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# Proteomic analysis of *Metarhizium anisopliae* secretion in the presence of the insect pest *Callosobruchus maculatus*

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Crop improvement in agriculture generally focuses on yield, seed quality and nutritional characteristics, as opposed to resistance to biotic stresses. Consequently, natural antifeedant toxins are often rare in seed material, with commercial crops being prone to insect pest predation. In the specific case of cowpea (*Vigna unguiculata*), smallholder cropping is affected by insect pests that reproduce inside the stored seeds. Entomopathogenic organisms can offer an alternative to conventional pesticides for pest control, producing hydrolases that degrade insect exoskeleton. In this study, protein secretions of the ascomycete *Metarhizium anisopliae*, which conferred bioinsecticidal activity against *Callosobruchus maculatus*, were characterized via 2D electrophoresis and mass spectrometry. Proteases, reductases and acetyltransferase enzymes were detected. These may be involved in degradation and nutrient uptake from dehydrated *C. maculatus*. Proteins identified in this work allowed description of metabolic pathways. Their potential applications in biotechnology include both novel compound development and production of genetically modified plants resistant to insect pests.

## INTRODUCTION

*Callosobruchus maculatus* is the main pest of cowpea (*Vigna unguiculata*), a common smallholder crop in Latin America and Africa (Delincee *et al.*, 1998). During its larval stage, this weevil feeds on cotyledon material, causing significant economic losses through reduced seed quality and quantity (Credland *et al.*, 1986). Control of *C. maculatus* currently relies upon application of chemical insecticides, biological control with bacteria, protozoa, nematodes, viruses and fungi (Kaya & Gaugler, 1993; Legner, 1995; Becker, 1998; Scholte *et al.*, 2004; Jackai & Adalla, 1997; Galvan *et al.*, 2006), and post-harvest treatment with ionizing radiation (Delincee *et al.*, 1998). In recent years, the applicability of entomopathogenic fungi has been examined for biological control of coleopteran pests (Adane *et al.*, 1996; Rice & Cogburn, 1999; Bourassa *et al.*, 2001; Kassa *et al.*, 2002). For example, strains of *Metarhizium anisopliae* from diverse geographical origins have been characterized according to their virulence against storage Bruchidae pests of maize, beans, rice and other agricultural crops (Cherry *et al.*, 2005; Murad *et al.*, 2006, 2007). These fungi secrete hydrolytic enzymes involved in insect attack, and offer potential for development of bioinsecticides and/or construction of pest-resistant genetically modified plants (Murad *et al.*, 2006, 2007). In a previous study, we screened 10 *M. anisopliae* isolates for virulence against *C. maculatus*, and performed enzymic and 2D gel electrophoretic analyses of fungal secretions in the absence and presence of dehydrated *C. maculatus* (Murad *et al.*, 2006). The results indicated that *M. anisopliae* strain CG34 has potential as a source of proteins for control of *C. maculatus*. Other authors have also utilized proteomic approaches in fungi for discovery of novel compounds with biological control properties. For example, Grinyer *et al.* (2004) characterized secretions in *Trichoderma atroviride* (formerly *Trichoderma harzianum*). This mycopathogenic fungus inhibits growth of diverse

Abbreviation: PMF, peptide mass fingerprinting.

Supplementary data are available with the online version of this paper: two supplementary figures, showing Bradford analysis of protein concentration of fungal secretions and the *M. anisopliae* growth curve in the presence and absence of dehydrated *C. maculatus*, and a supplementary table presenting *in silico* evidence of the presence of secretion signal peptide in proteins secreted by *M. anisopliae* in response to the presence of *C. maculatus* adult insects.

phytopathogens, offering an alternative to traditional chemical control. A novel aspartic proteinase has been identified in *T. atroviride* secretions, induced by cell wall material from the pathogen *Rhizoctonia solani* (Suarez *et al.*, 2005). Hydrolytic enzymes have also been detected in *T. atroviride*, active against *R. solani* (Grinyer *et al.*, 2005). Two-dimensional gels have also been utilized to identify molecular mechanisms involved in degradation of exoskeleton material in the grasshopper *Melanoplus sanguinipes*, using proteinases secreted from *M. anisopliae* and *Beauveria bassiana* (Bidochka & Khachatourians, 1994). Proteomic approaches have also been applied to elucidate physiological processes in *Aspergillus fumigatus* (Carberry *et al.*, 2006). This fungus synthesizes approximately 250 proteins, of which 110 are related to energy metabolism, with constitutive expression in carbohydrate uptake and metabolism. A 2D LC-MS/MS (liquid chromatography mass spectrometry/mass spectrometry) approach has also been utilized to analyse protein expression in *Saccharomyces cerevisiae* during responses to nutritional stress (Kolkman *et al.*, 2006).

Based upon such studies, we continued the work described previously (Murad *et al.*, 2006), analysing secretions in the virulent *M. anisopliae* strain CG34. Important enzymes involved in fungal carbohydrate/nitrogen metabolism were identified, providing novel insights into the physiology of insect host colonization by *M. anisopliae*. These offer potential for development of new tools for improved crop protection to insect pests.

## METHODS

**Cowpea weevil colonies.** *C. maculatus* colonies were maintained in flasks which contained cowpea seeds and incubated at 28 °C with 60–70 % relative humidity. Secretion induction in *M. anisopliae* was carried out using adult weevils removed from colonies 24 h after oviposition. Insects were dehydrated at 49 °C for 48 h and macerated.

