

# Specific induction of a cuticle-degrading protease of the insect pathogenic fungus *Metarhizium anisopliae*

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**The insect pathogenic fungus *Metarhizium anisopliae* produces several extracellular cuticle-degrading proteases and evidence is consistent that one of these, a chymoelastase PR1, is a determinant of pathogenicity. We have shown previously that the wide-domain regulatory circuits of carbon and nitrogen derepression regulate PR1 production. In the present work we have established in addition that PR1 is specifically induced by insect cuticle, but not by other soluble or insoluble proteinaceous substrates. The feeding of elastin or collagen to derepressed established mycelium (starved for carbon and nitrogen) did not enhance PR1 production significantly and the soluble proteins BSA and gelatin rapidly and completely repressed PR1. The carbohydrate polymers cellulose and xylan gave derepressed basal levels of PR1. However, addition of locust cuticle enhanced PR1 production to a level approximately 10-fold that of derepressed mycelium. In order to establish if the enhancing effect of insect cuticle on PR1 production was due to specific induction or merely a reflection of enhanced growth on this insoluble dual carbon and nitrogen source, ergosterol was used as a measure of fungal growth. Expressing enzyme activity per mg dry weight showed that PR1 production in cuticle cultures increased approximately five- and ninefold after 12 and 24 h growth compared with elastin-grown cultures. Thus, the substantial increase in PR1 production on cuticle was shown not to be a function of fungal growth and this confirms that PR1 is induced by a component of insect cuticle; we believe this is the first report of induction by a specific substrate for any microbial protease.**

**Keywords:** *Metarhizium anisopliae*, insect pathogen, protease regulation

## INTRODUCTION

Insect pathogenic fungi, and in particular the Deuteromycete *Metarhizium anisopliae*, have great potential for use in pest control. Infection occurs directly through the insect exoskeleton or cuticle and penetration is effected by a combination of mechanical pressure and enzymic degradation (Charnley, 1984). Biochemical studies (reviewed by Charnley & St Leger, 1991) have provided strong evidence that an extracellular chymoelastase, PR1, is essential for cuticle penetration and is a determinant of pathogenicity (St Leger *et al.*, 1988a). Briefly, protease is first in a sequence of cuticle-degrading enzymes produced *in vitro* on host cuticle and PR1 is adapted to degrade extensively cuticular protein (St Leger *et al.*, 1987a). PR1 is the only cuticle-degrading enzyme produced in high

amounts by all pathogenic isolates of *Metarhizium* (St Leger *et al.*, 1987b). It is produced in high levels by the fungus during host penetration (St Leger *et al.*, 1987c) and specific inhibition of PR1 delays disease symptoms and mortality of the tobacco hornworm *Manduca sexta* (St Leger *et al.*, 1988a).

Understanding the role of PR1 in cuticle penetration is facilitated by studying its regulation. It has previously been reported that PR1 is produced under conditions of carbon and nitrogen starvation (St Leger *et al.*, 1988b, 1991). Proteases are similarly derepressed in many *Aspergillus* species (Cohen, 1981) and *Schizophyllum commune* (Sessoms & Lilly, 1986). However, we report here an additional level of regulation of PR1 by *M. anisopliae*, that of specific induction by insect cuticle but not by other insoluble or soluble proteinaceous substrates.

