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Genetic variation in the cuticle-degrading protease activity of the entomopathogen *Metarhizium flavoviride*

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Abstract

Extracellular proteases have been shown to be factors of virulence in fungal entomopathogenicity. We examined the production of the cuticle-degrading extracellular proteases chymoelastase (Pr1) and trypsin (Pr2) in isolates of the fungus *Metarhizium flavoviride*. Fungal growth was in a mineral medium (MM) containing nitrate, and in MM supplemented with either cuticle from *Rhammatocerus schistocercoides* or with the non-cuticular substrate casein. The substrates used for growth influenced the expression of both analyzed proteases, the highest protease activities of nearly all isolates having been observed in the medium containing insect cuticle, with more Pr1 than Pr2 being produced. There was a natural variability in the production of cuticle-degrading proteases among isolates, although this was less evident for Pr2. Our data support the hypothesis that the production of Pr1 on insect cuticle is a useful characteristic for the analysis of intraspecific variability of *M. flavoviride* isolates.

Key words: Metarhizium flavoviride, *Rhammatocerus schistocercoides*, chymoelastase (subtilisin) (Pr1), trypsin (Pr2).

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Introduction

The South-American grasshopper, *Rhammatocerus schistocercoides,* is a major problem in Central Brazil, causing severe agricultural losses (Lecoq and Pierozzi, 1994). The entomopathogenic fungus *Metarhizium flavoviride* has been suggested as a biological control agent for locusts and grasshoppers (Prior *et al*., 1992), and it is known that some *M. flavoviride* isolates are highly virulent against *R. schistocercoides* (Magalhães *et al*., 1997). Fungal proteases are the enzymes most frequently considered critical in facilitating invasive mycosis of insects (Charnley and St. Leger, 1991), the studies on cuticle-degrading proteases in entomopathogenic fungi having mainly focused on *Metarhizium anisopliae*, which produces a chymoelastase (Pr1) (St. Leger *et al*., 1987a), a metalloprotease (St. Leger *et al*., 1994), a trypsin-like enzyme (Pr2) belonging to the serine protease group and a cysteine protease (Pr4) (Cole *et al*., 1993).

The most extensively studied protease of *M. anisopliae* is Pr1, whose role in host invasion was clearly demonstrated by St. Leger *et al*. (1988). According to Charnley and St. Leger (1991), this enzyme provides the best understood model of the fungal entomopathogenicity determinant . Gillespie *et al*. (1998) found that the Pr1 protease from different isolates of *Metarhizium* hydrolyzes different types of insect cuticle to different degrees, although there was no correlation between Pr1 activity and the mean lethal time of the isolates in the desert locust (*Schistocerca gregaria*).

The role of the Pr2 protease in insect parasitism was not yet elucidated, although St. Leger *et al*. (1994) reported that Pr2 as well as the other cuticle-degrading proteases may complement each other in the splitting of peptide bonds in the insect cuticle. Results obtained by Gillespie *et al*. (1998) are consistent with a role of Pr2 in the induction or activation of Pr1, since Pr2 was detected before Pr1 in a culture containing ground locust cuticle. Both Pr1 and Pr2 are also produced by other entomogenous fungi, including *Beauveria bassiana* (St. Leger *et al*., 1987b; Gupta *et al*., 1992), *Nomuraea rileyi,* and *Verticillim lecanii* (St. Leger *et al*., 1987b).

The only study on the production of cuticle-degrading proteases by *M. flavoviride* used cuticle of the desert locust, *S. gregaria* (Gillespie *et al*., 1998). We compared

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the production of Pr1 and Pr2 in *M. flavoviride* isolates, after growth on *R. schistocercoides* cuticle, with to protease production on non-cuticular substrate (casein and nitrate).

Materials and Methods

Organisms and culture conditions

Seven isolates of *Metarhizium flavoviride* (syn. *Metarhizium anisopliae* var. *acridum*) were obtained as liquid nitrogen-stored stock cultures from the Cenargen/Embrapa-Brazil collection of entomopathogenic fungi: CG423, CG430, CG431, CG288 (isolated from *Schistocerca pallens* in Brazil), CG442 (ARSEF 3391; IMI 324673, isolated from *Zonocerus elegans* in Tanzania), CG441 (isolated from *Ornithacris cavroisi* in Nigeria), and CG291 (ARSEF 324, isolated from *Austracnis guttulosa* in Australia).

