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Comparative analysis of the *Metarhizium anisopliae* secretome in response to exposure to the greyback cane grub and grub cuticles

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ABSTRACT

Metarhizium anisopliae is a well-characterized biocontrol agent of a wide range of insects including cane grubs. In this study, a two-dimensional (2D) electrophoresis was used to display secreted proteins of *M. anisopliae* strain FI-1045 growing on the whole greyback cane grubs and their isolated cuticles. Hydrolytic enzymes secreted by *M. anisopliae* play a key role in insect cuticle-degradation and initiation of the infection process. We have identified all the 101 protein spots displayed by cross-species identification (CSI) from the fungal kingdom. Among the identified proteins were 64-kDa serine carboxypeptidase, 1,3 beta-exoglucanase, Dynamin GTPase, THZ kinase, calcineurin like phosphoesterase, and phosphatidylinositol kinase secreted by *M. ansiopliae* (FI-1045) in response to exposure to the greyback cane grubs and their isolated cuticles. These proteins have not been previously identified from the culture supernatant of *M. anisopliae* during infection. To our knowledge, this the first proteomic map established to study the extracellular proteins secreted by *M. ansiopliae* (FI-1045) during infection of greyback cane grubs and its cuticles.

Introduction

The greyback cane grub, *Dermolepida albohirtum* (Waterhouse) (Coleoptera: Scarabaeidae) is considered the most serious pest of sugarcane in tropical areas of Queensland, Australia (Robertson *et al.* 1997). Damage is caused by the larvae feeding on the roots of the sugarcane plant leading to retarded growth and in extreme cases, plant death. The fungus *Metarhizium anisopliae* grows naturally in soils throughout the world and causes disease in various insects (Zimmermann 2007). In Australia, it is applied as a biopesticide for use against various cane grub species (Milner 2000) and is the active ingredient in the biopesticide 'BioCane™' (Milner *et al.* 2002).

Entomopathogenic fungi exhibit many characteristics that determine virulence towards their hosts, including the production of degradative enzymes. Hydrolytic enzymes such proteases and chitinases are produced during fungal penetration through the cuticle. Among them, fungal proteases are considered to play a significant role in cuticle-degradation and are essential for the initiation of the infection process (St. Leger *et al.* 1987, 1996). One of the best-studied proteases of which the function in host invasion has been clearly established is subtilisin-like serine protease (Pr1) of *M. anisopliae* (St. Leger *et al.* 1986a, b). During the early stages of pathogenesis, Pr1 degrades insect cuticular proteins (St. Leger *et al.* 1996; Freimoser *et al.* 2003) and has been ultrastrucutrally localized

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in the host cuticle during early phase of penetration (St. Leger et al. 1996). A trypsin-like serine protease (Pr2) also appears during the early stages of colonization, suggesting that it has some role in cuticle-degradation complementary to that of Pr1 (St. Leger et al. 1987). Pr1 and Pr2 proteases have been identified in various entomopathogenic fungi including *M. anisopliae*, *M. flavoviride*, *Beauveria bassiana*, and *Paecilomyces fumosoroseus* (St. Leger 1995; Joshi et al. 1997; Bidochka & Meltzer 2000; Shah & Pell 2003). Inhibition of protease activity or use of protease deficient mutants resulted in decreased virulence against insects (Bidochka & Khachatourians 1990; Wang et al. 2002). Several authors have described Pr1 and Pr2 activity in *Metarhizium* growing in liquid minimal medium supplemented with different insect cuticles (St. Leger et al. 1986a, b; Bidochka & Khachatourians 1994; Pinto et al. 2002). The synthesis of extracellular proteases (Pr1 and Pr2) is controlled by several regulatory paths that include repression and induction by the carbon sources (St. Leger et al. 1987; Bidochka & Khachatourians 1988). In addition, *M. anisopliae* produces several chitinolytic enzymes which act after the proteases have considerably digested the cuticular proteins thereby exposing the chitin part of the cuticle (Shah & Pell 2003; Krieger de Moraes et al. 2003).

Proteins represent more than 50 % of the weight of the insect cuticle and insect larvae have a soft thin cuticle (epicuticle) over most of their body. Major portion of the insect larvae is made up of muscle which contains approximately 70–75 % water, 20–22 % protein, 4–8 % lipid, 1 % ash, and no carbohydrates. Two-dimensional (2D) gel electrophoresis has uncovered over 100 different cuticular proteins that differ in their molecular weight and isoelectric point (Andersen et al. 1995; St. Leger et al. 1996; Freimoser et al. 2003). However, there are differences in the ability of entomopathogenic fungal proteases to degrade different types of insect cuticle because these cuticle types differ in their protein composition (Shah & Pell 2003). In this study, we have examined the production of various proteins by *M. anisopliae* in the presence greyback cane grubs and isolated cane grub cuticles, extending our knowledge about protein production by this fungus with a view of establishing novel biotechnological tools to use for greyback cane grub control.

