges overlapped, at pH 5.5, 7.5 and 8.5, the point given is the average of the two values obtained.

morpholino]ethanesulphonic acid) buffer at pH 6.5. MES was preferred to the sodium phosphate buffer used in the other extractions, to avoid precipitation of metal phosphates. Mycelial extract was dialysed twice for 12 h at 0 °, each time in 2000 ml of 100 mM Tris/HCl buffer, pH 7.5, containing 200 μM EDTA. The activity of the dialysed extract against four substrates was measured before and after dialysis. The assays of dialysed extract were then repeated with the addition of a solution of each of seven divalent metal chlorides. For each assay 5 μl of a 100 mM metal chloride stock solution was added to 1 ml of the dialysed extract, thereby making a concentration of 500 μM added metal in the extract. Then 100 μl of the mixed extract and metal chloride was used in the assay, with the dilution of the extract adjusted to maintain a total volume of 1 ml.

The manganese concentration dependence of activity against Arg-pNA was investigated by adding 5, 10, 50, 100 and 200 μl of 100 mM manganese chloride to the assay, adjusting the dilution of the extract to maintain the volume of the assay mixture at 1 ml. Concentrations of manganese in these assays were thus 0.5, 1.0, 5.0, 10 and 20 mM.

Isoelectric focusing

Isoelectric focusing was performed in a Rotofor cell (BioRad). Pooled mycelium from 16 mycelial mats each grown on 50 ml peptone medium in a 250 ml flask for 5 wk, was homogenised in ice-cold sodium phosphate buffer (pH 6.5). The extract was dialysed overnight against 2000 ml of 100 mM Tris/HCl, pH 7.5, at 0 °. The 53 ml of extract obtained was mixed with 2 ml ampholyte, pH range 3–10 (BioRad). The extract-ampholyte mixture was transferred to the pre-cooled Rotofor column (5 °) immediately after mixing. Power was then applied and maintained at 12 W until 1 h after a plateau value of voltage indicated that the pH gradient had stabilised. Twenty samples were then collected by bleeding the column through manifold needles into ice-cold sample tubes, and their pH values immediately measured with a microprobe to record the pH gradient. Activities in the samples were assayed with four substrates. The activity of the extract against Arg-pNA was measured both before and after dialysis, and before and after mixing with ampholyte, to check that activity was still present after these procedures.

RESULTS

pH sensitivities of activities

The activities against substrates of differing composition, and their pH optima, inhibitor sensitivities and pI values, indicated a number of distinct proteinase activities present in the mycelial extract. Peptidase and proteinase substrates hydrolysed were Arg-pNA, Leu-pNA, Bz-Phe-Val-Arg-pNA, Bz-Tyr-pNA, Suc-Ala-Ala-Pro-Leu-pNA, Suc-Ala-Ala-Pro-Phe-pNA, and Suc-Ala-Ala-Val-Ala-pNA. Other substrates which were not hydrolysed were Glu-pNA, Ac-Ala-pNA, Ac-Leu-pNA, Bz-Arg-pNA, Suc-Ala-Ala-Val--pNA and Suc-Phe-pNA. Substrates blocked at the amino end by either a succinyl ('Suc') or benzoyl ('Bz') group, if hydrolysed indicate the activity of an endopeptidase or proteinase capable of breaking bonds in the middle of polypeptide chain (Beynon & Bond 1994). Four of the activities found were further characterised

Table 1. Percentage inhibition of enzyme activities of mycelial extract against five synthetic protease substrates. Molarities and concentrations are those in the assay mixture.

	Conc. in assay	Leu-pNA	Arg-pNA	Suc-$(Ala)_2$-Pro-Phe-pNA	Bz-Phe-Val-Arg-pNA	Suc-$(Ala)_2$-Pro-Leu-pNA
Inhibitor						
PMSF	1 mM	–	–	41	–	100
EDTA	1 mM	50	–	–	–	–
1,10-phenanthroline	1 mM	55	–	–	–	–
E64	100 μM	70	–	–	–	70
amino-benzamidine	1 mM	–	*24	–	–	nd

* Inhibition of activity against Arg-pNA by aminobenzamidine was obtained only after 20 min pre-incubation of inhibitor with mycelial extract before adding substrate. No inhibition was obtained with pepstatin, iodoacetate, iodoacetamide, TLCK, TPCK, soybean trypsin inhibitor, lima bean trypsin inhibitor or trypsin/chymotrypsin inhibitor.

Table 2. (a) The effect of removal of metal ions by double dialysis against 200 μM EDTA followed by readdition of various divalent metal cations (to 500 μM). (b) Concentration/reactivation curve for Mn^{++} mediated reactivation of activity against Arg-*p*NA. Activities are in nmoles min^{-1} ml $extract^{-1}$ in both (a) and (b).

