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PRODUCTION, REGULATION AND RELEASE OF EXTRACELLULAR PROTEINASE ACTIVITY IN BASIDIOMYCETE FUNGI

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Agaricus bisporus, *Coprinus cinereus* and *Volvariella volvacea* were grown on defined liquid media under conditions of proteinase induction and derepression. Growth of the organisms was compared with protein utilization and extracellular proteinase activity. All three species utilized protein as sole source of C, N and S. Total proteinase activity was not repressed by supplementation of protein-containing medium with glucose, ammonium or sulphate. The production of *Coprinus* proteinase activity was also derepressed in protein-free media in the absence of sulphate. Protein utilization commenced before release of significant extracellular proteinase activity. The *Coprinus* and *Volvariella* activities were associated with hyphal growth but the *Agaricus* proteinase activity was not.

In fungi, as in bacteria, extracellular proteinases are produced by many species from each of the major taxa (Matsubara & Feder, 1971; North, 1982). Although much information is available about the physicochemical properties of fungal extracellular proteinases, little is known about the mechanisms controlling their production. Most work on the mechanism of biosynthesis of extracellular proteinases in fungi has dealt with *Aspergillus* species (Cohen, 1972, 1981) and *Neurospora crassa* (Hanson & Marzluf, 1973; Drucker, 1975).

In *Aspergillus* species, proteinase production is controlled by derepression in the absence of a carbon, nitrogen or sulphur source (Tomonaga, Ohama & Yanagita, 1964; Cohen, 1973*a*, 1981). These organisms secrete at least four proteinases, three of which are stable and functionally different from the intracellular proteinases (Cohen, 1973*b*). Derepression involves de novo synthesis (Cohen, 1973*b*). Proteinase production in *Neurospora* is controlled by induction and repression (Hanson & Marzluf, 1973; Drucker 1973, 1975). As in *Aspergillus*, induction involves de novo synthesis, and the secreted proteinases exhibit different properties from the intracellular enzymes (Heiniger & Matile, 1974). The proteinases are induced when protein is present with one of the major elements (carbon, nitrogen, sulphur) otherwise absent. In the presence of protein and all three alternative sources of the major elements proteinase production becomes repressed (Drucker, 1972; Hanson & Marzluf, 1973). Peptides and amino acids do not cause induction (Hanson & Marzluf, 1973) but certain amino acids inhibit proteinase synthesis when added to media deficient in one of the major elements (Cohen & Drucker, 1977).

An ericoid mycorrhizal fungus, *Hymenoscyphus ericae*, used protein as a source of both nitrogen and carbon; proteolytic activity being produced in the presence of alternative carbon sources (Bajwa & Read, 1985; Spinner & Haselwandter, 1985). Some ectomycorrhizal fungi have also been shown to use protein as a source of nitrogen (Abuzinadah & Read, 1986*a*), to the benefit of their symbionts (Abuzinadah & Read 1986*b*; Abuzinadah, Finlay & Read, 1986), although little or no proteinase activity has been detected in culture filtrates of ectomycorrhizal fungi (Lundeberg, 1970). The saprotrophic basidiomycetes *Agaricus bisporus*, *Volvariella volvacea* and *Coprinus cinereus* have been shown to use protein readily as a source of carbon, nitrogen or sulphur (Kalisz, Moore & Wood, 1986) and, as judged from the continued catabolism of protein in the presence of glucose or ammonium, appear to be free of the catabolite repression seen in lower fungi (Cohen, 1980). This is probably of importance to these litter-degraders as protein, in the form of proteins or peptides bound to lignin, microbial protein and plant protein, probably represents the most abundant nitrogen source available to these organisms in their natural habitats and is clearly a valuable carbon source.