**Production of the secretome.** *M. anisopliae* strain CG34 was obtained from the Entomopathogenic Fungal Collection, Embrapa Recursos Genéticos e Biotecnologia (Brazil). This strain was previously determined by our group as the most virulent against *C. maculatus* (Murad *et al.*, 2006). Following initial incubation for 10 days at 28 °C on potato dextrose agar (10 % potato, 0.1 % D-glucose and 2.5 % agar at pH 7.0), a conidial spore suspension ($10^7$ spores $ml^{-1}$) was prepared and inoculated into TM medium, containing 0.1 % bactopeptone, 0.03 % urea, 0.2 % $KH_2PO_4$, 1.4 % $(NH_4)_2SO_4$, 0.03 % $MgSO_4.7H_2O$, 0.3 % glucose, and $FeCl_3$ as trace element. After 3 days incubation at 38 °C and 130 r.p.m., mycelia were washed several times with sterile distilled $H_2O$ and transferred to an induction medium comprising minimal medium (0.2 % $KH_2PO_4$, 0.03 % $MgSO_4$, 1.4 % $(NH_4)_2SO_4$ and $FeCl_3$ as trace element), together with 0.5 % dehydrated *C. maculatus*. Mycelia were reincubated for 3 days with agitation at 29 °C and at 130 r.p.m. Culture broth was filtered through 0.3 μm Millipore filters, dialysed against water with a 1.0 kDa cutoff at 4 °C, lyophilized and stored at −80 °C. In evaluation of fungal growth conditions, mycelial dry weight was measured, and average pH values determined. A growth time-course was also determined, with secretions collected during the exponential phase only (see Supplementary Fig. S1, available with the online version of this paper). Viable cells were counted using a Neubauer chamber, in order to guarantee that experiments were not contaminated with intracellular proteins following lysis. As a negative control, *M. anisopliae* was also grown in the absence of dehydrated insects, using otherwise identical growth conditions.

**Quantification of protein secretion.** The Bradford method (Bradford, 1976) and the fluorescent Qubit kit (Invitrogen) were used for protein quantification. In the first method, 1.0 ml Bradford reagent (100 mg Coomassie brilliant blue, 50 ml 95 % ethanol and 100 ml 85 % phosphoric acid) was added to a 100 μl sample. Distilled water was used as a negative control. $OD_{595}$ was measured by spectrophotometry, using BSA to construct the standard curve. The second method was conducted according to the manufacturer's instructions.

**Gel electrophoresis analyses.** Isoelectric focusing and molecular mass separation were conducted according to the protocol of Gorg *et al.* (1988), using 13 cm immobilized pH gradient (IPG) strips with a pH range of 3–11 and a Multiphor II electrophoresis system (GE Healthcare). A 2000 μg portion of each sample was resuspended in 0.005 M Tris/EDTA buffer containing 1 mM PMSF and 1 mM E-64 protease inhibitor, divided into four equal volumes and further precipitated using a 2D Clean-Up kit (GE HealthCare). Precipitate containing 500 μg total protein was resuspended in a 250 μl solution of 2 % CHAPS, 8 M urea, 7 mg DTT $ml^{-1}$ and 2 % IPG buffer. Strips were hydrated in this solution for 16 h. Isoelectric focusing was performed in gradient mode for 30 min at 500 V, 30 min at 1000 V, 90 min at 35 000 V and 380 min at 3500 V, at 2 mA and 5 W. After the first dimension, strips were equilibrated in a solution of 6 M urea, 1 % DTT and 2 % SDS for 15 min and then applied to gels. Mini-gel and second-dimension separation were performed in $18 \times 16 \times 0.1$ cm SDS-PAGE 12.5 % gels, as described by Laemmli (1970), with bromophenol blue used as tracking dye. Electrophoresis was conducted on a Hoefer system (GE HealthCare) at 250 V, 40 mA and 10 W for 5 h. Gels were silver stained and repeated in triplicate. The broad-range pI marker (GE Healthcare) was also used for pI identification on gels. Gels were evaluated in technical triplicate and biological duplicate.

Gels were digitzed using an HP Scanjet model 8290 scanner and further analysed with Bionumerics software v. 4.5 (Applied Maths). Calibration with a grey scale was necessary to transform grey levels into values for each pixel of the gel picture. This was conducted using a calibration curve. All gel images were analysed as .tif files. The six gel images were placed in one folder and the wizard detection method proposed by the software was used for spot detection. Automatically detected spots were manually checked, with manual addition or removal according to size (>0.2 cm), shape (circular) and density (>2 pixels $cm^{-1}$). Following the detection procedure, a normalization step was conducted to assign common protein identities to identical spots derived from different images. For this procedure, a reference gel was constructed, using automatic matching options. For each sample, when a protein was detected in all gel images, this protein was automatically added to the reference gel.

