Production and Localization of Proteinases in Colonies of Timber-decaying Basidiomycete Fungi

By C. E. VENABLES AND S. C. WATKINSON*

Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RA, UK

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Proteinase activity was detected in culture filtrates of eight wood-decaying basidiomycete fungi and compared in terms of ability to clear skimmed milk agar. All the fungi were proteolytic, but to differing extents. Five were compared using azocasein hydrolysis as a measure of proteolytic activity at the centres and margins of agar-grown colonies and it was found that in *Coriolus versicolor* the marginal mycelium was the more strongly proteolytic, while in all the other fungi proteolysis by central mycelium was greater. The time course of changes in proteolytic activity in culture filtrates of *C. versicolor* and *Serpula lacrimans* grown on the surface of liquid media was compared over 4 weeks, and differences were found which suggested that *C. versicolor* mycelium inactivates its proteinases after secreting them, but that *S. lacrimans* does not. The results are interpreted in terms of the likely role of proteinases in the nitrogen economy of these fungi when growing on their wood substrates.

INTRODUCTION

Timber-decaying fungi whose sole substrate is wood and which have no other source of nutrients must obtain all their cellular nitrogen from this source. Protein is the most abundant form of nitrogen in wood, according to the analysis by Laidlaw & Smith (1965) of *Pinus sylvestris* sapwood, though amino acids, nucleic acids, inorganic compounds and alkaloids are also present. Thus proteinases are likely to be among the extracellular enzymes with which such fungi degrade and utilize their substrate. Both extracellular and intracellular proteinases are produced by various fungi, and one species may produce several different proteinases although each is active under different conditions (North, 1982). Extracellular proteinases have received most attention and have been found to be controlled by both induction and repression mechanisms. Basidiomycete proteinases are less well explored than those of Ascomycetes and Deuteromycetes. Control of proteinase production in the basidiomycete timber-decaying fungus *Trametes (Coriolus) versicolor* during nitrogen starvation has been investigated by Staszczak & Nowak (1984). Proteinases from both medium and mycelium grown under conditions of nitrogen starvation and sufficiency were separated by gel electrophoresis. Under nitrogen starvation the pattern of bands changed. Of five extracellular proteinase bands, one disappeared and another intensified. The changes accompanied a shift to secondary metabolism and the onset of ligninolysis.

In the nitrogen nutrition of timber-decaying fungi, proteinases probably have functions both in primary attack on wood protein, and in acting on senescent mycelium to retrieve fungal protein. (Regulatory functions are also likely, but are not considered here.) Location within the whole colony should indicate which of the two functions a proteinase is performing. Enzymes involved in primary attack would be expected at or near the growing colony margin, like the proteinase of *Aspergillus niger* (Yanagita & Nomarchi, 1967). Those concerned with protein retrieval would be active in older, stationary-phase mycelium at the centre of colonies.

The rate of net protein breakdown has been shown in *Serpula lacrimans* to depend on the age of the mycelium and the concentration of available nitrogen in the medium (Venables &
extracellular proteolysis was found near growing mycelium of C. bisporsus, Coprinus cinereus and Volvariella volvacea showed differences between these species in the time and location of maximum proteolytic activity: most extracellular proteolysis was found near growing mycelium of C. cinereus and V. volvacea but in A. bisporsus older, non-growing mycelium was the most proteolytic.

The aim of this work was to investigate the occurrence of proteolytic activity in a range of timber-decaying Basidiomycetes, and to begin to analyse the spatial distribution of proteolytic activity in whole colonies with a view to understanding its role in the physiology of these fungi.

METHODS

Cultures. The following fungal isolates were kindly supplied by the Princes Risborough Laboratory of the Building Research Institute: Serpula lacrimans (Schum. ex Fr.) Karst (11E), Coniophora puteana (Schum. ex Fr.) Murrill (108N), and Lentinus lepideus (Fr. ex Fr.) (7H), all causing brown rot of timber; Coriolus versicolor (L. ex. Fr.) (28A), and Pleurotus ostreatus (Jacq. ex Fr.) Quel (40C), both causing white rot. Isolates of Stereum sanguinolentum (A. & S. ex Fr.) Fr. and (white rot) and Phlebia (Peniophora gigantea (Fr. ex Fr.) Donk (brown rot) were supplied by the Plant Pathology Department of the Forestry Commission. Cultures were maintained on 2% (w/v) malt agar at 25 °C but grown on distilled water agar prior to their use as inoculum discs on skimmed milk agar plates, in order to avoid carrying over nitrogen compounds which might repress proteolytic activity.