Little is known about the regulation of protein degrading systems of basidiomycetes. Enzymes

secreted by *Agaricus* growing on compost include laccase, cellulases, xylanase, proteinases, phosphatases and laminarinase (Wood & Fermor, 1985). When growing on bacteria or protein as sole nitrogen and carbon sources *Agaricus* produces acid, neutral and alkaline proteinase activities (Fermor & Wood, 1981). *Coprinus* fruit body tissues produce proteinases in their autolytic 'ink' (Iten, 1970) but no study has been made on proteinase production by vegetative mycelia of either *Coprinus* or *Volvariella*.

The present study was undertaken to determine the conditions under which total extracellular proteinase activity is expressed.

MATERIALS AND METHODS

Organisms and culture conditions

Agaricus bisporus strain D791, the Indonesia strain of *Volvariella volvacea* and Birmingham dikaryon strain (ATCC 42721) of *Coprinus cinereus* were cultured, manipulated and harvested as described before (Kalisz *et al.*, 1986)

Assay of extracellular proteinase

Extracellular proteinase was assayed at pH 7·0 (phosphate buffer) and pH 9·0 (glycine-NaOH buffer), using a proteinase halo test (Cohen, 1981) on milk agar plates composed of 1% (w/v) skimmed milk (Oxoid), 0·1 M buffer in 1·8% (w/v) agar (Lab 'm' MC2). A sample (50 μl) was added to wells 3 mm diam and 8–9 mm deep, and the plates incubated at 25 °C for 22 h. The diameter of clearance zones around the wells was measured and the concentration of enzyme calculated using Sigma Grade VI pancreatin as a standard. One unit of activity was defined as the mount of enzyme exhibiting equivalent activity to 1 μg of pancreatin.

In this account proteinase enzyme activity recovered from culture filtrates is described either as 'volume activity' (= units or kilo-units per flask of 25 ml of medium irrespective of the amount of mycelium) or as 'specific activity' (= units per mg dry weight of mycelium).

Mycelial dry weight and residual protein in the medium were measured as described by Kalisz *et al.* (1986).

RESULTS

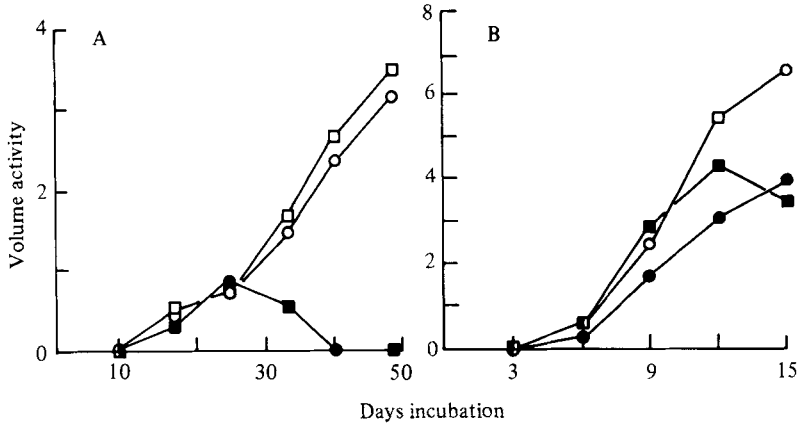
Experiments were carried out with twelve different medium constitutions; eight of the mixtures contained protein (as 1% insoluble casein) and in one this was the sole source of C, N and S; the other seven had all possible combinations of single, double and triple supplementation with additional carbon (1% glucose), nitrogen 30 mM NH_4Cl) or sulphur (0·05% $MgSO_4 . 7H_2O$). Of the remaining four mixtures which lacked protein, one contained all three major elements (as glucose, ammonium and sulphate) while each of the other three lacked one or other of these.

Growth characteristics and the pattern of protein utilization have been reported in detail elsewhere (Kalisz *et al.*, 1986); some basic features are summarized in Table 1. A most remarkable observation was that growth yield more than doubled in medium containing both glucose and protein, and substrate conversion ratios (with the reservation that they are based on substrate removal from the medium) increased, implying more efficient use of the greater quantity of supplied carbon.