**Protein digestion.** All spots were excised from gels using a scalpel and placed into 1.5 μl microtubes. A protein in-gel digestion with Gold sequencing-grade trypsin (Promega) was conducted following the procedure of Shevchenko *et al.* (1996). A 300 μl volume of 100 % acetonitrile was added to tubes for 5 min. Supernatant was removed and spots were dried in a SpeedVac for 5 min. Samples were incubated for 60 min at 56 °C in a solution containing 50 μl 10 mM DTT and 100 mM $NH_4HCO_3$. The solution was replaced with 50 μl 55 mM iodoacetamide and 100 mM $NH_4HCO_3$ and incubated in darkness for 45 min. Spots were rinsed twice with MilliQ water (Millipore) for 10 min, and exposed to 100 μl 100 % acetonitrile for 5 min. Excess acetonitrile was removed and again spots were dried on

a SpeedVac for 5 min. Protein digestion was carried out using 650 ng trypsin diluted in 50 µl 50 mM $NH_4HCO_3$ and 6 mM $CaCl_2$, with overnight incubation at 37 °C. The supernatant was used for mass spectrometry analysis.

**Mass spectrometry.** The peptides derived from tryptic digestion were analysed as described by Henzel *et al.* (1993), using an UltraFlex II MALDI-TOF/TOF instrument (Bruker Daltonics). Each sample (2 µl) was mixed in 6 µl 0.1 % α-cyano-4-hydroxycinnamic acid, 0.1 % trifluoroacetic acid and acetonitrile (1 : 1). A volume of 0.5 µl was applied to a MALDI plate and dried at room temperature. Spectrometry was operated in linear mode for MS acquisition and reflected mode for MS/MS acquisitions using modulated power with 200 random shoots. Data were saved in standard Bruker's software format.

**Protein identification.** Spots were identified using peptide mass fingerprinting (PMF) and *de novo* sequencing. The mass list for each sample was analysed using the program MASCOT v2.1.0 (Matrix Science; http://www.matrixscience.com), assuming one mis-cleavage, carboxymethylation and methionine oxidation as modification. The lists of masses were compared against the non-redundant NCBI database. Results were evaluated by comparing the molecular mass and pI of the most scored hit with data observed in 2D gels. *De novo* sequencing was conducted manually, subtracting the masses from *y* series and comparing them to amino acid masses. Sequences were then compared to the Swiss-Prot database (http://www.expasy.org) using the MPSrch tool from the European Institute of Bioinformatics (EBI). Only protein hits with molecular mass and pI values similar to those found on gels, together with coverage, similarity and tryptic digestion pattern, were considered for identification. Protein spots with lower scores were considered unidentified and were discarded.

## RESULTS AND DISCUSSION

SDS-PAGE was carried out to evaluate *M. anisopliae* secretions in response to the presence of *C. maculatus* insects (Fig. 1). Induced and non-induced secretions showed clear differences in protein patterns. Fresh minimal medium and medium lacking insect exoskeleton, used as a negative control, showed no protein bands. When *M. anisopliae* grown in the presence of insects was transferred to fresh minimal medium without insect shells (last lane, Fig. 1), a clear modification in secretion patterns was observed. Protein secretion was similar to that observed under non-induced conditions, which supports the conclusion that certain proteins are expressed in response to the presence of insects. However, a number of proteins detected in *M. anisopliae* on minimal medium without insect shells were similar to proteins observed on induced medium. Two explanations may help to elucidate such findings. Firstly, the fungus may continue to express proteins induced by the presence of insect shells for some time after transfer to fresh medium. A second hypothesis is that following transfer, different proteins with similar molecular masses, not observed in non-induced and induced media, are also expressed in response to reduction in carbon and nitrogen sources. Similar data were previously observed by Hess *et al.* (2006) in yeasts subjected to ammonia and potassium limitation. Following growth of *M. anisopliae* strain CG34 on the induction medium containing dehydrated *C. maculatus*, as well as in an identical broth lacking insects, 2D gels were produced for characterization of protein expression (Fig. 2). Secretions were collected during the exponential phase to reduce cell lysis, senescence and autophagy (see Supplementary Figs S1 and S2, available with the online version of this paper). Microscopic analyses showed that approximately 98 % of fungal cells were intact, with entire hyphal structures (data not shown). Each gel was loaded with 500 µg total secreted proteins for both treatments, representing a total mycelial mass of 1.87 g in induced conditions and 10.15 g in non-induced conditions. Whether induced or non-induced, *M. anisopliae* strain CG34 expressed more proteins in the current study than observed previously (Murad *et al.*, 2006). Marked differences in protein yield may reflect utilization of a higher protein concentration in combination with a thinner gel (1.0 mm rather than 1.5 mm) and a 2D Clean-Up kit to enhance protein yield. In total, 30 additional spots were observed on 1.0 mm gels with different pIs and molecular masses (Fig. 2). Moreover, correlation curves of 2DE calculated with the Bionumerics software Image analysis superposition improved reliability. These curves showed that biological replicates were extremely reproducible, indicating a correlation rate of 0.92 (data not shown). Moreover, when 2D gels from technical replicates were compared, the correlation rate increased to 0.95 (data not shown). By contrast, when induced and non-induced gels were compared *in silico*, only a correlation rate below 0.12 was obtained, suggesting that the gels were extremely different and, therefore, not comparable.

