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Regulation of production of proteolytic enzymes by the entomopathogenic fungus *Metarhizium anisopliae*

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Abstract. Synthesis of chymoelastase and trypsin by the entomopathogenic fungus *Metarhizium anisopliae* occurs rapidly (< 2 h) during carbon and nitrogen derepression in minimal media. Enzyme levels were enhanced when minimal media were supplemented with insect cuticle or other insoluble polymetic nutrients (e.g. cellulose) that were insufficient to produce catabolite repression. Addition of more readily utilized metabolites (e.g. glucose or alanine) repressed protease production confirming that production is constitutive but repressible. Operational control of protease release involves synthesis rather than secretion because catabolite repression reduced endocellular activity (associated with a sedimentable vacuole containing fraction) as well as extracellular enzyme levels. Studies with metabolic inhibitors indicated that production of Pr1 and Pr2 does not require DNA synthesis. However, synthesis is substantially reduced by inhibitors of transcription (actinomycin D and 8-azoguanine) and translation (cyclohexamide and puromycin).

Inhibition by 8-azoguanine is relieved by guanine. These results imply that the operative steps in protease regulation involve *de novo* synthesis of mRNA. Inhibition of enzyme production by an AMP analogue adenosine 5′-0-thiophosphate implies an involvement for AMP-dependent enzyme systems in derepression. However, neither exogenous cAMP nor an inhibitor of cAMP phosphodiesterase relieved catabolite repression by glucose or NH_4Cl. Use of *o*-vanadate to inhibit plasmalemma ATPase confirmed that secretion of chymoelastase-like protease and trypsin-like protease via the cell membrane is an active process.

Key words: Chymoelastase – Trypsin – *Metarhizium anisopliae* – Entomopathogen – Regulation of synthesis

The entomopathogenic fungus *Metarhizium anisopliae* produces extracellular chymoelastase (Pr1) and trypsin-like (Pr2) proteases both in culture and *in situ* during penetration of host cuticles (St. Leger et al. 1987a, b). The chymoelastase solubilizes procuticular proteins more effectively than other endoproteases obtained from *M. anisopliae* or commercial enzymes and is likely to be a major determinant of pathogenicity (St. Leger et al. 1987a, b). To help understand the role of proteases in cuticle penetration it is desirable to determine how their synthesis is regulated. This will give an insight into how production may be regulated by cuticle components and will also enable critical comparison of enzyme production by mutants or isolates which differ in pathogenicity.

The comparatively few critical investigations conducted on regulation of production of fungal proteases have emphasized the importance of catabolite repression (CR) whether the protease is constitutive e.g. from several *Aspergillus spp.* (Cohen 1981), or induced by protein e.g. from *Neurospora crassa* (Drucker 1972). Unfortunately most other studies, including those involving entomopathogens (Kucera 1981) have not considered the effects of CR. Because of the difficulty in interpreting these results, a more rigorous approach was applied in this study to determine the mode of protease regulation in *M. anisopliae*.

Materials and methods

Organisms and growth

The fungal isolate (*Metarhizium anisopliae* ME1), culture media, rearing conditions for the desert locust, *Schistocerca gregaria*, and for the tobacco hornworm, *Manduca sexta*, and preparation of insect cuticles were described by St. Leger et al. (1987b).

Transfer experiments

Standardized fungal inocula from 3-day sucrose/basal salts cultures (St. Leger et al. 1986a) were incubated for up to 24 h in sterile (120° C, 15 min) media (pH 5.6) lacking either carbon (C), nitrogen (N), carbon and nitrogen (CN), sulphur (SO_4) or phosphorus (PO_4), and the results compared with those from complete medium (CM) that contained (w/v): 1% sucrose, 0.2% NH_4Cl, 0.1% KH_2PO_4, 0.05% $MgSO_4$, and additional trace elements (Cooper and Wood 1975).

To test potential inducers of protease production – CN or CM media were supplemented with carbon sources presterilized in an atmosphere of ethylene oxide. The pH of the medium in all experiments remained between 5.6 and 6.2.

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Abbreviations: Pr1, chymoelastase-like protease; Pr2, trypsin-like protease; VAN, sodium *o*-vanadate; BSA; bovine serum albumin; CR, catabolite repression

Table 1. Protease production by *Metarhizium anisopliae* 5 h after transfer of growing mycelium to cultures containing either a carbon and nitrogen source or nutrient limiting conditions