## METHODS

**Growth and culture conditions.** *M. anisopliae* isolate ME1 (origin: pecan weevil) originally obtained from Tate and Lyle, Group Research and Development Laboratory, Reading, UK, was used throughout this work and was cultured routinely on 1/4-strength Sabouraud's dextrose agar (SDA) containing 0.25% (w/v) yeast extract at 27 °C. Conidial suspensions were prepared from actively growing cultures by washing with 0.05% Tween 80. The resulting suspensions were centrifuged at 3000 g for 5 min and then resuspended in sterile distilled water. Liquid cultures were grown either in modified basal salts plus trace elements (Cooper & Wood, 1975), which contained per litre, 1 g  $\text{KH}_2\text{PO}_4$ , 0.5 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  and 10 ml trace element solution which comprised 0.2% (w/v)  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 1% (w/v)  $\text{ZnSO}_4$ , 0.02% (w/v)  $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ , 0.02% (w/v)  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  and 0.02% (w/v)  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , plus 50 mM MES (a non-metabolizable buffer) pH 6, or in complete medium (CM), which consisted of Modified Czapek Dox (Oxoid) supplemented with 2 g l<sup>-1</sup> of each of casein hydrolysate, mycological peptone, yeast extract and malt extract. Carbon/nitrogen sources were supplied at 1% (w/v) because BSA concentrations above 1% repress protease production (I. C. Paterson, unpublished). All polymeric carbon/nitrogen sources with the exception of insect cuticle were from Sigma. Comminuted cuticle from adult desert locusts (*Schistocerca gregaria*) was prepared essentially as described by Andersen (1980). Approximately 100 locusts were frozen at -20 °C for 1 h and then homogenized in a Waring blender in 1% (w/v) potassium tetraborate. The cuticle pieces were washed extensively in distilled water, stirred overnight in 1% (w/v) potassium tetraborate and then dried at 37 °C. The cuticle pieces were then milled to a fine powder in a Glen-Creston hammer mill (DEH-48) using a 0.5 mm sieve. The powder was washed in 1% potassium tetraborate and finally in distilled water, allowed to settle and any floating material removed. This method retains cuticular protein.

**Transfer experiments.** CM, 100 ml in 250 ml Erlenmeyer flasks, was inoculated with  $4 \times 10^6$  conidia and incubated at 27 °C in an orbital shaker (150 r.p.m.) for 3 d in order to establish extensive fungal growth. Cultures developed as uniform submerged mycelial balls with no sporulation evident; the morphology of fungal cultures remained constant over the duration of the experiments. The culture was sieved through two layers of sterile muslin. Retained mycelium was washed with sterile basal salts medium, transferred to 100 ml basal salts medium and then incubated for a further 24 h under the same conditions as above to ensure complete catabolite derepression. Potential inducers of protease were then added at 1% (w/v) and PR1 activity in the culture supernatant was assayed for up to 24 h.

**Enzyme assays.** PR1 was assayed by monitoring at 410 nm the release of nitroanilide (NA) from the peptide substrate succinyl-Ala-Ala-Pro-Phe-NA essentially as described by St Leger *et al.* (1987a) except that 100 mM Tris/HCl buffer pH 8 was used. Activity is expressed as nmol NA released ml<sup>-1</sup> min<sup>-1</sup>.

**Chemicals.** All chemicals, unless otherwise stated, were from Sigma. HPLC grade solvents were from Rathburns.

**High performance liquid chromatography (HPLC).** HPLC was performed on a Gilson system comprising two 303 pumps, a 811B dynamic mixer, a 401 dilutor, a 231 sample injector, a Rheodyne injection valve fitted with a 20 µl loop and a Gilson 118 UV dual wavelength detector. The system was controlled by the Gilson 714 manager on an Opus PC.

**Fungal growth assessment.** In order to measure dry weight,

mycelium from liquid cultures was collected over a Buchner funnel and the mycelial mat dried to a constant weight at 70 °C. The dried mycelium was cooled to room temperature in a desiccator before weighing.

The procedure used for the extraction and quantification of ergosterol was a modification of the method described by Seitz *et al.* (1979). Mycelium from 100 ml liquid culture was collected over a Buchner funnel and homogenized for 1 min on a 'Pulse Matic 16' osterizer in 40 ml methanol/dichloromethane (2:1, v/v). After 30 min the suspension was filtered over a Buchner funnel and the residue washed with 40 ml dichloromethane and then 40 ml 2 M KCl in 0.5 M potassium phosphate buffer (pH 7.4). The filtrate and the washings were combined and the aqueous phase discarded. The organic phase was evaporated to dryness under reduced pressure at 40 °C in a rotary evaporator. The solid was saponified in 30 ml 1 M KOH in 95% (v/v) ethanol for 1 h at 70 °C. Once cool, 60 ml distilled H<sub>2</sub>O was added and the non-saponified fraction extracted with three 100 ml washings of light petroleum. The three washings were combined and evaporated to dryness under reduced pressure at room temperature in a rotary evaporator. The solid obtained was stored under nitrogen in sealed tubes at 4 °C until required. Ergosterol was separated and quantified by reverse-phase HPLC using a Rainin C18 column with detection at 282 nm. A mobile phase of 95% (v/v) methanol and flow rate of 2 ml min<sup>-1</sup> gave good separation of the ergosterol peak from other extracted sterols; ergosterol standard (Fluka) gave a retention time of 10.5 min. Ergosterol converts to ergocalciferol (vitamin D) upon exposure to UV light (Tuksida, 1980); therefore all samples were stored in the dark. Ergocalciferol standard (Fluka) had a retention time of 7.5 min and could easily be distinguished from the ergosterol peak. A calibration curve was produced using ergosterol standard.