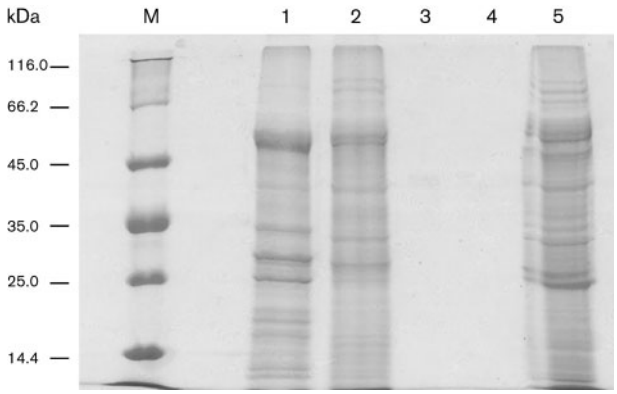


**Fig. 1.** SDS-PAGE analyses of *M. anisopliae* secretion in the presence (induced, lane 1) and in the absence (non-induced, lane 2) of *C. maculatus* insects. Fresh medium (lane 3) and medium containing only insects (lane 4) were used as negative controls. *M. anisopliae* was also transferred to fresh medium after induction and evaluated after 48 h (lane 5). Each sample was evaluated at a standard concentration of 30 µg $ml^{-1}$. M, molecular mass markers.

Spots marked on the induced gel (Fig. 2) were excised and, following gel trypsin digestion, peptides were identified on the basis of mass spectrometry (Table 1), pI and relative

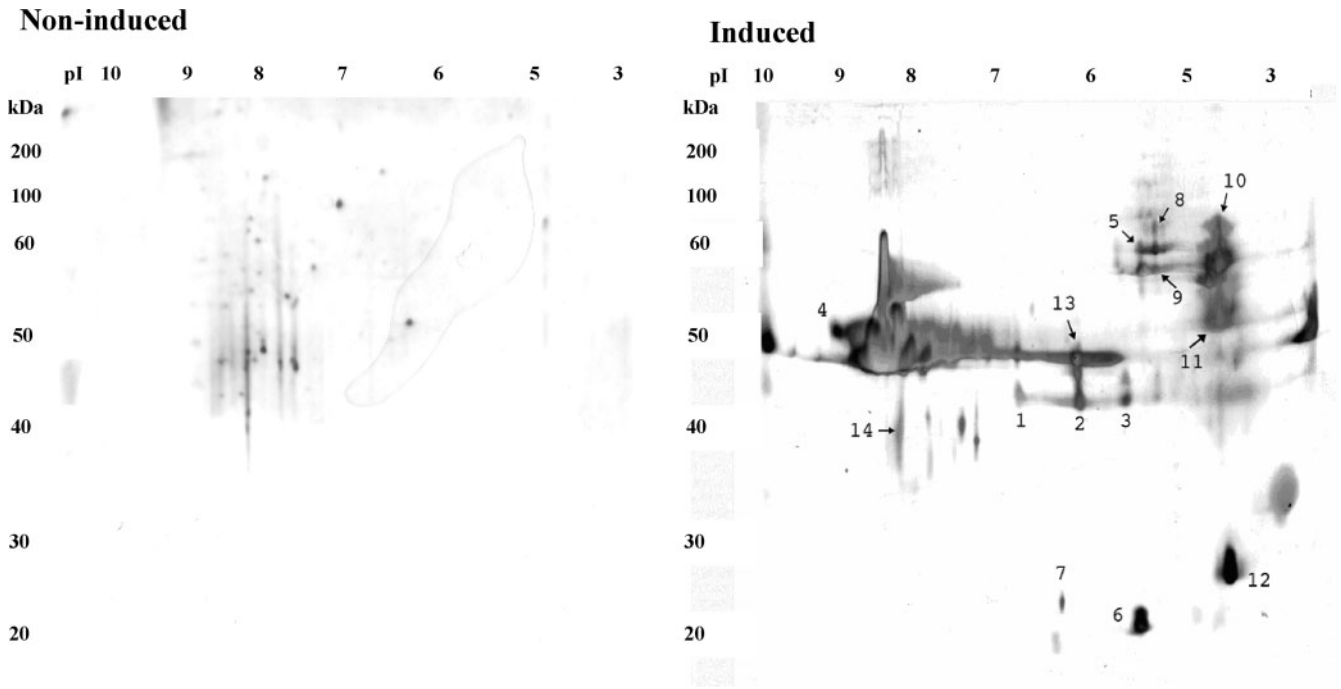


**Fig. 2.** Two-dimensional electrophoreses of *M. anisopliae* secretion in the presence (induced) and in the absence (non-induced) of *C. maculatus* insects. Numbers and arrows indicate analysed and identified spots. A broad-range pI marker from GE Healthcare and a theoretical molecular mass obtained from MS data were used. Gels were silver stained. 2D gels were performed in triplicate, with a correlation value higher than 0.9. Gels were evaluated in technical triplicate and biological duplicate.

