

Regulation of Extracellular Chitinases and Proteases in the Entomopathogen and Acaricide *Metarhizium anisopliae*

Caroline Krieger de Moraes, Augusto Schrank, Marilene Henning Vainstein

Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Campus do Vale, P.O. Box 15005, 91501-970 Porto Alegre, RS, Brazil

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Abstract. *Metarhizium anisopliae* infects insects and ticks via a combination of specialized structures and cuticle degradation. Hydrolytic enzymes are accepted as key factors for the penetration step. The search for pathogenicity determinants has demonstrated that the process is multifactorial. Host specificity is an important factor to be addressed. The study of the enzymes produced during infection is important to discover those with a role in the process. To address some of the enzymes that take part during the infection of the tick, *Boophilus microplus*, we have analyzed the secretion of proteases and chitinases in single and combined carbon/nitrogen sources as compared with such complex substrates as chitin and *B. microplus* cuticles. Two chitinases, endo- and N-acetylglucosaminidases, and two proteases, subtilisin and trypsin-like proteases, were analyzed. Enzyme activities were detected in all carbon sources tested, but higher levels were found when combinations of carbon sources were used. A major 30-kDa protein apparently secreted during *M. anisopliae* growth on all carbon/nitrogen sources tested was demonstrated by SDS-PAGE.

Metarhizium anisopliae is a well-known, broad-range arthropod pathogen, which has been used in experimental systems for biological control of several insect pests and ticks [4, 5, 17]. During the fungal penetration through the host cuticle, hydrolytic enzymes such as proteases, chitinases, and lipases are produced and secreted and are important for the initiation of the infection process [13, 18]. *M. anisopliae* produces distinct extracellular serine proteases, such as subtilisin-like proteases, trypsin-like proteases, metalloproteases, as well as several families of exo-acting peptidases that are believed to be important for host cuticle degradation [17, 19]. The subtilisin protease family Pr1 is the main enzyme produced by *M. anisopliae* during the infection process, although the trypsin-like protease Pr2 is the first to appear during in vitro growth on the cuticle [5]. Antiserum against Pr1 protease interferes with penetration of the host cuticle and reduces infection, indicating that the level of active Pr1 may determine the capacity of the fungus to cause disease [15]. The addition of multiple copies of the *pr1A* gene under the control of a constitutive promoter increased the virulence of the transfor-

mants, suggesting that this subtilisin protease may be an important determinant for the development of engineered biopesticides [20]. However, the disruption of this gene did not affect the overall infectivity. This and other results suggest that the infection process is achieved by the synergistic action of the enzymes involved. *M. anisopliae* also produces chitinolytic enzymes that act after the pathogen's proteases have digested the cuticle protein components, exposing the chitin present in the arthropod cuticle. In fungi, chitinases have a physiological role in hyphal growth and morphogenesis [11]. The role of the chitinases in the host infection process is not yet fully understood.

A range of extracellular enzymes that can degrade the components of the insect cuticle are produced when *M. anisopliae* is grown in vitro with cuticle as the sole carbon and nitrogen source [3, 5]. The regulation of genes encoding cuticle-degrading enzyme isoforms is probably complex and may involve a combination of carbon/nitrogen induction and/or repression [12].

In the current work, the effects of different carbon sources, and/or the cuticle of the tick (*Boophilus microplus*), on the secretion of hydrolytic enzymes by *M. anisopliae* was investigated.

Table 1. Effect of carbon sources on the production of chitinases by *M. anisopliae*

