Characterization of an extracellular protease and its cDNA from the nematode-trapping fungus *Monacrosporium microscaphoides*

Miao Wang, Jinkui Yang, and Ke-Qin Zhang

Abstract: To better exploit the biocontrol potential of nematophagous fungi, it is important to fully understand the molecular background of the infection process. In this paper, several nematode-trapping fungi were surveyed for nematocidal activity. From the culture filtrate of *Monacrosporium microscaphoides*, a neutral serine protease (designated Mlx) was purified by chromatography. This protease could immobilize the nematode *Penagrellus redivivus* in vitro and degrade its purified cuticle, suggesting that Mlx could serve as a virulence factor during infection. Characterization of the purified protease revealed a molecular mass of approximately 39 kDa, an isoelectric point of 6.8, and optimum activity at pH 9 at 65 °C. Mlx has broad substrate specificity, and it hydrolyzes protein substrates, including casein, skimmed milk, collagen, and bovine serum albumin. The gene encoding Mlx was also cloned and the nucleotide sequence was determined. The deduced amino acid sequence contained the conserved catalytic triad of aspartic acid – histidine – serine and showed high similarity with two cuticle-degrading proteases (PII and Aoz1), which were purified from the nematode-trapping fungus *Arthrobotrys oligospora*. Research on infection mechanisms of nematode-trapping fungi has thus far only focused on *A. oligospora*. However, little is known about other nematode-trapping fungi. Our report is among the first to describe the purification and cloning of an infectious protease from a different nematode-trapping fungus.

Key words: extracellular serine protease, *Monacrosporium microscaphoides*, nematode-trapping fungus, nematocidal activity.

Résumé : Afin de bien exploiter le potentiel de la lutte biologique contre les champignons nématophages, il est important de bien comprendre la base moléculaire du processus infectieux. Dans cet article, l'activité nématocide de plusieurs champignons nématophages a été examinée. Une sérine protéase neutre (identifiée Mlx) a été purifiée par chromatographie à partir d'un filtrat de culture de *Monacrosporium microscaphoides*. Cette protéase a pu immobiliser le nématode *Penagrellus redivivus* in vitro et dégrader sa cuticule purifiée, suggérant que Mlx serve de facteur de virulence durant l'infection. La caractérisation de la protéase purifiée a révélé une masse moléculaire d'approximativement 39 kDa, un point isoélectrique de 6,8 et une activité maximale à pH 9, 65 °C. Mlx a une large spécificité de substrat et hydrolyse des substrats protéiques parmi lesquels on retrouve la caséine, le lait écrémé, le collagène et l'albumine de sérum bovin. Le gène codant Mlx a aussi été cloné et la séquence de nucléotides, déterminée. La séquence en acides aminés déduite contient la triade catalytique conservée constituée d'un acide aspartique, d'une histidine et d'une sérine et démontre une forte similarité avec deux protéases pouvant dégrader la cuticule (PII et Aoz1) qui ont été purifiées du champignon nématophage *Arthrobotrys oligospora*. Jusqu'à présent, la recherche sur les mécanismes infectieux de champignons nématophages s'est concentrée sur *A. oligospora*. Cependant, on connaît peu de choses sur les autres champignons nématophages. Notre rapport est parmi les premiers à décrire la purification et le clonage d'une protéase infectieuse d'un champignon nématophage différent.

Mots clés : sérine protéase extracellulaire, *Monacrosporium microscaphoides*, champignon nématophage, activité nématocide.

[Traduit par la Rédaction]

Received 12 May 2005. Revision received 31 August 2005. Accepted 1 September 2005. Published on the NRC Research Press Web site at http://cjm.nrc.ca on 27 January 2006.

M. Wang.[1] Laboratory for Conservation and Utilization of Bio-resources, Yunnan University, Kunming 650091, Yunnan, P.R. China, and College of Life Science, Henan University, Kaifeng 475001, Henan, P.R. China.
J. Yang[1] **and K.Q. Zhang.**[2] Laboratory for Conservation and Utilization of Bio-resources, Yunnan University, Kunming 650091, Yunnan, P.R. China.

[1]These authors contributed equally to this work.
[2]Corresponding author (e-mail: kqzhang111@yahoo.com.cn).

 doi:10.1139/W05-110 © 2006 NRC Canada

Introduction

Plant-parasitic nematodes can cause severe damage to world agriculture (Barker 1998), and nematophagous fungi have frequently been suggested as a biological agent to control the harmful nematodes. The molecular background of how nematophagous fungi infect nematodes is not fully elucidated. However, several research groups have shown that hydrolytic enzymes, including proteases, collagenases, and chitinases, are involved in penetrating the cuticle and (or) digesting the host cell (Butt 1990; Huang et al. 2004; Jansson and Nordbring-Hertz 1988; Schenck et al. 1980).