Table 1. *Comparison of approximate growth characteristics of three basidiomycetes grown in medium containing protein and/or glucose as sole carbon source*

	Organism		
C-source in medium	*A. bisporus*	*C. cinereus*	*V. volvacea*
A. Biomass (mg dry wt/flask of 25 ml medium)			
1% protein	45–50	75	55
1% glucose	45–50	75	55
1% protein + 1% glucose	175	220	165
B. Substrate conversion ratios (mg mycelium/mg substrate carbon)			
1% protein	0·7	0·6	0·5
1% glucose	0·8	0·9	0·74
1% protein + 1% glucose	±1	±1	±1

The values shown here are very much simplified from detailed data presented by Kalisz *et al.* (1986). The substrate conversion ratios refer only to substrate removal from the medium and are calculated on the assumption that protein constitutes 50% carbon by weight.



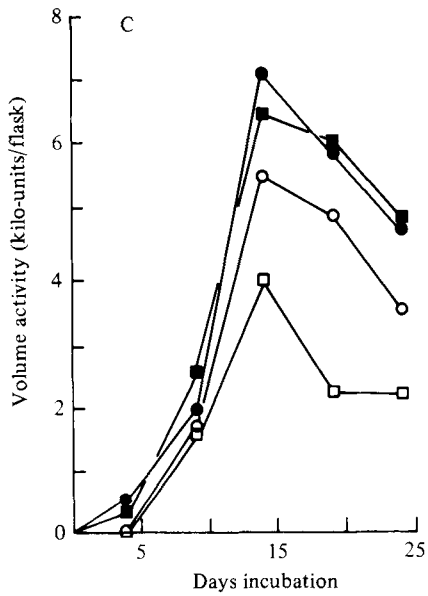


Fig. 1. Volume activity (in kilo-units per flask of 25 ml of medium) of extracellular proteinase in culture filtrates of (A) *Agaricus bisporus* (assayed at pH 7), (B) *Coprinus cinereus* (assayed at pH 9) and (C) *Volvariella volvacea* (assays at pH 7 and pH 9 gave the same activities) grown in media supplemented with protein only (solid circles), protein + glucose (open circles), protein + ammonia (solid squares), or protein + glucose + ammonia (open squares).

Extracellular proteinase

Extracellular proteinase was observed in cultures of *Agaricus bisporus* only under conditions of induction. No activity was found in media devoid of a protein source. Proteinase production was affected considerably by additional glucose, which potentiated a continued increase in volume activity, i.e. per flask of 25 ml of medium, that otherwise declined in glucose-free medium (Fig. 1A). Supplementation of the protein + glucose medium with ammonium effected a further slight increase in activity; additional sulphate had no effect. In media containing protein as sole carbon source, ammonium and sulphate addition did not affect proteinase activity. In all media, the activity detected at pH 7 was higher than that in assays performed at pH 9.

Proteinase volume activities (per flask of 25 ml of medium) varied considerably between replicate

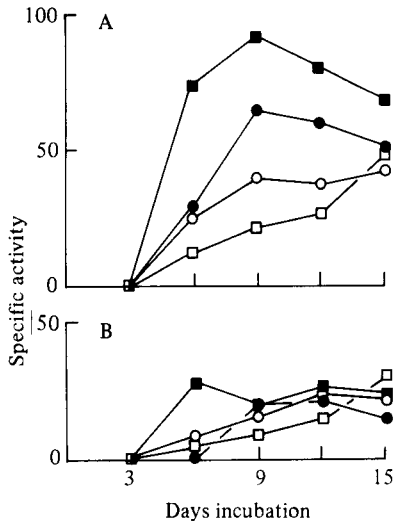


Fig. 2. Specific activity (units per mg dry weight of mycelium) of the proteinase activity of culture filtrates of *Coprinus cinereus* assayed at (A) pH 9 and (B) pH 7. The cultures were grown in medium supplemented with protein alone (solid circles), protein + ammonia (solid squares), protein + glucose (open circles), or protein + glucose + ammonia (open squares).