Proteinase assay. The assay used was based on that of Prestidge et al. (1971) for extracellular proteinase. Azocasein hydrolysis was determined at 25 °C in a reaction mixture containing 0-3 ml culture filtrate (two samples from two or more replicate culture filtrates strained through two layers of muslin), 0-1 ml buffer (see below), 0-1 ml deionized water and 0-5 ml 2% (w/v) freshly prepared azocasein (Sigma). Perchloric acid (2 ml; 7%, w/v) was added after 1 h to stop the reaction and the tubes were spun at 5000 g for 5 min in a benchtop centrifuge. In the control tubes, perchloric acid was added before the addition of the azocasein. Supernatant, 2-7 ml, was added to 0-4 ml 10 M-NaOH solution and after mixing, the absorbance was measured at 436 nm. Proteinase activity was expressed as mg azocasein hydrolysed during 1 h per ml of culture filtrate.

Choice of buffer. The optimum pH for proteolytic activity was established for each fungus using a range of buffers: 0-1 M-citrate/phosphate, pH 3-0-5-5: 0-05 M-MES/HCl, pH 5-9; and 1 M-Tris/HCl, pH 7-0. The pH value chosen for experiments on each fungus was that at which maximum proteolysis was shown in a preliminary experiment testing each fungus over a range of pH.

Growth on skimmed milk agar. Skimmed milk agar [1% (w/v) skimmed milk (Difco) and 1-5% (w/v) purified agar (Oxoid)], autoclaved at 121 °C for 15 min, was dispensed in 60 ml volumes to each of twenty-four 15 cm Petri-dishes. These were centrally inoculated with 8 mm discs of each fungus cut from colonies on water agar, three replicate plates for each fungus. The S. lacrimans plates were inoculated at 22 °C, the others at 25 °C. After 9 and 11 d the diameters of the colonies and the widths of the surrounding cleared agar zones were measured in two directions in an attempt to assess the proteolytic activity at the circumference of the colonies.

When fungal colonies neared the edge of the Petri dish the azocasein test was carried out as follows to compare the proteolytic activity of old (central) and young (marginal) hyphae. Three 12 mm discs of mycelium with underlying agar medium were cut with a cork borer from around the central inoculum disc and three from the margin of the colonies of all three plates. These discs were placed in test-tubes containing 0-1 ml of the appropriate buffer and 0-4 ml deionized water. Then 0-5 ml 2% (w/v) azocasein solution was added. One of the three discs in each case was treated as a control with the addition of perchloric acid before the azocasein.

Growth on liquid medium. Ser. lacrimans and Cor. versicolor were grown as floating colonies on liquid medium so that extracellular proteinase production could be assessed at twice-weekly intervals. Bacto-peptone medium (1% w/v, Bactopeptone, 0-3% yeast extract and 0-2% glucose per litre distilled water) was dispensed in 10 ml volumes to 25 ml conical flasks which were stoppered with foam bungs, covered with aluminium foil and autoclaved at 121 °C for 15 min. After cooling, the flasks were inoculated with floating 8 mm discs of mycelium plus agar cut submarginally from 2% malt agar plates. The flasks were incubated at 22 °C (Ser. lacrimans) and 25 °C (Cor. versicolor) and four flasks of each fungus harvested at 3 or 4 d intervals. The culture filtrate from each flask was strained through two layers of muslin and the mycelial mat separated from the agar disc and dried at 50 °C to constant weight. Samples of culture filtrate were tested for proteinase. On day 11, the culture filtrates from four flasks of each fungus were filtered separately and aseptically through Millipore filters (type GS, pore size 0-22 μm) to four sterile flasks using sterile 5 ml syringes. From day 14, samples of this mycelium-free culture filtrate were analysed as for proteinase activity as were samples from previously unsampled flasks with mycelium remaining. Proteolytic activity was calculated as units per ml culture filtrate.
RESULTS AND DISCUSSION

All the fungi tested showed some proteolytic activity but there were differences between fungi both in amount and in localization in the colony. Proteolysis was measured at the pH giving maximum activity: Coniophora puteana and Gloeophyllum trabeum, pH 4.0; Coriolus versicolor and Pleurotus ostreatus, pH 5.9; Lentinus lepideus, pH 7.0; Phlebia gigantea, pH 5.0; Serpula lacrimans, pH 5.5; and Stereum sanguinolentum, pH 3.0. Table 1 shows proteolysis by intact colonies of different fungi growing in contact with a substrate, and Table 2 shows proteolysis by excised mycelial discs taken from either the centres or the margins of colonies. Fig. 1 is based on measurement of proteolysis by culture filtrates from static liquid cultures of two fungi. It is necessary to bear in mind the different methods used to measure mycelial proteolytic activity when interpreting the results.