(a)

Activity (nmoles min^{-1} ml^{-1})	Arg-pNA, pH 8.5	Leu-pNA, pH 8.5	Suc-$(Ala)_2$-Pro-Phe-pNA, pH 6.5	Bz-Phe-Val-Arg-pNA, pH 2.5
Activity pre-dialysis	2.9	2.4	0.5	0
Activity post-dialysis	0.4	0.1	0	0
Activity post-dialysis, after re-addition of each of the metal ions below:				
Fe^{++}	0.7	0.4	0.1	0.6
Ca^{++}	0.2	0.1	0	0
Co^{++}	1.6	1.3	0.6	0
Cu^{++}	0.1	0.6	0	0
Mg^{++}	0.1	0	0	0
Mn^{++}	4.2	4.2	0.2	0
Zn^{++}	0	0.5	1.3	0

(b)

Concentration of $MnCl_2$ in assay (mM)	0	0.5	5	10	20
Activity (nmoles min^{-1} ml $extract^{-1}$)	0.6	0.6	0.9	10.8	18.5

by pH/activity curves (Fig. 1). Activity against the peptidase substrate Arg-pNA was optimal at around pH 8.5, and against Leu-pNA at pH 8.0. The proteinase activities differed greatly in pH optima. Suc-Ala-Ala-Pro-Phe-pNA was hydrolysed fastest at pH 6.5, but the highest rate of activity against Bz-Phe-Val-Arg-pNA was at pH 2.5, the lowest pH achievable in the assays, with a shoulder between pH 3.5 and 4.5 indicating a second activity peak. No hydrolysis of this substrate was found at pH 2.5 in the absence of added mycelial extract.

Inhibitor sensitivity of activities

The different inhibitor sensitivities of activities against five substrates (Table 1) confirmed their distinct characteristics. Activity with both Suc-Ala-Ala-Pro-Phe-pNA and Suc-Ala-Ala-Pro-Leu-pNA was inhibited by the serine proteinase inhibitor PMSF (phenylmethanesulphonyl fluoride), although only the activity against Suc-Ala-Ala-Pro-Leu-pNA was sensitive to the cysteine proteinase inhibitor E64 (L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane), which also inhibited activity against Leu-pNA. Activity with Leu-pNA had metallopeptidase characteristics, being inhibited by the chelators EDTA and 1,10-phenanthroline, and also by E 64. Activity with Arg-pNA was not affected by any inhibitors apart from aminobenzamidine, a reversible inhibitor of trypsin-like enzymes, that developed only after incubating the extract with the inhibitor for 20 min before adding the substrate and buffer to the assay (Table 1). Activity against Bz-Phe-Val-Arg-pNA could not be inhibited with any of the 13 inhibitors tried.

The effects of divalent metal ions

Dialysis to remove metal ions (Table 2) greatly decreased the activity of mycelial extract with both Arg-pNA and Leu-pNA. There was also a decrease with Suc-Ala-Ala-Pro-Phe-pNA, although the initial activity was too low for this effect to be unequivocal. Readdition of manganese, and to a lesser extent cobalt, resulted in an increase to higher than pre-dialysis levels of activity against both Arg-pNA and Leu-pNA. Neither activity was restored significantly by the readdition of zinc. Activity against Suc-Ala-Ala-Pro-Phe-pNA, a serine protease in terms of inhibitor sensitivity, was restored to higher than pre-dialysis levels by zinc, but not manganese. Activity on Arg-pNA measured in relation to manganese concentration (Table 2b) showed a sharp increase between 5 and 10 mM, and was still increasing at 20 mM.

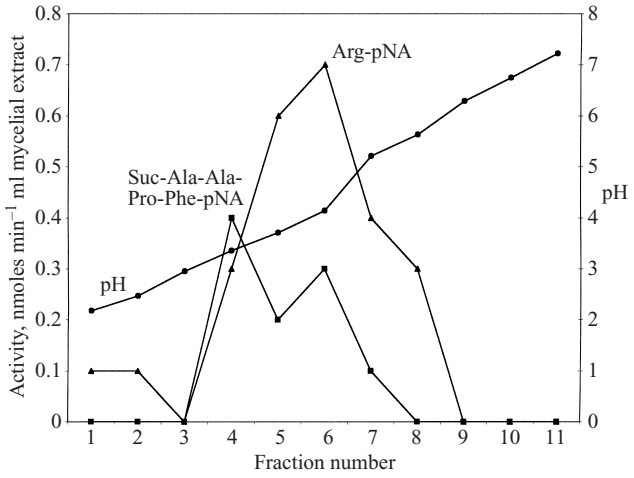

Fig. 2. Isoelectric focusing using a Rotofor cell. Substrates: ▲, Arg-pNA activity; ■, Suc-$(Ala)_2$-Pro-Phe-pNA; ●, pH gradient. Activities are given in nmoles min^{-1} ml extract^{-1}. Two replicate runs were carried out for each substrate (one representative run for each shown here). No significant activity against any other of the substrates in Fig. 1 was recovered from the column.