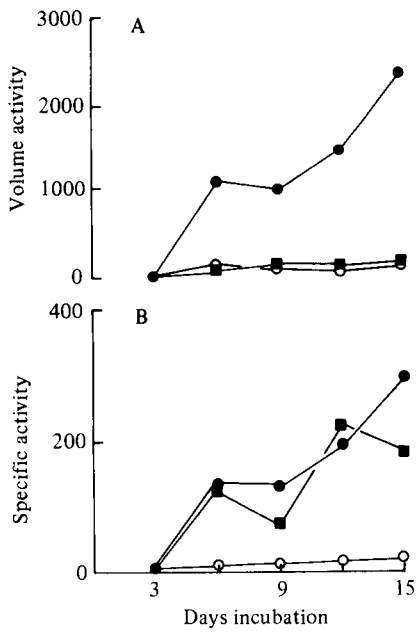


Fig. 3. Proteinase activity of culture filtrates of *Coprinus cinereus* grown in protein-free media under conditions of derepression. The media were deficient in sulphur (solid circles), carbon (solid squares), or nitrogen (open circles). Proteinase activity was assayed at pH 9 and is shown as (A) a volume activity (units per flask) and as (B) a specific activity (units per mg dry weight of mycelium).

cultures of *Coprinus cinereus*. However, proteinase activity was always considerably greater in media containing both protein and glucose than in the absence of glucose. In the absence of glucose, proteinase activity was considerably greater in the presence of additional ammonium; sulphate did not affect proteinase activity in media containing substrate protein. In most media, activity detected at pH 9 was greater than that found at pH 7. Results are summarized in Fig. 1B. The highest specific activity at pH 9 was observed using media containing protein as sole carbon source and supplemented with additional ammonium. In terms of specific activity in the pH 9 assay the greatest activities were observed in media containing protein as sole carbon source. Specific activity in the pH 7 assay was much the same on the different media (Fig. 2). Extracellular proteinase activity was also observed in *Coprinus* cultures grown under conditions of catabolite derepression (Fig. 3A), the highest activity being in media deficient in a sulphur source. The greatest proteinase specific activity observed in the absence of protein occurred in media deficient in a carbon or a sulphur source (Fig. 3B). Under these conditions of derepression the specific activity of the *Coprinus* proteinases assayed at pH 9 was about 3 times greater than the highest specific activity induced by substrate protein and the fact that starvation for sulphur and carbon, but not nitrogen, derepressed *Coprinus* extracellular proteinase is an interesting commentary on the metabolic value to the organism of substrate protein. Maximum activity in the assay done at pH 7 was more than 10-fold greater under conditions of derepression than induction.

In cultures of *Volvariella volvacea* the highest extracellular proteinase activity was observed in media containing protein as sole carbon source or as sole source of carbon and nitrogen; marginally lower activities were obtained when protein was the sole source of nitrogen and the lowest activities were observed on media containing protein + glucose + ammonium (Fig. 1C). The same pattern was observed when the results were expressed as specific activities. In *V. volvacea* proteinase activities detected in assays performed at either pH 7 or pH 9 were always similar. No proteinase activity was observed in media under conditions of derepression.

DISCUSSION

Under conditions of induction, *Coprinus* and *Volvariella* total proteinase formation was associated with mycelial growth. Large increases in proteinase activity paralleled rapid increases in mycelial biomass. The *A. bisporus* activity, how-