Materials and methods

Insect larvae

Third instar greyback cane grubs, *Dermolepida albohirtum* were dug from the soil below sugarcane stools in commercial fields around Townsville in Queensland, Australia (http://www.ga.gov.au/bin/gazd01?rec=155794) and each grub was packed in a single plastic tube for transportation to Sydney in a ventilated container. After arrival, the cane grubs were removed from the plastic tube and transferred into 100 ml sterile transparent plastic tubs (Technoplas, Australia) filled with 30 g of sterilized garden peat (Killarney peat moss, Australia). The cane grubs were fed with fresh pieces of carrot and held at room temperature (RT) (~22–25 °C). Around ten healthy cane grubs were immobilized by cotton dipped in chloroform (Ajax Finechem, Sydney) to anaesthetize the cane grubs. Cane grubs were then rinsed in sterile distilled water to remove peat particles attached to the body and transferred into sterile 15 ml tubes, freeze dried and pulverized using mortar and pestle. Cuticles were isolated by extracting the soft tissue from homogenized insects with potassium tetra borate (St. Leger et al. 1986a, b). Clean cuticle samples from the greyback cane grubs were prepared as described previously (St. Leger et al. 1987).

Fungal strain and cultivation conditions

Metarhizium anisopliae (FI-1045) was conidiated and maintained on potato dextrose agar plates (Difco Laboratories, MI, USA). Conidia were collected using 5 ml of 0.9 % (w/v) sodium chloride, 0.01 % (v/v) Tween 80 and 5 × 108 conidia were used to inoculate 50 ml of minimal medium (MM; 110 mM potassium phosphate, 38 mM ammonium sulphate, 2.4 mM magnesium sulphate, 4.1 mM calcium chloride, 2.9 mM manganese sulphate, 7.2 mM iron sulphate, 0.35 mM zinc sulphate, 0.71 mM cobalt sulphate, pH 5.5) supplemented with 2 % (w/v) glucose in a 250-ml Erlenmeyer flask, similarly to the method used by Grinyer et al. (2005). Cultures were grown at 28 °C on a shaker at 250 rpm for 48 h (pre-culture). The mycelia from each flask were collected, washed three times with 50 ml of Milli-Q water by invertion and centrifugation at 4000*g* for 10 min at 15 °C and re-inoculated in fresh MM with 1 % (w/v) pulverized greyback cane grub cuticles (Culture A) or 1 % (w/v) pulverized whole greyback cane grubs (Culture B). Cultures were grown for 48 h on a shaker at 250 rpm at 28 °C. Culture supernatants and mycelia were harvested at both 24 h and 48 h time points. Fungal and yeast protease inhibitor cocktail (0.05 % v/v) was added to the culture and allowed to incubate at RT for 20 min. Culture supernatants containing protease inhibitors were collected by centrifugation at 4000*g* for 10 min at 15 °C and stored at −20 °C until required. The mycelia were washed three times with 50 ml of Milli-Q water by invertion and centrifugation at 4000*g* for 10 min at 15 °C, collected and stored at −20 °C until use. The experiment was set up in triplicate but materials from only two cultivations were used.

Precipitation of proteins from culture supernatant

The *Metarhizium anisopliae* (FI-1045) culture supernatant was thawed and 13 ml were taken and spun at 21000g for 15 min at 10 °C. Ammonium sulphate was added to the supernatant to give an 80 % saturated solution that was stirred overnight at 4 °C to allow protein precipitation. The precipitated proteins were pelleted by centrifugation at 21 000*g* for 15 min at 4 °C. Precipitated proteins were re-suspended in 1 ml of re-suspension solution [7 M urea, 2 M thiourea, 4 % (w/v) CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate)(detergent C-3023: Sigma), 40 mM Tris, 5 mM tributylphosphine, 10 mM acrylamide, 1 mM phenylmethylsulphonyl fluoride (PMSF), 0.1 % protease inhibitor tablet] and incubated at RT for 90 min to allow complete reduction and alkylation of proteins. The reduction and alkylation reactions were quenched with 10 mM dithiothreitol before insoluble material was removed by spinning at 21 000*g* for 10 min. The solution was desalted (buffer exchanged) further by ultra-filtration with 7 M urea, 2 M thiourea, 4 % (w/v) CHAPS in Ultrafree™