	Enzyme activity	
	Pr1 (nmol nitroanilide/ml · min)	*Pr2* (nmol nitroanilide/ml · min)
+C (1%) + N (0.2%)[a]	0	0.01 ± 0[b]
−CN	9.8 ± 1.33	2.5 ± 0.14
−C + N (0.2%)	0	0.01 ± 0
+C (1%) − N	0.05 ± 0.01	0.01 ± 0
$-PO_4 - SO_4$	0	0.01 ± 0
−CN + locust cuticle (1%)	23.2 ± 3.47	6.8 ± 1.18
−CN + *Manduca* cuticle (1%)	19.4 ± 2.25	6.5 ± 0.42
−CN + elastin (1%)	15.4 ± 2.34	6.1 ± 0.63
−CN + collagen (1%)	12.6 ± 1.49	5.4 ± 0.40
−CN + BSA (1%)	0.9 ± 0.01	5.0 ± 0.26
−CN + cellulose (1%)[c]	15.3 ± 1.7	2.8 ± 0.18
−CN + laminarin (1%)	10.3 ± 1.6	3.1 ± 0.21

[a] C = sucrose; N = NH_4Cl
[b] Mean protease activity (nmol nitroanilide/ml · min) for 3 replicates ± SD. The results are representative of two similar experiments
[c] Insoluble crystalline cellulose

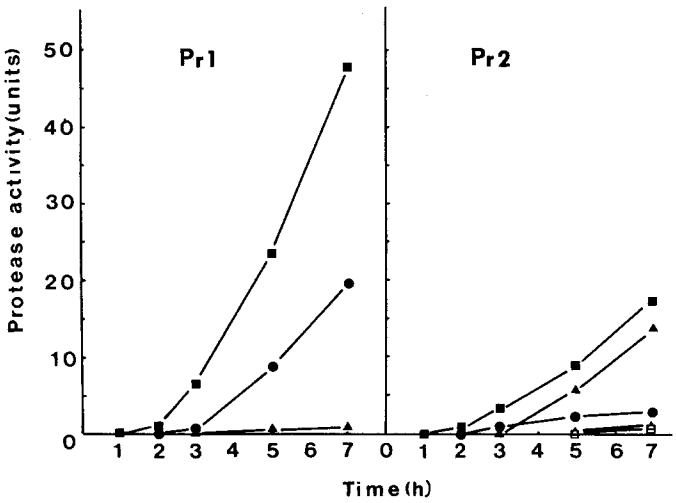

Fig. 1. Extracellular *Pr1* and *Pr2* production by *Metarhizium anisopliae* growing on 1% ground locust cuticle (*squares*), 1% bovine serum albumin (*triangles*), and starved of carbon and nitrogen (−CN) (*circles*). Open symbols indicate *Pr2* production in the presence of cyclohexamide (10 µg/ml). Cyclohexamide completely inhibited production of *Pr1*

Preparation of vacuole-rich fractions

The method used was adapted from that of Stevens and McLennan (1983). Mycelium was harvested on Whatman No. 1 filter paper, washed with distilled water and aspirated to dryness. Mycelium (5 g) was added to 100 ml of 1 M sorbitol in 10 mM K phosphate buffer (pH 5.8) to which was added 40 mg of Novozym 234. After gently shaking for 2 h at 30° C, the mycelium was sedimented at 2,500 × *g* for 5 min and washed by resuspending and centrifuging 3 times in 50 ml of 1 M sorbitol. The mycelium was then suspended

Table 2. The effect of antimetabolites on protease production by *M. anisopliae* after 5 h in medium containing locust cuticle (1%)

Inhibitor	Mode of action	Concentration (µg/ml)	Enzyme production[a] % *Pr1*	% *Pr2*
None	–	–	100	100
Cyclohexamide	Inhibits initiation,[b] elongation and termination of protein chains	10	0	1.8
Puromycin	Analogue of acceptor[b] aminoacyl t-RNA, causes premature release of partially formed protein	20	21	33
		100	17	28
Actinomycin D	Inhibits DNA primed[b] RNA polymerase	20	74	76
		100	24	38
8-azoguanine	Competitive guanine[b] analogue, incorporated into RNA	20	2	7
		100	1.5	5
Cytosine arabinoside	Inhibits DNA[b] polymerase	100	100	100
Hydroxyurea	Inhibits ribonucleotide[b] reductase	100	100	100
Adenosine 5′-*o*-thiophosphate Li_2	Substrate and inhibitor[b] for AMP dependent enzyme systems	100	4.1	1.7
NaCN	energy blocker[b]	10	0.5	0.6
Na *o*-vanadate	Inhibitor of plasmalemma ATPase in fungi[c]	50	2.1	8.4

[a] Results are the mean of 3 replicates. The experiment was repeated once with similar results
[b] Dawson et al. (1986)
[c] Bowman and Slayman (1979)

in 50 ml of cold (4° C) 1 M sorbitol in 10 mM TES buffer (pH 7.5) (buffer A). The suspension was homogenized using a teflon-glass homogenizer and unbroken mycelium removed by centrifugation (600 × *g*, 5 min). The supernatant was then centrifuged at 15,000 × *g* for 20 min. The pellet was washed by resuspending and centrifuging in 50 ml buffer A, disrupted by shaking (10 min, 4° C) in 10 ml K phosphate buffer (10 mM) containing 0.25% Triton X-100 (buffer B) and centrifuged at 15,000 × *g* for 20 min. N-Acetylglucosaminidase which is localized in vesicles in *M. anisopliae* (unpublished results) and other fungi (Hoch et al. 1979) was used as a marker.