Carbon source	Chitinolytic activity U ($\mu\text{g protein}^{-1} \text{h}^{-1}$)	
	Diacetylchitobiose	Tetraacetylchitotetraose
GlcNAc 1.5%	0.619 \pm 0.012 ^b	0.958 \pm 0.048 ^c
GlcNAc 1.0%	0.665 \pm 0.040 ^b	1.074 \pm 0.050 ^c
GlcNAc 0.5%	1.380 \pm 0.030 ^a	1.302 \pm 0.040 ^b
GlcNAc 0.25%	0.732 \pm 0.010 ^b	0.581 \pm 0.010 ^d
Glucose 0.8%	0.201 \pm 0.020 ^d	0.146 \pm 0.006 ^e
Tick cuticle 0.8%	0.090 \pm 0.009 ^e	0.026 \pm 0.002 ^g
Chitin 0.8%	0.073 \pm 0.003 ^e	0.055 \pm 0.004 ^{f,g}
Tick cuticle 0.8% plus GlcNAc 1.0%	1.300 \pm 0.040 ^a	1.825 \pm 0.050 ^a
Tick cuticle 0.8% plus GlcNAc 0.2%	0.455 \pm 0.020 ^c	1.059 \pm 0.020 ^c
Tick cuticle 0.8% plus Glucose 0.8%	0.247 \pm 0.003 ^d	0.078 \pm 0.004 ^{f,g}
Chitin 0.8% plus GlcNAc 1.0%	0.709 \pm 0.020 ^b	0.105 \pm 0.003 ^{f,g}
Chitin 0.8% plus GlcNAc 0.2%	0.373 \pm 0.010 ^{c,d}	0.209 \pm 0.010 ^e
Chitin 0.8% plus Glucose 0.8%	0.602 \pm 0.010 ^b	0.074 \pm 0.005 ^{f,g}

The results are means of three replicates.

Means followed by the same letter in each column are not significantly different according to Duncan's Multiple Range Test ($\alpha = 0.05$).

Table 2. Effect of carbon sources on the production of proteases by *M. anisopliae*

Carbon source	Proteolytic activity U ($\mu\text{g protein}^{-1} \text{h}^{-1}$)	
	Suc-(Ala) ₂ Pro-Phe-AMC	CBZ-Arg-Arg-AMC
GlcNAc 1.0%	48 \pm 0.70 ^b	50.5 \pm 0.70 ^d
GlcNAc 0.5%	160 \pm 0.50 ^d	19 \pm 0.41 ^g
Glucose 0.8%	27 \pm 0.70 ^j	67.5 \pm 0.70 ^b
Tick cuticle 0.8%	229.5 \pm 0.70 ^b	60.5 \pm 0.70 ^c
Chitin 0.8%	155.5 \pm 0.50 ^{d,e}	121.5 \pm 0.70 ^a
Tick cuticle 0.8% plus GlcNAc 1.0%	59.15 \pm 0.19 ^g	42.5 \pm 0.12 ^e
Tick cuticle 0.8% plus GlcNAc 0.2%	39 \pm 0.33 ⁱ	35.5 \pm 0.50 ^f
Tick cuticle 0.8% plus Glucose 0.8%	149.5 \pm 0.70 ^e	71 \pm 0.70 ^b
Chitin 0.8% plus GlcNAc 1.0%	330.5 \pm 0.70 ^a	45.6 \pm 0.70 ^e
Chitin 0.8% plus GlcNAc 0.2%	88.5 \pm 0.70 ^f	37.5 \pm 0.14 ^f
Chitin 0.8% plus Glucose 0.8%	191 \pm 0.41 ^c	43.5 \pm 0.70 ^e

The results are means of three replicates.

Means followed by the same letter in each column are not significantly different according to Duncan's Multiple Range Test ($\alpha = 0.05$).

Materials and Methods

Organism and culture conditions. *Metarhizium anisopliae* E6, from the Microbial Genetics Group Collection (Escola Superior de Agronomia Luiz de Queiroz, USP, Brazil), was maintained and spores were produced as described [9]. For the experiments, spores (10^6 ml^{-1}) were grown in 100 ml of liquid Cove's medium supplemented with yeast extract (0.05%). As carbon sources, glucose (0.8%) or *N*-acetylglucosamine, GlcNAc (0.5% or 1%) was added. Complex substrates such as chitin 0.8%; tick cuticle (*B. microplus*) 0.8%; combination of tick cuticle or chitin 0.8% plus different GlcNAc concentrations (0.2% or 1%); and tick cuticle or chitin 0.8% plus glucose 0.8% were also tested. After 3 days of incubation at 28°C on a rotating shaking platform (180 rpm), the mycelium was removed by filtration on Whatman # 1 filter paper. Prior to use for enzymatic assays, the filtrates were dialyzed against 10 mM acetate buffer (pH 5.4). The total protein content was determined by the Bradford method [2], with known concentrations of BSA as standard. Reducing sugar concentration was determined by using dinitrosalicylic reagent and D-glucose as standard [8].