**Fast atom bombardment mass spectrometry.** A VG Analytical 70 70E mass spectrometer was utilized with a mass spectrometry services data system to obtain mass spectra of standard and extracted ergosterol. Xenon gas was used in the atom gun running at 8 kV with 3-nitrobenzyl alcohol as the matrix.

## RESULTS

### Effect of different carbon and nitrogen sources on protease production

Mycelium from 3 d CM cultures was incubated for 24 h in basal salts medium lacking both C and N; after this period of starvation different C and N sources (1%, w/v) were added and PR1 activity was assayed for up to 24 h (Table 1). Addition of elastin, collagen, cellulose or xylan did not enhance PR1 production above basal levels (-C -N) by 16 h or 24 h and the soluble proteins BSA and gelatin rapidly and completely repressed PR1 production. However, addition of locust cuticle enhanced PR1 production to a level approximately 10 times that of control (-C -N) flasks after 16 h and approximately 6 times after 24 h; no effect was apparent after 8 h. Some enhancement was evident with elastin and collagen 8 h after adding the protein, but PR1 levels subsequently fell to that of the control flasks. Experiments designed to measure the binding of PR1 to insoluble substrates demonstrated that the enhanced PR1 levels in cuticle-containing cultures reflected enzyme production and were not an artifact of the differential binding of enzyme to substrate (data not shown).

**Table 1.** PR1 production by derepressed mycelium of *M. anisopliae* on a range of polymeric substrates

C/N source (1%, w/v)	Enzyme activity*		
	8 h	16 h	24 h
Control (-C-N)	211 ± 15	293 ± 15	580 ± 33
Cuticle	140 ± 50	2842 ± 76	3355 ± 107
Elastin	560 ± 28	298 ± 11	245 ± 13
Collagen	725 ± 40	396 ± 21	311 ± 27
BSA	0	0	0
Gelatin	0	0	0
Cellulose	97 ± 18	89 ± 28	112 ± 32
Xylan	191 ± 17	271 ± 12	291 ± 22

\* Mean activity ± SD from three replicates 8, 16 and 24 h after addition of C/N source to mycelium starved of C and N for 24 h. Enzyme activities are expressed as nmol nitroanilide released ml<sup>-1</sup> min<sup>-1</sup> from Suc-Ala-Ala-Pro-Phe-NA and are representative of three similar experiments.

**Table 2.** Effect of carbon, nitrogen or sulphur deficiency on PR1 production by *M. anisopliae*

C/N source (1%, w/v)	Enzyme activity*	
	8 h	16 h
-C-N+cuticle	159 ± 31	818 ± 66
-C+cuticle	0	0
-N+cuticle	57 ± 16	255 ± 27
-S+cuticle	0	0
-C-N+BSA	0	0
-C+BSA	0	0
-N+BSA	0	0
-S+BSA	0	0
-C-N	9 ± 4	41 ± 6
-C	0	0
-N	0	0
-S	0	0

\* Mean activity ± SD from three replicates 8 and 16 h after the transfer of mycelia to basal salts deficient in one of C, N or S, in the presence or absence of cuticle or BSA (1%, w/v). Enzyme activities are expressed as nmol nitroanilide released ml<sup>-1</sup> min<sup>-1</sup> from Suc-Ala-Ala-Pro-Phe-NA and are representative of three similar experiments.