Enzyme assay

Pr1 activity vs. succinyl-(alanine)$_2$-proline-phenylalanine-*p*-nitroanilide [Suc-(Ala)$_2$-Pro-Phe-NA] and Pr2 activity vs. benzoyl-phenylalanine-valine-arginine-*p*-nitroanilide (Ben-Phe-Val-Arg-NA) were assayed as described by St. Leger et

Table 3. Specific enzyme activities in cell fractions. Vacuole rich fractions were prepared from wall digested mycelium using 1% sorbitol as stabilizer. Mycelium was grown for 7 h in complete medium (CM), or was starved of carbon and nitrogen (−CN) with or without BSA[a]

	Specific activities (units/mg protein)								
	Pr1			*Pr2*			N-Acetylglucosaminidase		
	CM	−CN	−CN +BSA	CM	−CN	−CN +BSA	CM	−CN	−CN +BSA
Supernatant from disrupted mycelium (centrifuged at 15,000/*g*)	7.25[b]	50.79	17.41	58.91	209.98	272.41	7.61	11.81	8.25
Supernatant from pellet following disruption with Triton X-100	9.29	88.32	23.26	88.73	299.61	325.42	10.47	18.06	11.32

[a] *Pr1* and *Pr2*, nmol nitroanilide/ml · min; N-acetylglucosaminidase, nmol nitrophenol/ml · h
[b] Results are representative of 3 similar experiments

al. (1987a). N-acetylglucosaminidase activity (vs. *p*NP-N-acetyl-β-D-glucosamine) was assayed as described by St. Leger et al. (1986b).

Chemicals. Except for Novozym 234 (Novo Enzyme Industries, Denmark) and VAN (Fisher Scientific, Rochester, NY, USA) all chemicals were from Sigma, St. Louis, MO, USA.

Results

Transfer experiments

Exponentially growing mycelium was transferred from sucrose/basal salts medium to CM or media deficient in essential nutrients (Table 1). Little Pr2 and no Pr1 was produced extracellularly over 24 h in CM, $-PO_4$ or $-SO_4$ media. In some experiments inoculum was obtained from cultures grown on reduced levels of PO_4 (0.03% KH_2PO_4) or SO_4 (0.02% $MgSO_4$) but this did not effect subsequent enzyme production on $-PO_4$ or $-SO_4$ media. However, limitation of C and N (−CN) derepressed enzyme production; modifying the concentration of C or N demonstrated that both were independently capable of repressing protease production even in the absence of the alternative repressing catabolite. Addition of insoluble non-repressing protein substrates to −CN media enhanced production of both Pr1 and Pr2 with activities apparent < 2 h after transfer (Fig. 1). Highest enzyme activities were produced in media containing locust and *Manduca* cuticle. However, cellulose also enhanced production of Pr1 indicating that induction by proteins is probably not a major factor. Production of Pr1 and Pr2 were repressed when sucrose (1%), alanine (1%), N-acetylglucosamine (1%) or NH_4Cl (0.2%) were added to media containing cuticle or cellulose showing that repression overrides the enhancing effect of polymeric substrates. The production of extracellular enzymes by starved mycelium or mycelium provided with cuticle or bovine serum albumin (BSA) was not due to the release of preformed enzyme molecules since cyclohexamide (10 μg/ml) almost completely inhibited enzyme release (Fig. 1).

Further studies with metabolic inhibitors were conducted on mycelium transferred to media containing cuticle and supplemented with analogues or inhibitors known to inhibit an enzyme activity or some phase of protein, RNA or DNA synthesis. Production of Pr1 and Pr2 continues *de novo* in the presence of inhibitors of DNA synthesis (cytosine arabinoside and hydroxyurea) but was reduced by the transcriptional inhibitors actinomycin D and 8-azoguanine. Inhibition of enzyme production by 8-azoguanine (20 μg/ml) was largely overcome (> 72% in 5 replicates) by guanine (100 μg/ml). Inhibitors of mRNA translation (cyclohexamide and puromycin) also substantially reduced enzyme production.

Using NaCN as an energy blocker confirmed the energy dependence of protease production. In addition, protease release in −CN media (not shown) as well as in cuticle-containing media was inhibited by VAN, a specific inhibitor of the plasmalemma ATPase in fungi. This suggests that secretion *via* the cell membrane is an active process.