Enzymatic assays. Chitinolytic activity was determined with *N,N'*-diacetylchitobiose (4 mM) to detect *N*-acetyl- β -D-glucosaminidase and *N,N',N'',N'''*-tetraacetylchitotetraose (4 mM) to detect endochitinase activities. The reaction mixture was: 40 μl 0.2 mM acetate buffer (pH 5.4); 10 μl of each substrate; and 120 μl of sample. After 1 h of incubation at 37°C, the amount of *N*-acetylglucosamine released was determined by the method described by Reissig et al. [10]. One unit of chitinase was defined as the amount of enzyme that releases 1 μmol GlcNAc min^{-1} at 37°C. Proteolytic activity was determined with the following fluorogenic peptides as substrates: suc-(Ala)₂-Pro-Phe-A-4-UM (1 mM) to determine subtilisin and metalloprotease activities, or N-CBZ-Arg-Arg-A-4-UM (1 mM) to determine trypsin activity. These assays were performed in a total volume of 200 μl (100 μl of culture supernatant; 95 μl of 50 mM Tris-HCl pH 7.4; 5 μl of substrate) for 1 h at 37°C. The hydrolysis was monitored by measuring the fluorescence at a λ emission of 420 nm and a λ excitation of 320 nm in a TKO 100 minifluorimeter.

Electrophoresis. SDS-PAGE was carried out with 12% acrylamide running gels [6]. After electrophoresis, the gels were stained for 2 h in