Proteolysis measured by clearing of skimmed milk agar

The width of the cleared zones in skimmed milk agar (Table 1) must depend not only on the diffusion rate and activity of proteinase but also on the rate at which the colony extends to cover the zone which has been cleared. Thus a relatively narrow cleared zone around a relatively large colony, as seen in G. trabeum and Cor. versicolor, need not mean that these fungi are less actively proteolytic than those with a wider cleared zone and smaller colony. However, legitimate comparisons may be made between fungi with similar sized colonies and indicate that Con. puteana and Pl. ostreatus cleared the medium faster than St. sanguinolentum. Colonies of Ser. lacrimans were exceptional in the way that the width of the cleared zone increased faster than the rate of extension of the mycelium. This fungus thus appears to differ from the others in the physiology and/or biochemistry of its proteolysis. Possible differences could be in the rate of diffusion of the proteinase, or in the location and timing of proteinase production or inactivation. Results described below suggest that Ser. lacrimans is able to continue to produce proteinase from older mycelium after initial growth has ceased.

Proteolysis by mycelium from different parts of colonies

The azocasein test on excised discs of mycelium was used to compare proteolytic activity in old and young regions of a single colony on agar sampled at the same time (Table 2). Proteolysis by discs taken from the centres and margins of colonies was assayed for five different fungi. As this involved cutting the hyphae and transferring excised discs to assay tubes it is possible that some of the proteolytic activity measured was not truly extracellular as in the previous experiment. Two very different patterns of localization of proteolysis were found. In colonies of Cor. versicolor the activity measured was three- to fourfold greater in mycelium taken from the margin than in mycelium from the centre, while in the four other fungi activity was much greater in the central older mycelium than at the colony edge. Further work is necessary to interpret these results, in particular to establish whether there is a difference in proteolytic activity of different regions of the colony in vivo in intact mycelium, and if so whether the proteinase is intracellular or secreted into the medium.

The location of proteolysis in the colony is likely to be important in regulating nitrogen turnover as the fungus advances through its wood substrate. Proteinases produced by growing hyphae probably solubilize wood protein for assimilation, while those produced in older mycelium may break down cellular protein for possible translocation and re-use. Other wood-decaying enzymes may be affected by the activity of proteinases in their vicinity. If the suppression of ligninolysis by nitrogen compounds in the medium, which Fenn & Kirk (1981) have demonstrated for Phanerochaete chrysosporium, is a feature of white rot fungi in general, it might be expected that in these fungi extracellular proteolysis would need to be spatially separate from ligninolysis since proteinase produces free amino acids which could repress ligninolytic enzyme activity. It may thus be necessary for white rot fungi such as Cor. versicolor to limit protein breakdown in older mycelium.
Table 1. *Diameters of colonies and width of cleared zone on skimmed milk agar plates*

Values are the means of two measurements (taken perpendicularly to each other) from each of three replicate plates.

<table>
<thead>
<tr>
<th>Fungus</th>
<th>Mean width of cleared zone diameter (mm)</th>
<th>Mean colony diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9 d</td>
<td>11 d</td>
</tr>
<tr>
<td><em>Coriolus versicolor</em></td>
<td>3-0</td>
<td>4-5</td>
</tr>
<tr>
<td><em>Pleurotus ostreatus</em></td>
<td>21-2</td>
<td>24-7</td>
</tr>
<tr>
<td><em>Coniophora putea</em></td>
<td>12-0</td>
<td>10-8</td>
</tr>
<tr>
<td><em>Gloeophyllum trabeum</em></td>
<td>2-8</td>
<td>5-0</td>
</tr>
<tr>
<td><em>Lentinus lepideus</em></td>
<td>3-5</td>
<td>3-7</td>
</tr>
<tr>
<td><em>Serpula lacrimans</em></td>
<td>8-3</td>
<td>23-2</td>
</tr>
<tr>
<td><em>Phlebia gigantea</em></td>
<td>3-3</td>
<td>2-7</td>
</tr>
<tr>
<td><em>Stereum sanguinolentum</em></td>
<td>3-8</td>
<td>5-3</td>
</tr>
</tbody>
</table>

Table 2. *Proteolytic activity at centres and margins of fungal colonies*

Results are the means and standard errors of two readings from each of three replicate plates. Proteolytic activity is expressed as mg azocasein hydrolysed per 12 mm disc of mycelium plus agar, during 1 h.