**Table 1.** Proteins identified from *M. anisopliae* secretions

PMF was conducted using the software Mascot. MPSrch score, pI and molecular mass were used for identification. *De novo* sequences were compared to the Swiss-Prot database using MPSrch from the European Bioinformatics Institute. MPSrch score, pI and molecular mass were also used for identification.

| Spot | Cover (%) | Score | Theoretical mass (Da) | Theoretical pI | Hits access code | Identification | Similarity |
|---|---|---|---|---|---|---|---|
| 1 | 15 | 54 | 24 839 | 5.39 | XP_749174 | PMF | Acetyltransferase, GNAT family (*Aspergillus fumigatus*) |
| 2 | 2 | 47 | 43 725 | 6.65 | ZP_01313438 | KGYVYTR | Putative methionine $\gamma$-lyase |
| 3 | 21 | 44 | 43 533 | 9.57 | gil85090383 | PMF | ATPase (*Desulfuromonas acetoxidans*) |
| 4 | 10 | 45 | 51 382 | 8.67 | NP_015228 | PMF | Mitochondrial tyrosyl-tRNA synthetase (*Saccharomyces cerevisiae*) |
| 5 | 16 | 34 | 57 243 | 5.99 | CAB63907.1 | PMF | Subtilisin-like protease PR1H (*Metarhizium anisopliae*) |
| 6 | 41 | 51 | 14 535 | 5.12 | Q4R5J1 | PMF | Dehydrogenase |
| 7 | 37 | 44 | 16 057 | 7.75 | NP_084164.1 | PMF | Zinc finger protein 142 (*Mus musculus*) |
| 8 | 5 | 40 | 55 108 | 4.93 | XP_572174.1 | PMF | $\alpha$-Amylase (*Cryptococcus neoformans*) |
| 9 | 10 | 46 | 51 760 | 4.42 | AA_119890705 | PMF | Regulation protein of Ty1 (*Saccharomyces cerevisiae*) |
| 10 | 9 | 39 | 56 143 | 4.74 | XP_599034.3 | PMF | A-kinase (*Bos taurus*) |
| 11 | 4 | 72 | 38 022 | 5.47 | Q1F2S0_9CHLR | KAFWVFDMPNR | Amidohydrolase 2 (*Metarhizium anisopliae*) |
| 12 | 24 | 67 | 26 615 | 5.68 | gil4768909 | PMF | Trypsin proteinase (*Metarhizium anisopliae*) |
| 13 | 2 | 37 | 48 093 | 6.84 | Q2P5Z4_XANOM | AFTYTR | Putative aminotransferase |
| 14 | 4 | 56 | 37 017 | 6.73 | A1ZIA1_9SPHI | KQVTYHVYYINR | Oxidoreductase |

mass observed on gels. Our data, in addition to previous reports (Murad *et al.*, 2006; Kolkman *et al.*, 2006; Lomako *et al.*, 2004; Lesage & Bussey, 2006), indicate a clear nutrient uptake strategy, suggesting metabolic pathways involved in degradation of compounds from liquid culture. Fig. 3 illustrates the probable chitin degradation cycle for strain CG34. Following chitin hydrolysis by endo/exo chitinases (Murad *et al.*, 2006; Kang *et al.*, 1999) (EC 3.2.1.14), as detected by enzyme assays in our previous study (Murad *et al.*, 2006), the synthesized *N*-acetyl-D-glucosamine may be phosphorylated by an *N*-acetyl-D-glucosamine kinase (spot 10). Chitin may be deacetylated by a chitin deacetylase (Nahar *et al.*, 2004) (EC 3.5.1.41), and D-glucosamine units may be acetylated once again by a