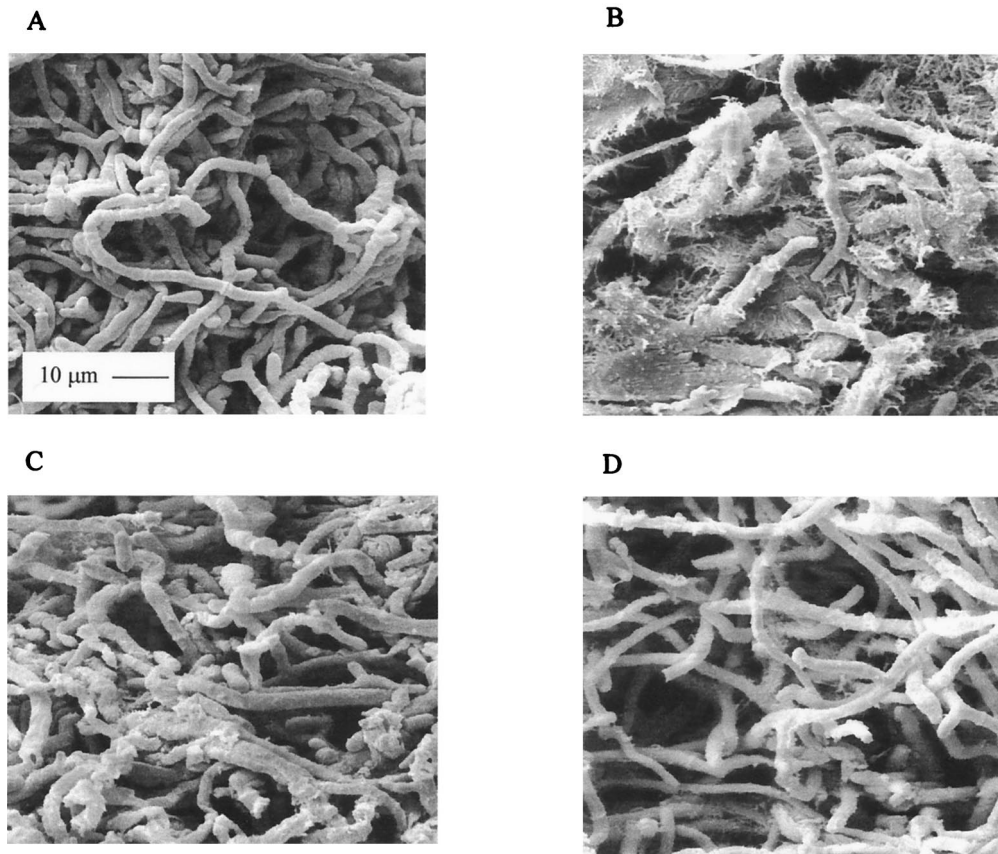


Fig. 1. *Metarhizium anisopliae* mycelia grown in Cove's medium supplemented with different carbon sources: (A) glucose; (B) chitin; (C) 1% GlcNAc; (D) tick cuticle. Specimens were examined in a Zeiss DSM 940 A scanning electron microscope at 15 kV \times 2000.

0.25% Coomassie Brilliant Blue-R dye/40% methanol/10% acetic acid and were destained in the same solution without the dye.

Scanning electron microscopy (SEM). The mycelia were fixed in Trump's fixative [7], post-fixed for 1 h in 0.1 M cacodylate buffer at pH 7.4 with 1% osmium tetroxide/2% glutaraldehyde. Samples were dehydrated in a battery of acetone solutions (30%, 50%, 70%, 90%, and 100%), dried at critical point, coated with gold, and examined in a Zeiss DSM 940 A scanning electron microscope.

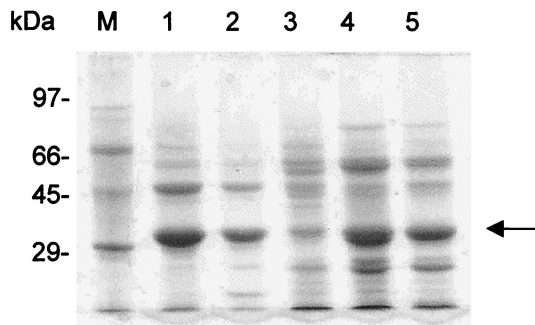
Protein purification. Purification of a protein with chitinase activity was carried out with ion-exchange column chromatography. The culture filtrates from media supplemented with chitin 0.8% plus GlcNAc 0.2% or with chitin 0.8% plus glucose 0.8% were freeze-dried and resuspended in 20 ml Milli-Q water. The concentrated samples were applied to a DEAE-Sepharose column (0.8 \times 15 cm) equilibrated with 50 mM acetate buffer (pH 5.4) at a 2-ml \cdot min $^{-1}$ flow rate. The column was washed with equilibration buffer (100 ml) and with a 160-ml linear NaCl 1 M gradient. Five-milliliter fractions were collected and assayed for chitinase activity as previously described.

Results and Discussion

Metarhizium extracellular hydrolytic enzymes are important for the degradation of host cuticle during infection, assisting penetration and providing nutrients for further

growth [1, 17]. The effects of different carbon sources on chitinase and protease secretion by *M. anisopliae* were tested in medium supplemented with simple or complex carbon sources individually or in combination. As shown in Tables 1 and 2, *M. anisopliae* produced chitinases and proteases in all media tested; however, the amount of secreted enzymes varied. The highest levels of *N*-acetyl- β -D-glucosaminidase and endochitinase activities were found in the supernatants from 0.5% *N*-acetylglucosamine (GlcNAc) alone and with combinations of tick cuticle plus 1.0% GlcNAc. When chitin or tick cuticles were added to the media, without GlcNAc, both chitinase activities were detected at basal levels, suggesting an induction of chitinolytic enzymes by GlcNAc. When different concentrations of GlcNAc were used as carbon sources, endochitinase activity was detected even when the fungus was grown in high concentration of this monomer. According to St Leger et al. [13], GlcNAc might cause catabolic repression of chitinases when in excess of the immediate growth requirements of the organisms, and *N*-acetyl- β -D-glucosaminidase production could be related to cellular growth.