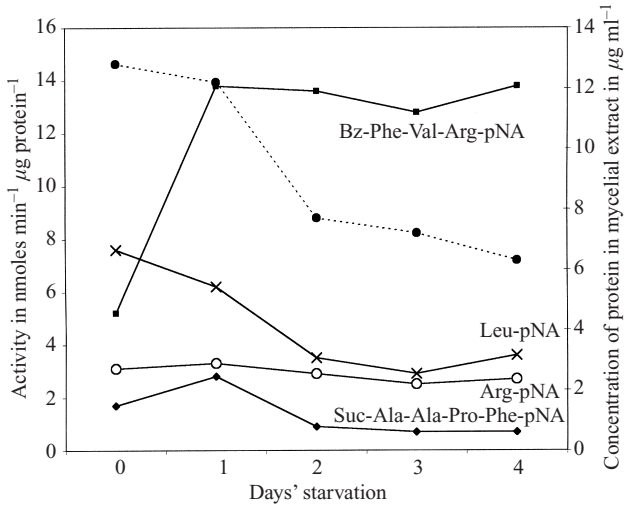

Fig. 3. Activity against four substrates in extracts of mycelium that had been starved for 0, 1, 2, 3 and 4 days, and total protein as μg/ml of mycelial extract. Results are means from duplicate extracts, each assayed in triplicate. Activities are given in nmoles min^{-1} μg of protein^{-1}. Substrates: ■, Bz-Phe-Val-Arg-pNA; X, Leu-pNA; O, Arg-pNA; ◆, Suc-Ala-Ala-Pro-Phe-pNA. Dotted line indicates total protein.

Isoelectric points

Isoelectric focusing (Fig. 2) revealed that activity against Arg-pNA has pI 3.4, and activity against Suc-Ala-Ala-Pro-Phe-pNA has pI 4.3. Activity against Bz-Phe-Val-Arg-pNA and Leu-pNA was not recovered from this procedure.

Differential effects of starvation on activities

Two of the enzyme activities increased as a result of starving the mycelium prior to assay (Fig. 3). Activity against Bz-Phe-Val-Arg-pNA was more than doubled in mycelium that had been starved for 1, 2, 3 or 4 d. Activity against Suc-Ala-Ala-Pro-Phe-pNA was nearly doubled in mycelium starved for one day, but decreased again after 2, 3 or 4 d starvation. Starvation halved activity against Leu-pNA after 2 d, and it was also low at 3 and 4 d starved cultures. The activity against Arg-pNA was relatively unaffected by starvation, remaining steady in all treatments.

DISCUSSION

Two of the enzyme activities characterised showed the type of regulation that we sought, increasing on starvation – the activities against Suc-Ala-Ala-Pro-Phe-pNA and Bz-Phe-Val-Arg-pNA. They would therefore both appear to be candidates for a role in the breakdown of intracellular protein. However, the very low pH optimum of Benz-Phe-Val-Arg-pNA activity suggests a vacuolar location and a lysosomal role in dead or dying cells, or in extracellular proteolysis, rather than in regulating the cellular processes of growing, healthy mycelium. In its inhibitor insensitivity it shows the robustness characteristic of extracellular enzymes. *Serpula lacrymans*, unlike other wood decaying basidiomycetes, exudes a widely-diffusing and potent proteinase (Venables & Watkinson 1989b). Its mycelium also autolyses very readily under nitrogen starvation on carbon-rich media, with accompanying acidification of the culture medium to pH 2.8. This destructive proteolysis might be the role of the highly active acid endoprotease identified by its hydrolysis of the substrate Bz-Phe-Val-Arg-pNA. The serine protease detected with Suc-Ala-Ala-Pro-Phe-pNA seems the more likely candidate for an enzyme with the function of releasing amino acid in a controlled manner from stored protein without cell damage. Starvation induced a transient activation of this enzyme, in keeping with a physiological role in healthy mycelium which has an average pH in *S. lacrymans* of 6.5. Its activity was highest in mycelium starved for only one day, and after longer periods of starvation activity was much lower. Inhibition by PMSF tends to characterise this activity as a serine protease, a type known from many fungi (North 1982), and known to play many physiological roles, for example in attack on insects by entomopathogenic fungi. The results suggest a role for metals in activation of the serine protease-type activity found in *S. lacrymans*. Dialysis for 12 h reduced activity, and readdition of zinc restored it. Serine proteinases typically have a stabilising metal and it is possible that zinc plays this role. Addition of chelators did not inhibit activity, which could be interpreted to mean that the metal has a stabilising rather than a catalytic role. Investigation of *S. lacrymans* intracellular proteases by SDS–PAGE and azocaseinase assay showed relatively low serine protease activity, although a serine protease sensitive to PMSF was found in the wood decay fungus *Coriolus versicolor* (Wadekar, North & Watkinson 1995). A serine proteinase characterised from *Agaricus bisporus* fruiting bodies (Burton *et al.* 1993) was implicated in protein hydrolysis for mobilisation of amino acids from stipe to cap during maturation of harvested sporophores (Burton *et al.* 1997), a similar function to that postulated for the *S. lacrymans* serine proteinase described here. Other serine proteinases are