Conidia were obtained by harvesting sporulated spread plates of minimal medium (MM) (Pontecorvo *et al*., 1953), containing nitrate as nitrogen source, and MM supplemented with either 1.0% casein (MM+cas) or 0.5% (w/v) insect cuticle (MM+cut) prepared from adult *Rhammatocerus schistocercoides* using an aqueous solution of 1% potassium tetraborate (Andersen, 1980). The prepared cuticle was added to previously sterilized MM and autoclaved for 5 min at 115 °C. For enzyme production, conidia were inoculated into 100 mL of liquid MM+cas and MM+cut at a concentration of 1 x 10^7 mL^{-1} and incubated as shake cultures at 150 rpm, 28 °C, for 72 h. The mycelia were harvested by filtration and the supernatants assayed for enzyme activity.

Enzyme assays and protein determination

In this paper, all *M. flavoviride* chymoelastase-like and trypsin-like activities are referred to as Pr1 and Pr2, respectively. Chymoelastase (Pr1) activity was assayed using succinyl-$(alanine)_2$-proline-phenylalanine-*p*-nitroanilide, and trypsin (Pr2) activity by using benzoyl-phenylalanine-valine-arginine-*p*-nitroanilide, as described by Gupta *et al*. (1992). Assays were performed with three replicates for each sample. Protein content was measured using Coomassie Brilliant Blue G-250 (Bradford, 1976).

Statistical analyses

Statistical analyses were performed using the Statistical Analysis System software (SAS Institute Inc., 1989). The experiment was set up as a randomized complete block design, with two replicates. The effect of treatment combinations (isolates and media) was assessed by factorial analysis. The variance analyses were followed by a comparison of the proteolytic activity means, using the Tukey test.

Results and Discussion

Chymoelastase (Pr1) activity was present in all the supernatants analyzed, and was highly expressed in the presence of insect cuticle (Figure 1). Isolate CG288 had the lowest activity, and isolate CG441 had the highest activity, about 27 times that of CG288 on the media tested.

All the isolates, with the exception of CG288, showed a significantly different Pr1 activity ($p < 0.01$) as a function of the growth substrate (Table I), the enzyme activity being about 2 to 8 times higher in MM+cas and about 4 to 20 times higher in MM+cut than in MM. The high levels of Pr1 found in MM+cut cultures may be due to the induction of Pr1 by insect cuticle components, since Paterson *et al*. (1994) determined that the Pr1 activity of starved *M. anisopliae* mycelium is specifically induced by cuticle from *S. gregaria*.

Among the Brazilian isolates, there was a significant enhancement in the Pr1 activity of CG423 after growth on *R. schistocercoides* cuticle, which may be related to its high virulence against this grasshopper species (Magalhães *et al*., 1997), since quantitative differences in *in vivo* protease production between isolates can influence virulence (Gillespie *et al*., 1998).

Trypsin (Pr2) was also produced by all isolates (Figure 2), but at lower levels than Pr1and with less variability between isolates than Pr1 (Table I). All isolates except CG288 and CG291 produced significantly different levels of trypsin ($p < 0.01$) as a function of the substrate used for growth (Table I), the highest production having been in MM+cut. Paterson *et al*. (1993) showed that *M. anisopliae* Pr2 is nonspecifically induced by any protein, if the fungus is starved of nitrogen, but not if starved of carbon in the presence of nitrogen. In our experiments with MM+cut, the Pr2 activity was only 29% (isolate CG442) and 24% (isolate CG423) of the Pr1 activity, similar results having been

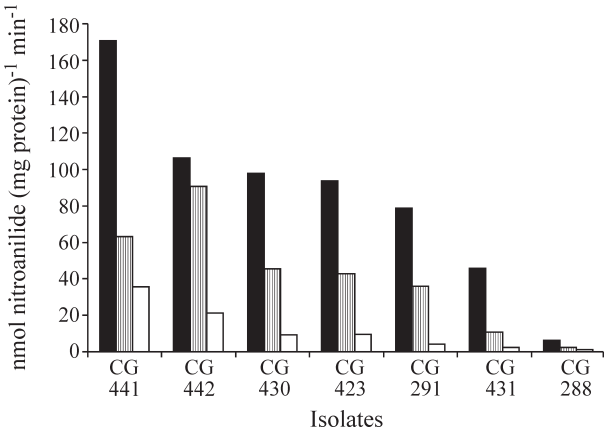

Figure 1 - Chymoelastase (Pr1) activity expressed as nmol nitroanilide released mg^{-1} protein min^{-1} from succinyl-$(alanine)_2$-proline-phenylalanine-*p*-nitroanilide, in culture supernatants from *Metarhizium flavoviride* isolates grown in minimal medium (□) and minimal medium amended with casein (1%) (vertical lines) or insect cuticle (0.5%) (■). The results are from two experiments with three replicates for each sample.