A



B

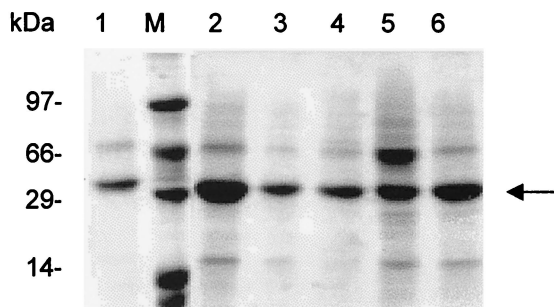


Fig. 2. SDS-PAGE on a 12% gel of the proteins secreted by *M. anisopliae* grown in different media. (A) M, molecular mass marker; 1, 0.8% cuticle + 1% GlcNAc; 2, 0.8% cuticle + 0.2% GlcNAc; 3, 0.8% cuticle + 0.8% glucose; 4, 0.8% chitin + 1% GlcNAc; 5, 0.8% chitin + 0.2% GlcNAc; 6, 0.8% chitin + 0.8% glucose. (B) M, molecular mass marker; 1, 0.8% cuticle; 2, 0.8% chitin; 3, 0.8% glucose; 4, 1% GlcNAc; 5, 0.5% GlcNAc. Arrow indicates the 30 kDa protein.

In media supplemented with glucose or GlcNAc, low amounts of reducing sugar were detected in the culture supernatants after 3 days of growth (data not shown), indicating that the repressing carbon source was consumed, resulting in enzyme synthesis. No differences among mycelia grown on different carbon sources were apparent by SEM analysis, and hyphae hydrolysis was not observed (Fig. 1). These data indicate that the enzymatic activities resulted from protein secretion and were not due to autolysis of the hyphae.

High levels of subtilisin activity were observed in cultures supplemented with tick cuticle or chitin plus 1.0% GlcNAc, and lower levels were observed in cultures containing glucose, 1.0% GlcNAc or tick cuticle plus 0.2% GlcNAc. The highest level of trypsin activity was found in medium with added chitin 0.8% (Table 2). Since arthropod cuticles comprise about 70% protein,

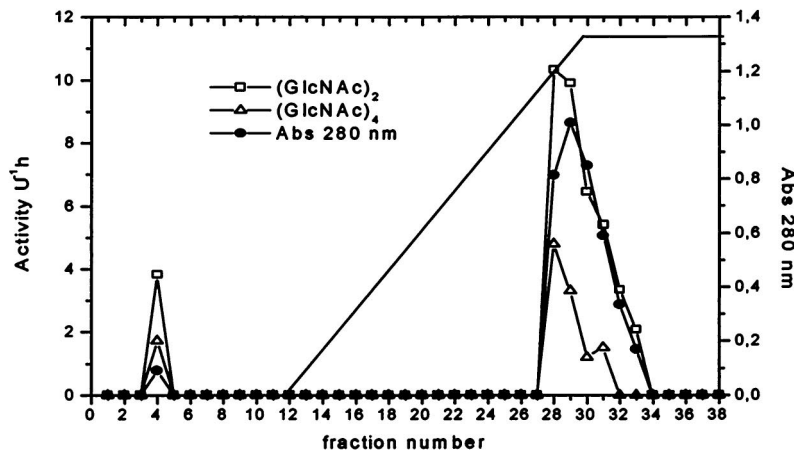
this enzyme activity may have an important role in host penetration [5]. Several experiments have used insect cuticles as substrates to analyze how host cuticles influence production of cuticle-degrading enzymes. *M. anisopliae* produced distinct types of proteases during growth on cockroach cuticle, and four isoforms of Pr1 were separated from other proteases by narrow-range isoelectric focusing (IEF) [14, 16]. Charge differences among Pr1 isoforms probably affect cuticle-degrading ability, since St. Leger *et al.* [16] have shown that electrostatic binding of Pr1 is a prerequisite for cuticle hydrolysis.

Analysis of total extracellular protein by SDS-PAGE (Fig. 2) showed that *M. anisopliae* secretes a large number of proteins when grown on media supplemented with different substrate sources. An approximately 30-kDa protein was induced independent of the individual (Fig. 2A) or combined (Fig. 2B) source used, except for glucose (Fig. 2 A, lane 3).