involved in attack on the insect host by entomopathogenic fungi (Khatchatourians 1996). It appears that serine proteinases are widespread in fungi, and play a variety of physiological roles.

The two peptidase activities stimulated by readdition of cobalt and manganese after dialysis, and active against Leu-pNA and Arg-pNA, had similar, slightly alkaline pH optima, and neither was activated by starving the mycelium. Inhibition by the chelators EDTA and 1,10-phenanthroline confirmed the metalloproteinase character of the activity against Leu-pNA. All three effective inhibitors of this enzyme were optimally effective at pH 4.5 (data not shown). Leu-pNA activity was greatly reduced by starvation, and the fact that it decreases when growth stops, but is high in well-nourished mycelium, suggests a role for this enzyme in active mycelial growth. An example of a proteases playing a part in active growth are those known to be required for chitin synthetase activation for wall growth in *Mucor rouxii* and *Candida albicans* (North 1982, Gooday 1995).

The other peptidase activity, against Arg-pNA, although similar to that against Leu-pNA in being activated by readdition of manganese and cobalt after double dialysis, was distinguished from it in being unaffected by the chelators EDTA or 1,10-phenanthroline. Moreover, it was inhibited only by aminobenzamidine, which did not inhibit activity on Leu-pNA. Its activity was unaffected by the nutritional state of the mycelium.

The large effect of readdition of manganese on Leu-pNA and Arg-pNA hydrolysing activity after dialysis is noteworthy. It may not however be representative of physiological activation of the enzymes. Manganese dependent proteases are rare (Frausto da Silva & Williams 1991), with only cytosolic proline aminopeptidase and proline dipeptidase reported (Auld 1995). It is therefore most likely the activation of these enzymes by manganese and cobalt seen in our experiments was an artefact, and that zinc is the metal bound under physiological conditions. Some Zn metalloproteinases with two zinc atoms can be reconstituted with one manganese and one zinc atom to give far higher activity, as was shown by Carpenter & Vahl (1973) with a bovine leucine aminopeptidase. Our reactivation curve, where activity against Arg-pNA continued to increase as manganese was raised up to the high level of 20 mM, indicates a relatively weak binding of manganese atoms. Zinc is bound 35 times more strongly than manganese by zinc metalloproteases (Carpenter & Vahl 1973), and cytosolic zinc concentration is typically twenty times that of manganese (Auld 1995). Therefore, we consider that manganese binding may not significantly affect activity *in vivo*, even if it gives an enzyme with above-normal activity in assays.

We conclude that there is evidence for two intracellular proteinase activities implicated in mycelial development in *Serpula lacrymans*, one active under acid and the other under neutral conditions, which can be assayed with the substrates Bz-Phe-Val-Arg-pNA and Suc-Ala-Ala-Pro-Phe-pNA respectively. We speculate that the role of the acid proteinase is lysosomal, in proteolysis during hyphal death, and may play a part in creating the empty, aseptate 'vessel' hyphae of mycelial cords. It may also be responsible for the unusually active extracellular proteolysis which is characteristic of *S. lacrymans* compared with other wood decay fungi (Venables & Watkinson 1989b). *S. lacrymans* acidifies Czapek-Dox medium to pH as low as 2.5, the optimum pH for this enzyme. The neutral proteinase appears to have the characteristics expected in an intracellular proteinase concerned with the mobilisation of protein stores within a living mycelial system. Its pH optimum is the same as the pH of fungal cytoplasm, and it is only transiently activated in response to starvation, rather than remaining high during starvation-induced autolysis. This enzyme is therefore the most appropriate for further investigation of the activation pathway of nitrogen reallocation during mycelial foraging in this fungus.

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We dedicate this publication to the memory of David Wood.

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