Among the hydrolytic enzymes, proteases are of special interest because it is believed that proteases are important for infecting nematodes and that they are produced during the first step of infection (Dackman et al. 1989; Tunlid and Jansson 1991). So far, six extracellular proteases (PII, Aoz1, VCP1, pSP-3, P32, and Ver112) have been isolated and characterized as virulence factors from nematophagous fungi (Bonants et al. 1995; Lopez-Llorca and Robertson 1992; Segers et al. 1994; Tunlid et al. 1994; Yang et al. 2005; Zhao et al. 2004). Furthermore, two other proteases, PRB1 and PRA1, from the mycoparasitic fungus *Trichoderma harzianum* appear to participate in virulence against the nematode *Meloidogyne javanica* (Sharon et al. 2001; Suarez et al. 2004).

It has been suggested by Åhman et al. (2002) that the level of proteolytic activity may correlate with the nematode control abilities of fungi. Therefore, one way to improve the biocontrol efficiency of nematophagous fungi is to overexpress the protease-encoding genes in these organisms. This strategy had been applied to the genes *PII* and *prb1*, and improved fungal pathogenicity was achieved (Åhman et al. 2002; Sharon et al. 2001). Extensive study of the nematocidal proteases from different fungi and comparing the amino acid sequences may reveal changes and (or) similarities that are responsible for nematocidal activity in nature and could offer a promising basis for deeper exploitation of the fungi as biocontrol agents.

We are interested in revealing the molecular background of nematode infection by fungi and as such aim to improve the abilities of fungi to attack and kill nematodes. Previous studies on nematode-trapping fungi mainly focused on *Arthrobotrys oligospora*, of which the enzymes PII and Aoz1 were purified and extensively studied. However, trials with nematode-trapping fungi of the genus *Monacrosporium* have also given some interesting results (Siddiqui and Mahmood 1996). In our study, we surveyed several nematode-trapping fungi of the *Arthrobotrys* and *Monacrosporium* genera. The *Monacrosporium microscaphoides* strain isolated from field soil from Yunnan Province (China) showed similar protease activity and nematocidal activity as that of the *A. oligospora* strains. We describe the isolation and biochemical characterization of a serine protease (Mlx) produced by this strain. In addition, the amino acid sequence derived from the gene encoding Mlx is presented and compared with the amino acid sequences of other reported serine proteases from nematophagous fungi.

Materials and methods

Isolation and screening of strains for activities against nematodes and proteases production

During a survey of nematophagous fungi in China, a collection of nematode-trapping fungi was isolated from soil samples collected in the western area of Yunnan Province according to the method described by Liu and Zhang (2003). Five species (*Arthrobotrys brochopage*, *Arthrobotrys guizhouense*, *Monacrosporium cystosporium*, *Monacrosporium elegans*, and *Monacrosporium microscaphoides*) of the collection were screened for activities against nematodes and the production of proteases.

The natural infection of the nematode *Penagrellus redivivus* by these strains was examined on agar plates following the bioassay method described by Åhman et al. (2002). The protease and nematocidal activities of the cultures filtrates were assayed with the methods as described below. A protease-inducing (PI) medium was used for liquid cultures (26 °C, 200 $r \cdot min^{-1}$) of the selected nematophagous fungi. PI medium comprises 0.1% glucose, 0.1% gelatin, 0.1% $(NH_4)_2SO_4$, 0.001% $FeSO_4$, 0.05% $MgSO_4$, 0.2% KH_2PO_4, 0.05 g phenylalanine$\cdot L^{-1}$, and 0.05 g valine$\cdot L^{-1}$ supplemented with potato extract and saprophytic nematode (*P. redivivus*). (Potato extract: boil 100 g of peeled, diced potatoes in 500 mL of distilled water for 0.5 h and decant the infusion and use in 1 L of the above medium. *Penagrellus redivivus*: collect nematodes from semiliquid oat medium using the Baerman funnel technique and add to the above-described medium at a concentration of 10 000 nematodes$\cdot L^{-1}$).

Cultures of *M. microscaphoides* and *P. redivivus*

Monacrosporium microscaphoides was maintained in the dark on potato dextrose agar at 26 °C and cultured in PI medium as described above. The nematode *P. redivivus* was grown axenically in semiliquid oat medium at 28 °C as described by Luo et al. (2004).

Determination of extracellular protease activity

The protease activity of the purified fractions was qualitatively analyzed by using the casein-plate method as described by Zhao et al. (2004).