### Effect of nutrient limitation on protease production

Mycelium from 3 d CM cultures was transferred to basal salts deficient in one of C, N or S plus or minus either BSA or cuticle. The medium used was buffered basal salts plus trace elements plus sucrose (1%, w/v) or NH<sub>4</sub>Cl (0.2%, w/v). Medium deficient in S contained MgCl<sub>2</sub> instead of MgSO<sub>4</sub>·7H<sub>2</sub>O; similarly chlorides were substituted for the sulphates in the trace elements. Protease activity was measured 8 and 16 h after transfer.

PR1 was repressed completely in all media containing N and greatly reduced in media containing C (Table 2). No PR1 was produced in media lacking N (but containing C) unless cuticle was present, in which case PR1 levels were approximately 30% of those produced in -C-N media plus cuticle. No PR1 was produced in media lacking only S. Again, basal levels were detected in the absence of both C and N.

### Ergosterol as a measure of mycelial biomass in *M. anisopliae*

It was necessary to establish if the enhancing effect of cuticle on PR1 production was a result of specific induction or merely a reflection of enhanced growth of *M. anisopliae* on this dual carbon and nitrogen source. However, separation of mycelium from insoluble substrates is impracticable, which precludes comparisons of mycelial dry weight. The assay methods commonly used for measuring fungal biomass on solid substrates are chitin and ergosterol estimation (Matcham *et al.*, 1984). Chitin is also an integral part of insect cuticle structure and thus ergosterol content was considered as a means of estimating biomass in this study. It was first essential to establish the validity of this assay for growth of *M. anisopliae*.

Ergosterol was extracted from mycelium and analysed by HPLC. The chromatogram showed a peak with the same retention time as the ergosterol standard, and there were no interfering peaks (data not shown). Most other UV-absorbing components did not bind to the column. Spiking the sample with ergosterol standard did not give rise to any extra peaks. The fast atom bombardment mass spectra of extracted and authentic ergosterol revealed similar molecular masses and fragmentation patterns (data not shown). Sterols extracted from insect cuticle and elastin by the method used with mycelium were subjected to HPLC, but no peaks with retention times similar to ergosterol or ergocalciferol were detected.

Mycelium from 3 d CM cultures was transferred to -C-N basal salts medium and incubated for 24 h. The mycelium was then transferred to either CM (to give a level and rate of growth approximately equivalent to that on insect cuticle) or to -C-N basal salts (control) and incubated for a further 24 h. The dry weight and ergosterol content of the mycelium were measured at various times to determine the relationship between ergosterol and fungal biomass; the linearity of the graph demonstrated that the relationship between ergosterol content and fungal growth in CM remained constant at approximately 1.4 µg per mg dry weight of mycelium, thus enabling ergosterol to be used as a valid measure of fungal biomass in cultures containing insoluble substrates. However, extended periods of nutrient starvation (-C-N) resulted in a reduction in ergosterol yield per mg dry weight; after 36 and 48 h starvation ergosterol yields fell to 1.2 and 0.95 µg mg<sup>-1</sup> respectively.

**Table 3.** Fungal biomass and PR1 production by *M. anisopliae*

Culture conditions*	Fungal growth†		PR1 production	
	Ergosterol (µg)	Dry wt (mg)	Enzyme activity‡	Activity (mg dry wt) <sup>-1</sup>
CM 3 d	361	260	0	0
-C-N 0 h	435	310	136.3	0.44
-C-N + 12 h	396	330	201.4	0.61
-C-N + 24 h	285	300	286.4	0.95
Cuticle + 12 h	600	429	2090.6	4.87
Cuticle + 24 h	975	696	3245.2	4.66
Elastin + 12 h	540	386	384	0.99
Elastin + 24 h	855	611	307.9	0.5

\* Mycelia were grown in CM for 3 d, starved of C and N for 24 h in basal salts (shown here as -C-N 0 h) before addition of cuticle or elastin or continued starvation.

† Dry weights for cultures containing cuticle or elastin were estimated from ergosterol content.

‡ Enzyme activities are expressed as nmol nitroanilide released ml<sup>-1</sup> min<sup>-1</sup> from Suc-Ala-Ala-Pro-Phe-NA and represent the means of three replicates.