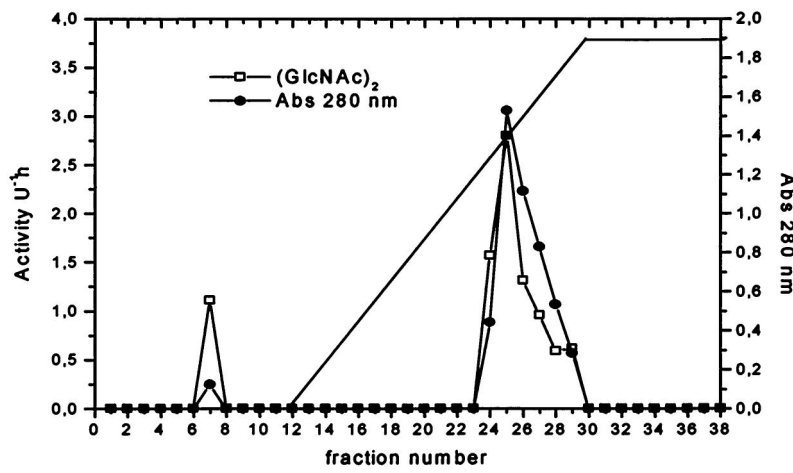
The partially purified proteins with chitinase activity from different culture filtrates of *M. anisopliae* differed in charge since they eluted at different salt concentrations from the DEAE-Sepharose column (Fig. 3). Two peaks with chitinolytic activity were observed when *M. anisopliae* was grown in medium containing 0.8% chitin plus 0.2% GlcNAc (Fig. 3A). According to Pinto *et al.*, [9], the enzyme present in the medium with added 0.8% chitin plus 0.2% GlcNAc also has endo-exo chitinase activity. No activity against tetraacetylchitotetraose was observed when the fungus was grown in medium with added chitin plus glucose (Fig. 3B), suggesting that glucose inhibits the enzyme that hydrolyzes such substrate. Analysis by SDS-PAGE from both purifications showed the presence of a 30-kDa protein (Fig. 3C). However, in order to determine whether this protein is the same as the chitinase purified by Pinto *et al.* [9], further analysis must be performed.

The multiplicity of *M. anisopliae* enzymes provides a major challenge to determine the role played by a particular enzyme in the adaptation to a new environment or in pathogenicity. When *M. anisopliae* was grown in media with added cuticle extracted from locust (*Schistocerca gregaria*), subtilisin, metalloprotease, and trypsin activities were found [5]. On the other hand, protease activities found in *M. anisopliae*-grown cultures on cockroach cuticle (*Blaberus giganteus*) were subtilisin, carboxipeptidase, and trypsin [17]. Our plan is to purify the enzymes secreted in culture media so that the effects of the observed protein secretion will be better analyzed by using antibody against purified chitinases and proteases. The variability of enzymes increases the range of tools naturally available to degrade the cuticle and to develop biotechnological procedures for pest control. However, the physiological role of proteases and

A



B



C

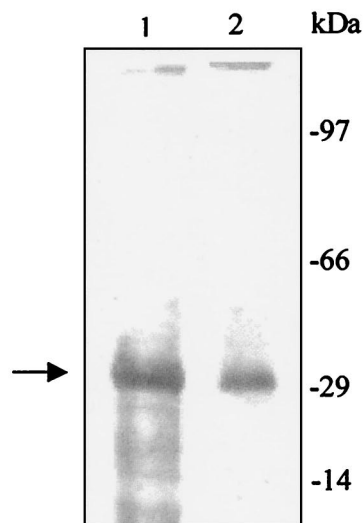


Fig. 3. Partial purification of *M. anisopliae* secreted chitinases on DEAE-Sepharose column (0.8 × 15 cm). Concentrated sample was pumped into a column at a flow rate of 2 ml h⁻¹. The column was washed with 100 ml 50-mM sodium acetate pH 5.4 and 160 ml linear NaCl 0–1 M gradient. Fractions of 5 ml were collected. (A) chitin + 0.2% GlcNAc; (B) chitin + glucose. (C) Silver-stained SDS-PAGE 12% gel of the partially purified proteins. Lane 1, fraction 26 from medium containing chitin + GlcNAc as carbon source; lane 2, fraction 28 from medium containing chitin + glucose. Arrow indicates the 30-kDa protein.

mainly of chitinases remains to be investigated in order to fully determine the importance of these enzymes in pathogenicity.

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