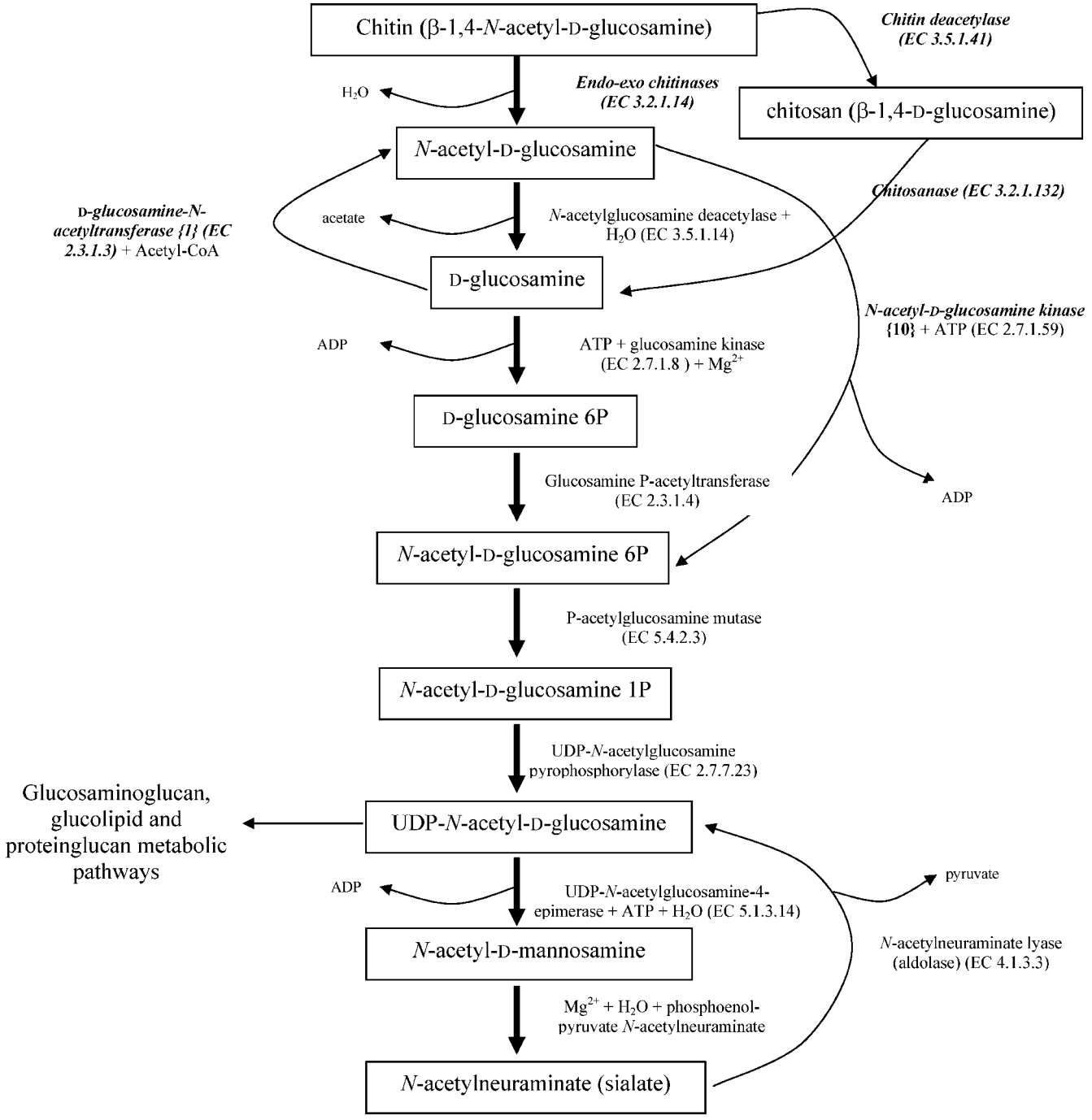


**Fig. 3.** Chitin degradation pathway in *M. anisopliae*. Bold italic type indicates enzymes described in fungi in this report or by other authors. Numbers in { } indicate the identified spots from Fig. 2 and further described in Table 1.

D-glucosamine *N*-acetyltransferase (spot 1) (EC 2.3.1.3) followed by a further phosphorylation by *N*-acetyl-D-glucosamine kinase (spot 10). Lesage & Bussey (2006) reviewed cell wall assembly mechanisms in *S. cerevisiae*, another ascomycete, reporting that several enzymes are necessary, including chitin deacetylases. Chitosan, which is a deacetylated derivative of chitin, is much more water soluble, facilitating adsorption of this compound by fungal cell walls. Although it is not clear how prevalent chitosan is among fungi, appearing mainly in zygomycetes, this polymer has now been reported in cell walls in ascomycetes. For example, chitosan has been detected during both sporulation, in the case of *S. cerevisiae*, and vegetative growth, in the case of *Cryptococcus neoformans* (Banks *et al.*, 2005).

The phosphorylated product *N*-acetyl-D-glucosamine 6-phosphate is important for synthesis of UDP-*N*-acetyl-D-glucosamine and later conversion by other metabolic pathways to UDP-glucose. This may then serve for synthesis of glycogen, as previously described in fungi (Lomako *et al.*, 2004). *N*-Acetyl-D-glucosamine-6-phosphate may be metabolized into UDP-*N*-acetyl-D-glucosamine, following the described metabolic pathway (Fig. 3). Aldolase may transform *N*-acetyl-neuraminate into UDP-*N*-acetyl-D-glucosamine, producing pyruvate. Furthermore, UDP-*N*-acetyl-D-glucosamine is also an important substrate in the dolichol metabolic pathway, which results in the production of glycosaminoglycans, glycolipids and protein glucans. These molecules are used for energy storage and in cell walls (Sorensen *et al.*, 2003). Assigning potential roles to our identified proteins in metabolic pathways, we propose that UDP-*N*-acetyl-D-glucosamine is produced within the cell following the metabolism of *N*-acetyl-D-glucosamine-6-phosphate, with products utilized for energy metabolism. In addition, D-glucosamine can be transformed into glucose and used by fungi to construct their cell walls using $\beta$-1,3/1,6 glucose polymers, as described in *S. cerevisiae* (Lesage & Bussey, 2006). Moreover, Kolkman *et al.* (2006) used LC-MS/MS proteomic techniques to characterize differentially induced protein expression in *S. cerevisiae* following starvation. Their work showed that during carbon source limitation *S. cerevisiae* expresses genes that code for enzymes involved in carbohydrate metabolism, cellular machinery, fatty acid metabolism, glycolysis, oxidative phosphorylation and enzymes of the citric acid cycle. Our work indicates that *M. anisopliae* produced only enzymes involved in carbohydrate metabolism, but which may have an analogous metabolism in nutritional stress responses.