### Evaluation of insect cuticle as an inducer of PR1 production

Cultures containing cuticle or elastin, or lacking C and N (-C-N, control) were prepared as previously described. Elastin was chosen for comparison because, like locust cuticle, it is an insoluble protein substrate with a high proportion of alanine residues (Hojrup *et al.*, 1986). Ergosterol was extracted and PR1 activity measured 12 and 24 h after addition of either cuticle or elastin. The extracted ergosterol was quantified as described above and an estimate of fungal biomass was made (Table 3). Growth in control flasks was measured conventionally by dry weight determination, as extended periods of starvation result in reduced ergosterol yields (see above).

After 12 h growth on cuticle, PR1 activity was 10.4 times higher than in controls and 5.4 times higher than in cultures containing elastin. This corresponded with a 1.3- and a 1.1-fold greater biomass in cuticle cultures compared to control and elastin cultures respectively. After 24 h on cuticle, PR1 activity was 11.3 and 10.5 times higher than controls and elastin cultures respectively, with biomass being 1.38- and 1.07-fold greater. The high level of PR1 activity on cuticle compared with the other treatments occurred without a substantial increase in mycelial dry weight, showing that elevated PR1 levels do not result from increased fungal biomass. Analysis of enzyme activity per mg dry weight shows that protease production in cuticle cultures increased approximately eight- and fivefold after 12 and 24 h growth compared with controls, and five- and ninefold compared with elastin-grown cultures. Partial mycelial autolysis and subsequent

release of intracellular enzymes may have caused the slight increase in PR1 activity seen in control (-C-N) flasks because a slight fall in mycelial dry weight was observed; thus comparisons of PR1 activity per mg dry weight are probably more valid after 12 h than 24 h growth.

### DISCUSSION

A number of authors have suggested that protease regulation in fungi can be controlled by both induction and derepression mechanisms (reviewed by Cohen, 1980). There have been many reports of protease production being effected in fungi by exogenous protein under conditions of nutrient deprivation. *Neurospora crassa* produces both neutral and acid proteases if starved of either carbon, nitrogen or sulphur when grown on a protein substrate (Drucker, 1975; Cohen *et al.*, 1975). Similar results have been described for several *Candida* species (Crandall & Edwards, 1987; Banerjee *et al.*, 1991). Proteases of certain other fungi, e.g. *Mucor miehei* (Lasure, 1980), and the trypsin-like protease, PR2, from *M. anisopliae* (Paterson *et al.*, 1993) are induced non-specifically by any protein if the fungus is starved of nitrogen, but not if starved of carbon or sulphur in the presence of nitrogen. Maximal levels of PR1 are produced only when the fungus is grown on insect cuticle as sole source of carbon and nitrogen. No PR1 was produced in media lacking carbon or sulphur and PR1 was only produced in media deficient in nitrogen if insect cuticle was included in the growth medium, in which case PR1 levels were 30% of maximal levels (achieved on -C-N+cuticle). In all media the soluble protein BSA completely repressed PR1 production.

The exceptionally high levels of PR1 found only in cuticle-containing cultures suggest that PR1 is specifically induced by insect cuticle. An alternative explanation is that the rise in PR1 activity was due to an increase in fungal biomass. Difficulty in separating mycelium from solid substrates precluded the measurement of dry weight and therefore necessitated an alternative indicator of mycelial biomass. For this, the ergosterol content of the mycelium was determined and it was found to remain constant at 1.4 µg mg<sup>-1</sup> dry weight during growth in CM. This compares favourably with other reported yields from fungi grown in similar conditions, for example certain *Aspergillus* species (Seitz *et al.*, 1979; Shapiro & Gealt, 1982). However, extended periods of starvation for carbon and nitrogen resulted in lower yields, which in common with similar reductions seen in ascomycete salt marsh fungi (Newell *et al.*, 1987) could have been a result of partial mycelial autolysis.

The approximately ninefold increase in PR1 activity per mg dry weight in cuticle-containing cultures compared to controls cannot be explained by derepression alone and it is concluded that PR1 is specifically induced by some component(s), presumably proteinaceous, of insect cuticle. To the authors' knowledge, this is the first demonstration of the specific induction of a microbial protease. The significance of PR1 induction in pathogenesis is unclear. However, the specificity involved

presumably reflects adaptation by *M. anisopliae* to insect parasitism.

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