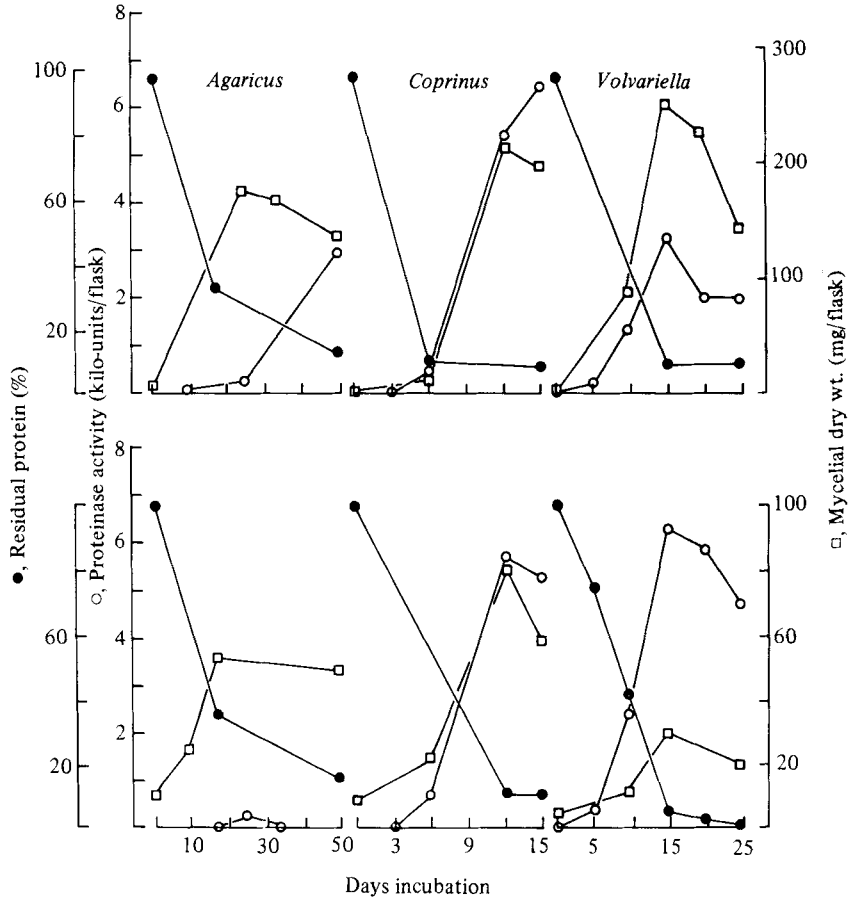


Fig. 4. Summary of the responses of the three basidiomycetes to growth in medium supplemented with protein + glucose (upper panel) or protein alone (lower panel). The display shows the mycelial biomass (open squares), a few representative values for residual supplied protein (closed circles; detailed data can be found in Kalisz *et al.*, 1986) and extracellular proteinase activity (assayed at pH 9) open circles.

ever, appeared to be produced in a non-growth-associated manner, with major increases occurring on transition to stationary growth, and ultimately, death phase (Fig. 4).

The proteolytic activities of all three organisms were first detected in the medium when most of the supplied protein had been utilized (Fig. 4). Adsorption of protein onto the mycelia was not demonstrated, and fungi are unlikely to assimilate intact protein molecules. Thus, the rapid decrease in protein concentration prior to the appearance of extracellular proteinase activity must be due to the hydrolytic action of proteolytic enzymes located at the cell surface but not dispersed in the medium. Failure to detect extracellular proteinase activity during this early growth phase is plainly not due to their being inhibited or inactive since their activity is inferred from the disappearance of the supplied substrate protein. It could be due to: (1) enzymes being bound to the substrate molecule during hydrolysis of peptide bonds; (2) the proteinases being bound to the outside of the fungal wall; or (3) the enzymes being located inside an extracellular layer or sheath attached to the cell wall. All three mechanisms have been demonstrated to occur in other circumstances in both bacteria and fungi.

The cellulases of *A. bisporus* and other organisms (Mandels, 1975; Ng, Weimer & Zeikus, 1977; Manning & Wood, 1983) are strongly adsorbed to cellulose at high substrate concentrations. It is possible, therefore, that proteinases may be bound to the protein substrate at high concentrations of the latter. In our experiments the pattern of change in proteolytic activity was similar in media

containing either soluble or insoluble protein (data not shown). If the enzymes had been bound to the soluble substrate, they would have been detected in the culture filtrates. The similarity of response argues against binding of basidiomycete extracellular proteinases to their substrates. We therefore conclude that immobilization to wall or 'sheath' layers is the most likely explanation, a circumstance that could also explain the lack of proteinase activity in culture filtrates of ectomycorrhizal fungi (Lundeberg, 1970).