Proteolytic enzymes are also produced by *M. anisopliae* during host colonization (St Leger *et al.*, 1997, 1986). Our data (Table 1) showed similarities with previous 2D identification of proteinases (Bidochka & Khachatourians, 1994), where degrading enzymes active against grasshopper (*Melanoplus sanguinipes*) cuticle were characterized from the entomopathogens *M. anisopliae* and *B. bassiana*. Several reports (St Leger *et al.*, 1987, 1994) have also described several trypsins and subtilisins from *M. anisopliae*. Together with our results, these findings make it clear that these enzymes are important for insect colonization by entomopathogens. It is widely known that numerous fungi utilize a nitrogen metabolism process different from that of plants and bacteria (Kneip *et al.*, 2007). Fungi are unable to extract nitrogen from the atmosphere, such activity being exclusive to bacteria (Kneip *et al.*, 2007). However, fungi are able to process nitrogen from other compounds: primarily from glutamine, glutamate and ammonium; and secondly from nitrite, nitrate, amino acids, purines, acetamide and acrylamide (Marzluf, 1981). For all these compounds, the synthesis of nitrogen-regulated enzymes can only occur if nitrogen catabolite repression (namely ammonium repression) is lifted. In other words, the need for nitrogen catabolization can be reduced by factors such as the presence of ammonium in the medium (Marzluf, 1981). The genetics of these characteristics were studied in *Aspergillus nidulans* and *Neurospora crassa*, and genes controlling the production of nitrite/nitrate reductase were identified (Marzluf, 1981). On this basis, given the high proteolytic activities seen in CG34 (Murad *et al.*, 2006) and the identified proteins, the metabolic pathway depicted in Fig. 4 is proposed. Proteins may be hydrolysed by a trypsin (St Leger *et al.*, 1996) (spot 12, Fig. 2) and by one subtilisin (St Leger *et al.*, 1994) (spot 5), producing free essential amino acids and small peptides that can be further assimilated. A methionine gamma lyase (spot 2) and amidohydrolase (spot 11) hydrolyse the $-NH_2$ groups found on N-termini and side chains of basic residues. $NH_3$ becomes a substrate for ammonia monooxygenase, which results in $NH_2OH$. This compound is then oxidized by hydroxylamine dehydrogenase to nitrite ($NO_2$), which is converted by nitrite oxidoreductase (spot 14) to nitrate ($NO_3^-$). A similar process has been described by Marzluf (1981) in *A. nidulans* and *N. crassa*. This is an important mechanism for reducing high ammonium levels, given that this compound is toxic to organisms such as *S. cerevisiae* (Hess *et al.*, 2006). Aminotransferase was another important enzyme identified in our study (spot 13). There are many types of aminotransferases. In our case, we appear to have identified both a D-alanine aminotransferase (EC 2.6.1.21), catalysing the reaction of D-alanine + 2-oxoglutarate to pyruvate + D-glutamate, as well as an aspartate aminotransferase (EC 2.6.1.1), which catalyses the reaction of L-aspartate + 2-oxoglutarate to oxaloacetate + L-glutamate, as previously found in *S. cerevisiae* (Cronin *et al.*, 1991). Kolkman *et al.* (2006) also showed that under limited nitrogen conditions, *S. cerevisiae* expresses genes responsible for amino acid metabolism, glycolysis, glycogen metabolism and several proteolytic enzymes, which also could be observed in *M. anisopliae* secretions (Fig. 4).

The identification of an $\alpha$-amylase (spot 8, Table 1) was a surprising result, considering that *M. anisopliae* showed only low $\alpha$-amylolytic activity in the presence of cowpea weevil (Murad *et al.*, 2006). In fact, this starch-hydrolysing enzyme was not expected to be identified, as insects do not

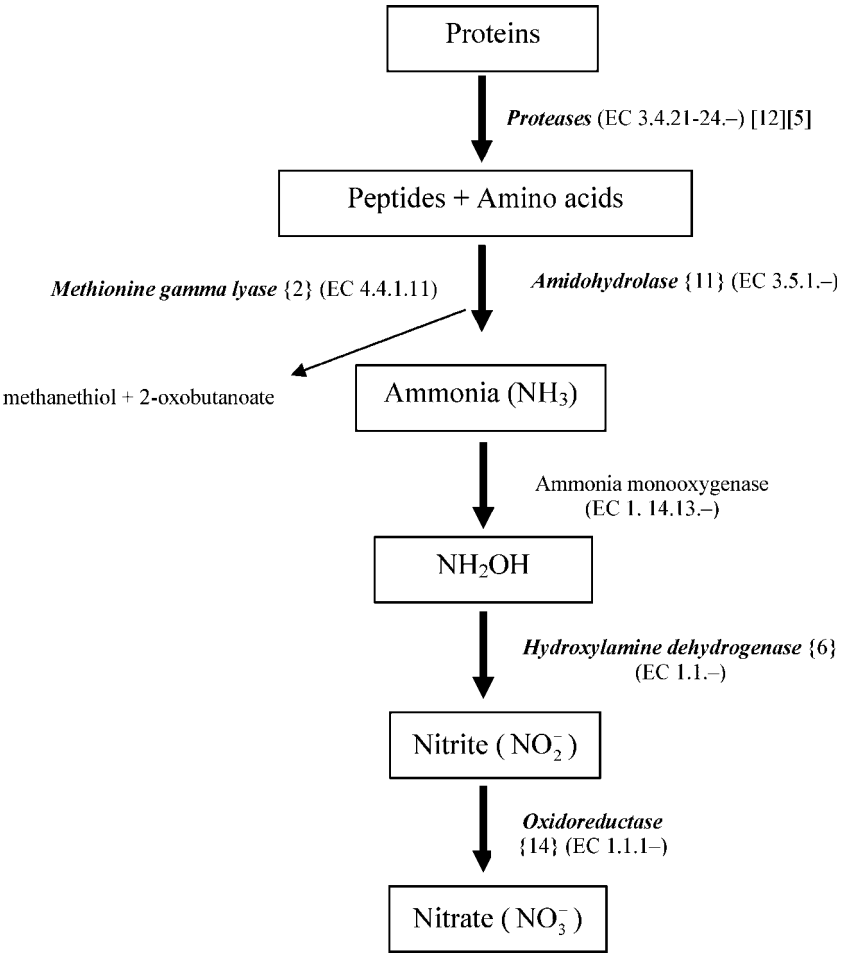


**Fig. 4.** Protein and ammonia degradation pathway in *M. anisopliae*. Bold italic type indicates enzymes described in fungi in this report or by other authors. Numbers in { } indicate the identified spots from Fig. 2 and further described in Table 1.