<table>
<thead>
<tr>
<th>Fungus</th>
<th>Proteolytic activity</th>
<th>Margin</th>
<th>Centre</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Coriolus versicolor</em></td>
<td>5-40 ± 0-02</td>
<td>1-50 ± 0-56</td>
<td></td>
</tr>
<tr>
<td><em>Gloeophyllum trabeum</em></td>
<td>0-36 ± 0-02</td>
<td>3-80 ± 0-02</td>
<td></td>
</tr>
<tr>
<td><em>Lentinus lepideus</em></td>
<td>0-05 ± 0-16</td>
<td>1-31 ± 0-08</td>
<td></td>
</tr>
<tr>
<td><em>Serpula lacrimans</em></td>
<td>0-14 ± 0-06</td>
<td>2-86 ± 0-08</td>
<td></td>
</tr>
<tr>
<td><em>Phlebia gigantea</em></td>
<td>0-08 ± 0-06</td>
<td>1-08 ± 0-18</td>
<td></td>
</tr>
</tbody>
</table>

*Proteolysis in culture medium from colonies of different ages*

The effect of the age of the mycelium, and its presence or absence, on the activity of proteinase in the medium was investigated for *S. lacrimans* and *C. versicolor*. Proteolytic activity of culture filtrates was measured over a four week period (Fig. 1). *S. lacrimans* continued to release proteinase after dry weight increase had ceased at 14 d. Mycelium of *C. versicolor* on the other hand began to autolysé at 10 d and proteolytic activity also declined from 14 d onwards. At the end of the experiment the dry weights were similar for the two fungi but the proteolytic activity in the medium was about five times greater in *S. lacrimans*. In order to see whether the decline in proteinase activity in *C. versicolor* medium occurred because the mycelium itself was able to inactivate proteinase in the medium, proteolytic activity was also measured in a parallel series of cultures of both fungi in which the mycelial mat was removed after 11 d. With *C. versicolor*, but not *S. lacrimans*, the level of activity declined faster and to a lower level when mycelium was present. A mechanism for the resorption of enzyme protein seems the most likely cause. However, even after mycelium had been removed, some decline in activity still occurred, persisting for 8 d until the level stabilized and remained constant or even slightly increased thereafter. One explanation could be that the fungus releases both proteinase and a further enzyme, involved in the breakdown of proteinase itself, whose residual activity causes a decline in proteolytic activity of the culture filtrate even for a period after removal of the mycelium. Proteinase of *C. versicolor* is less stable than laccase of *Agaricus bisporus* (Wood, 1980) which did not change in activity during 7 d in sterile culture filtrate.
Fig. 1. The time course of changes in fungal dry weight (○), and of proteolytic activity in the culture filtrate in the presence of mycelium (■) and after its removal on day 11 (▲). Each point represents the mean of three separate replicate cultures. Proteolytic activity in samples of culture filtrate was expressed as mg azocasein hydrolysed h⁻¹ (ml of culture filtrate)⁻¹.

This experiment tends to validate the results shown in Table 2 as showing a genuine difference in the pattern of proteinase secretion with the age of the mycelium. It also makes it probable that the progressive clearing of skimmed milk agar by *Ser. lacrimans* colonies showing little radial growth (Table 1) was due to outward diffusion of proteinase which continued to be produced and secreted by the older parts of the colony, and which in this fungus is not destroyed by the mycelium.

In applying these results to understanding the physiology of the colony *in vivo* it must be remembered that enzymes and their products are unlikely to diffuse so freely away from hyphae when they are growing in wood. The growth of fungal mats on liquid media is so far removed from the growth of mycelium on wood that direct comparisons cannot be made. Release of enzyme protein into a medium so that the fungus has effectively lost control of the enzymes would not appear to be a very beneficial adaptation to a nitrogen-poor substrate. The fungal sheath is probably instrumental in retaining enzymes and products of digestion close to the hyphae (see Palmer *et al.*, 1983a, b). Thus the technique of growing fungi in liquid culture although useful in analysing enzyme activities is not a very good model for the natural functions of extracellular enzymes since it allows diffusion away from the mycelium to a much greater extent. Experiments with skimmed milk agar approximate more closely to the natural substrate.

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REFERENCES