Many cell-wall bound enzymes have been reported in bacteria and fungi (Coles & Gross, 1967; Lampen, 1968; Eberhart & Beck, 1970; Law, 1980), including glucosidase in *Trichoderma reesei* (Kubicek, 1981); trehalase (Hill & Sussman, 1964), invertase (Chung & Trevithick, 1970) and proteinases (Mahadevan & Mahadkar, 1970) of *Neurospora*; and the cellulases of *V. volvacea* (Chang & Steinkraus, 1982). Enzymes have also been found inside layers of extracellular polysaccharide-rich sheaths (Palmer, Murmanis & Highley, 1983; K.-E. Eriksson, pers. comm.).

Naturally-immobilized enzymes would offer advantages to the organisms investigated here. The natural habitats of the mycelium of these fungi are solid substrates, mainly decaying plant materials, which are also inhabited by a large number of other micro-organisms. Competition for nutrients is likely to be intense. Surface located proteinases would only degrade proteinaceous material in the close proximity of the producing cell, ensuring efficient utilization of substrate protein. Secreted enzymes would catalyse the hydrolysis of proteins both close to and far from the mycelium which would be wasteful to the economy of the producing organism. On depletion of the protein substrate the wall-bound proteinases may be released by an as yet undefined mechanism to scavenge in the substratum.

In the three basidiomycetes studied, the major regulatory mechanism of total proteinase activity production appears to be induction, although in terms of specific activity *Coprinus* showed a quantitatively greater response to derepression. However, unlike the *Neurospora crassa* mechanism, repression does not over-ride induction of the proteinase in any of these organisms.

The *Coprinus* proteinase activity was also derepressed in medium deficient in one of the major elements. *A. bisporus* and *V. volvacea* proteinases were not detected in media deficient in protein. Since, under these conditions, no appreciable change in biomass occurred, the presence of the proteolytic activity which was detected may be due to release of intracellular proteinases on autolysis of cells. Proteinases of *Coprinus* detected in media deficient in a sulphur source were most probably released by intact cells, since the vast majority of cells examined by microscopy were found to be intact. In media deficient in a carbon source, i.e. carbon-derepression, specific activities of *Coprinus* proteinase activity were also extremely high, being several-fold greater than in media containing protein. Thus proteinase activity in this organism appears to be regulated by induction and sulphur- and carbon-catabolite derepression.

In the presence of protein, the highest proteinase volume activity, i.e. per flask of 25 ml of medium, in cultures of *Coprinus* and *Agaricus* was observed in media supplemented with glucose. Greatest volume activity of *V. volvacea* proteinase was detected in media lacking glucose. However, with the exception of *A. bisporus*, the highest specific activity of the proteinases was observed in media containing protein as sole source of carbon. The specific activity of *Coprinus* proteinase was increased by addition of ammonium to the medium. Proteinase specific activity in *Volvariella* cultures was unaffected by addition of ammonium in the absence of glucose, but was repressed in media supplemented with both glucose and ammonium. Addition of sulphate did not affect the specific activity of the proteinases. These results suggest that although the proteinases of these basidiomycetes were not totally repressed by other sources of the major elements, i.e. did not exhibit catabolite repression, the carbon and nitrogen sources partly regulated their activities.

The three organisms dealt with here occupy very similar habitats. The similarities between them may therefore represent expression of a 'basidiomycete type' of adaptation to such a habitat. The most striking of these are the apparent initial localization of the proteinase activity to the hyphal wall and the lack of carbon- and nitrogen-catabolite repression. Taken together with other studies (Kalisz *et al.*, 1986) this work clearly demonstrates that these basidiomycete fungi can utilize substrate protein via the production of extracellular proteinase activities and suggests that the regulation of proteinase production differs considerably from that previously described for fungi such as *Aspergillus* and *Neurospora*.

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