synthesize starch. We propose that at some point CG34 identified carbohydrate-binding enzymes as necessary for metabolism of *C. maculatus* shells, transcribing and secreting several of these into the culture media. The spot in question (spot 8, Fig. 2) showed only a low intensity, supporting this theory. Some additional proteins were also identified, although their possible mechanism of action in nutrient uptake remains unclear. Among these were an ATPase (spot 3, Fig. 2 and Table 1), tRNA synthetase (spot 4, Fig. 2 and Table 1), a zinc finger protein (spot 7, Fig. 2 and Table 1) and a Ty1 regulation protein (spot 9, Fig. 2 and Table 1).

Another important question raised by the results is whether the proteins reported here are indeed secreted by fungi, given that no signal peptides were detected. In order to resolve this question, an *in silico* search for signal peptides was performed using BLAST, FASTA 3 and Signal P, focusing on sequences representing these discovered enzyme classes (see Supplementary Table S1, available with the online version of this paper). All enzymes identified, with the exception of glucosamine 6-phosphate *N*-acetyltransferase and *N*-acetyl-D-glucosamine kinase, have indeed been observed in fungal secretions, as further described in Supplementary Table S1. The two exceptions, by contrast, have been detected by our group only in bacterial secretions. Reinforcing the data in the table, literature on fungal secretions supports our findings in *M. anisopliae*. For example, acetyltransferases have been observed in *S. cerevisiae* (Einerhand *et al.*, 1991), lyases in *Aspergillus niger* (Jenkins *et al.*, 1996), kinases in *S. cerevisiae* (Destruelle *et al.*, 1994), amidohydrolase in *Schizosaccharomyces pombe* (Albrecht *et al.*, 2000), a dehydrogenase in *Candida albicans* (Gil-Navarro *et al.*, 1997) and a oxidoreductase in *Neurospora crassa* (Germann *et al.*, 1988). Furthermore, proteolytic and chitinolytic enzymes have been identified in extracellular secretions of *M. anisopliae* (St Leger *et al.* 1996). *In silico* evaluations increased our knowledge of the proteins sequenced in our study, providing further support for the metabolic pathways proposed in Figs 3 and 4. Although other spots found in 2D gels showed similarities to common secreted proteins such as chitinases, proteinases and glucosidases (data not shown), due to low scores, these data were removed. Further analyses may, however, elucidate these specific classes.

In the last few years, gene expression data have been characterized for a number of biocontrol agents, as a step

towards development of disease-resistant genetically modified crop plants. For example, genes coding for endochitinases from the mycoparasitic fungus *T. harzianum* have been inserted into tobacco and potato, inducing disease resistance (Lorito *et al.*, 1998). High expression levels of the fungal genes were obtained from different plant tissues, without visible detrimental effects upon plant growth and development, and with resistance conferred against phytopathogens such as *Alternaria alternata*, *Alternaria solani*, *Botrytis cinerea* and *R. solani* (Lorito *et al.*, 1998). Brunner *et al.* (2005) developed a genetically modified strain of *Trichoderma atroviride* (SJ3-4), expressing the *A. niger* glucose oxidase-encoding gene, *GoxA*, under the control of a homologous chitinase (*nag1*) promoter. The modification of this strain improved its capacity as a biocontrol agent, with a threefold increase in spore germination inhibition in *B. cinerea*, together with an increased overgrowth and lysis of the plant pathogens *R. solani* and *Pythium ultimum* (Brunner *et al.*, 2005). These studies demonstrate the potential of heterologous genes encoding diverse protein products from fungal biocontrol agents. Candidate gene discovery therefore has promise in development of new disease- and pest-resistant engineered plants, as well as genetically modified biocontrol agents for improved efficiency of pest and pathogen control.

### Concluding remarks

In summary, this report provides new information with respect to the use of entomopathogenic fungi in the biological control of insect pests. A hydrolytic enzymic arsenal was characterized in *M. anisopliae* strain CG34, active in colonization and predation of *C. maculatus*. Given the capacity of this strain in control of bruchids, the enzymes characterized in this study represent a potential resource for development of biotechnological tools for control of insect pests.

Enzyme characterization also contributed to a clarification of the physiology of nutritional uptake and ammonium metabolism in filamentous fungi, with today's literature focused only on mechanisms in yeasts and bacteria. Enzymes such as methionine gamma lyase and nitrite oxidoreductase are clear targets for genetic manipulation and may serve, in the near future, for development of new biocompounds active against *C. maculatus*, together with genetic modification of plants for increased resistance to bruchid pests.

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