As cAMP may have diverse regulatory roles in fungi (Pall 1981) the effect of cAMP on protease production by *Metarhizium anisopliae* was studied. 5 mM exogenous cAMP or dibutyryl cAMP did not relieve CR caused by glucose (1%) or NH_4Cl (0.2%). Nor did addition of the cAMP phosphodiesterase inhibitor 3-isobutyl-1-methyl xanthine (IBMX; 5 mM), with or without cAMP, affect enzyme production. However, adenosine 5′-0-thiophosphate, an inhibitor of AMP dependent enzyme systems substantially reduced protease production (Table 2).

Intracellular distribution of enzymes

Crude vacuole-containing pellets were isolated by gentle homogenization of the mycelium in a stabilizing medium followed by differential centrifugation (Table 3). Pr1, Pr2 and the vesicle marker N-acetylglucosaminidase appeared in the supernatant and were released from vacuole pellets after disruption of membranes with Triton X-100. Controls washed in sorbitol (1%) did not release enzymes. There was a small, but reproducible difference in the specific activity of enzymes from the vacuole and supernatant fractions. Most probably enzymes are principally located in vacuoles and their activity in the supernatants derives from vacuoles disrupted during isolation. Lower levels of endocellular Pr1 and Pr2 were present in repressed (non-secreting) mycelium

from CM media compared with those found in starved mycelium. Bovine serum albumin (BSA) enhanced production of extracellular Pr2 but repressed Pr1 (Table 1, Fig. 1). This was also reflected in endocellular levels of enzymes from mycelium grown on BSA containig media.

Discussion

Previously we reported that proteases (Pr1 and Pr2) are produced rapidly by *Metarhizium anisopliae* in culture media and *in situ* on insect cuticle (St. Leger et al. 1986b, 1987b); their appearance precedes that of chitinase which is regulated by products of chitin degradation through an inducer-repressor mechanism (St. Leger et al. 1986a). The work described in this paper shows that synthesis of Pr1 and Pr2 occurs rapidly (< 2 h) by carbon and nitrogen derepression alone. Similar results with regard to derepression were found in *Aspergillus* spp. (Cohen 1981). *Neurospora crassa* (Cohen and Drucker 1977) and *Candida lipolytica* (Ogrydziak et al. 1977), although with the exception of some *Aspergillus* spp. (Cohen 1981) protease production was also derepressed upon limitation of sulphur – a feature not evident with *M. anisopliae*. It is noteworthy that sulphur-containing amino acids appear to be lacking from insect cuticle (St. Leger et al. 1986c). Since all proteins contain carbon and nitrogen these findings are consistent with the idea that a major function of the extracellular proteases is to make nutrients available from the cuticle. Enzyme levels were enhanced in cultures supplied with cuticle or other insoluble polymers (e.g. cellulose) that were insufficient to produce CR. Addition of more readily utilized metabolites (e.g. glucose or alanine) repressed protease production confirming that production is constitutive but repressible. Thus, rapid protease synthesis during pathogenesis is only likely in those host tissues where the concentration of readily metabolizable compounds is low. This is the case with insect cuticles as the components are largely insoluble until released by cuticle-degrading enzymes (St. Leger et al. 1986c, 1987a, b). However, repression could operate if ever the release from cuticle of degradation products exceeded fungal requirements.

Regulation of Pr1 and Pr2 is not identical; trace levels of Pr2 only are produced on CM and while the soluble protein BSA represses production of Pr1 it allows enhanced synthesis of Pr2 compared with −CN media. Also, while extracellular Pr1 levels in −CN medium exceeded that of Pr2, the reverse was true of endocellular activities; there being a greater percentage decrease for Pr1 than Pr2. Pr2 may be involved in cellular control mechanisms, catalyzing specific proteolytic inactivation and activation processes (St. Leger et al. 1987c) this endocellular role could account for it being less subject than Pr1 to CR.

Operational control of protease release is likely to involve synthesis rather than secretion as repression caused reduced endocellular levels of enzyme rather than accumulation as would be expected to occur if secretion was blocked but synthesis was unaltered. However, the involvement of *de novo* protein synthesis (shown by inhibition with cyclohexamide) in the rapid secretion of enzymes after repressed mycelium is placed in derepressing media implies the close coupling of synthesis and secretion. Inhibition studies suggest that regulation of proteases does not initially depend on DNA synthesis. However, inhibition by actinomycin D and 8-azoguanine implies that *de novo* synthesis of mRNA is involved in protease production with the operative steps in regulation being at the level of transcription.

This contrasts with bacteria where the lack of effect of transcriptional inhibitors has been used as evidence for the existence of stable protease-specific mRNA (see Law 1980, for review).

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