Table I - Mean values of chymoelastase (Pr1) and trypsin (Pr2) activities from *Metarhizium flavoviride* isolates grown in minimal medium plus cuticle (MM+ cut), minimal medium plus casein (MM+ cas), and minimal medium (MM), respectively. The results are from two experiments with three replicates for each sample.

Medium	M. flavoviride isolate						
	Pr1 activity						
	CG441	CG442	CG430	CG423	CG291	CG431	CG288
MM+cut	170.70aA	106.40aB	97.89aC	93.72aC	78.72aD	45.69aE	6.30aF
MM+cas	63.34bB	90.62bA	45.52bC	42.95bCD	35.99bD	10.68bE	2.32aF
MM	35.56cA	21.36cB	9.40cC	9.47cC	4.25cCD	4.25cCD	1.06aD
	Pr2 activity						
	CG442	CG441	CG288	CG291	CG431	CG430	CG423
MM+cut	31.29aA	24.15aB	22.05aB	18.47aC	22.13aBC	21.20aBC	22.64aBC
MM+cas	25.27bA	15.98cBC	20.07aB	20.72aAB	15.84bBC	15.66bBC	11.55bC
MM	12.40cB	19.65bA	6.43bC	1.22bD	1.95cCD	1.52cD	1.95cCD

Different letters represent a significant difference ($p < 0.05$) according to Tukey's test. Lower case letters indicate significant differences within a column, and capital letters within a row.

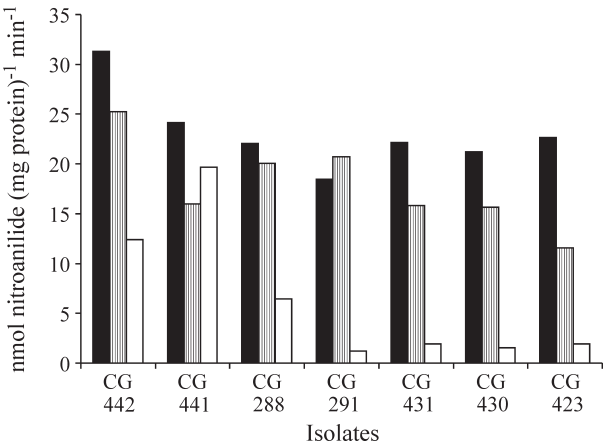

Figure 2 - Trypsin (Pr2) activity expressed as nmol nitroanilide released mg^{-1} protein min^{-1} from benzoyl-phenylalanine-valine-arginine-*p*-nitroanilide, in culture supernatants from *Metarhizium flavoviride* isolates grown in minimal medium (□) and minimal medium amended with casein (1%) (vertical lines) or insect cuticle (0.5%) (■). The results are from two experiments with three replicates for each sample.

observed for cuticle-degrading proteases produced by *M. anisopliae* against *S. gregaria* (Cole *et al*., 1993).

In our study, the substrates used for growth significantly influenced the expression of Pr1 and Pr2, except in isolate CG288 (Table I).We found a natural variability in the production of insect cuticle-degrading proteases among *M. flavoviride* isolates . Strain variability has also been observed in the production of proteases in *M. anisopliae* (St. Leger *et al*., 1986; Gillespie *et al*., 1998) and in *B. bassiana* (Gupta *et al*., 1992), while Leal *et al*. (1997) demonstrated significant variations in Pr1 sequences among strains of entomopathogenic fungi. It has also been described that *M. anisopliae* proteases comprise multiple isoforms (St. Leger *et al*., 1994).

A consistent pattern of Pr1 expression was apparent among isolates, with all isolates except CG423 and CG430 producing statistically different levels of Pr1 on cuticle, which gave a 100% homology on RAPD analysis (Martins, 1998). The production of Pr1 in a culture containing *R. schistocercoides* cuticle may be useful to analyze strain variability of *M. flavoviride* and related entomopathogenic fungi. Leal *et al*. (1997) used the PCR product derived from the *M. anisopliae* Pr1 gene to differentiate between many *Metarhizium* strains. Fungal strain variability in Pr1 production may be directly related to variability in virulence, because the regulation of the Pr1 gene expression may determine the capacity of the fungus to cause insect disease (St. Leger *et al*., 1988).

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