Quantitative analysis of protease activity was determined by a caseinolytic method modified from Walter (1984). An aliquot (250 µL) of appropriately diluted enzyme was added to 500 µL of 0.5% (*m*/*v*) casein solution (prepared in 20 mmol Tris–HCl$\cdot L^{-1}$, pH 7.0) in a 1.5 mL Eppendorf tube and subsequently incubated at 37 °C for 20 min. The reaction was stopped by adding 750 µL of 10% trichloroacetic acid. Undigested proteins were precipitated by centrifugation at 12 500*g* for 5 min. Then, 500 µL of the clear supernatant was mixed with 2.5 mL of 0.55 mol $Na_2CO_3 \cdot L^{-1}$ and 0.5 mL folin–hydroxybenzene agent followed by incubation at 30 °C for 15 min. The absorbance value of the resulting supernatant was measured at 680 nm against a blank control. A calibration curve was obtained using L-tyrosine as standard. One unit of protease (PU) activity was defined as the amount of enzyme that hydrolyzed the substrate and produced 1 mg of tyrosine in 1 min under the assay conditions.

Protein quantification

Protein content was determined according to Bradford (1976) using bovine serum albumin (BSA) as the standard.

Purification of extracellular protease

After 8 days of growth in the PI medium, the fungal cul-

© 2006 NRC Canada

tures were filtered through filter paper and subsequently concentrated 10-fold by ultrafiltration. The molecular mass cutoff of the used cassette was 5 kDa. This concentrated extract was designated as crude protease extract.

The crude protease extract was diluted below 2 mS·cm^{-1}, adjusted to pH 6.0, and applied to a Source 15Q PE 4.6/100 column (Amersham Pharmacia Biotech) that had been equilibrated with 10 mmol sodium phosphate·L^{-1} buffer. Bound proteins were eluted with a linear increasing NaCl gradient (0–1.0 mol·L^{-1}). Elution of proteins was monitored at 280 nm. Fractions of 2.0 mL were collected and qualitatively assayed for protease activity.

Fractions containing protease activity were analyzed by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS–PAGE) in 12% polyacrylamide gels. The molecular mass markers used were rabbit phosphorylase B (97 400 kDa), BSA (66 200 kDa), rabbit actin (43 000 kDa), bovine carbonic anhydrase (31 000 kDa), trypsin inhibitor (20 100 kDa), and hen egg white lysozyme (14 400 kDa). Gels were stained with Coomassie Brilliant Blue R-250 and destained using an aqueous mixture of 10% ethanol and 10% acetic acid.

Fractions with the same electrophoretic band pattern were pooled and mixed with 3.4 mol $(NH_4)_2SO_4$·L^{-1} sample buffer in a proportion of 3:2 (*v*/*v*). The sample was applied to a Hiprep 16/10 column (Amersham Pharmacia Biotech) that was equilibrated with 50 mmol sodium phosphate·L^{-1} buffer (pH 7.2) containing 1.7 mol $(NH_4)_2SO_4$·L^{-1}. The column was eluted with a linear decreasing $(NH_4)_2SO_4$ gradient (1.7–0 mol·L^{-1}). The flow rate was 1 mL·min^{-1}. Elution of proteins was monitored at 280 nm. Fractions of 0.5 mL were collected and qualitatively assayed for protease activity. After SDS–PAGE analyses, fractions with protease activity and a single band were pooled, dialyzed against distilled water, and lyophilized.

Isoelectric focusing

Isoelectric focusing was performed in a Multiphor II system (Pharmacia), according to the manufacturer's instructions, with the temperature set at 10 °C. The isoelectric point (pI) was estimated using a broad pI calibration kit (pH 3.5–9.3) (Pharmacia). The gel was stained with Coomassie Brilliant Blue R-250.

Characterization of purified protease

To determine the properties of the purified protease, the lyophilized protease was dissolved in 5 mmol sodium phosphate·L^{-1} buffer (pH 7.0) to a concentration of 0.436 µg·mL^{-1} and 2.576×10^{-3} PU·mL^{-1}, which is close to the activity concentration of the culture filtrate. The optimum temperature for the purified protease was determined by incubating the sample with casein at 20, 30, 40, 50, 60, 70, and 80 °C, and then the activity was quantitatively assayed. The optimum pH for the protease was determined by incubating 25 µL of the sample with 50 µL of casein and 425 µL of Britton Robinson universal buffer (pH 4–13) at the known optimum temperature (60 °C), followed by assaying for the activity. The thermal stability of the purified enzyme was investigated by incubation of the enzyme at 60 and 70 °C for 10 min, followed by assaying for the residual protease activity.

Proteolytic activity versus protein substrates and purified nematode cuticle were carried out using the method previously described by Tunlid et al. (1994). Nematode cuticle was prepared according to the method of Cox et al. (1981).

The effects of metal ions and various inhibitors were assayed at the optimum pH and temperature (60 °C, pH 9). Aliquots of the purified protease were preincubated with various inhibitors or metal ions (25 mmol·L^{-1}) and subsequently assayed for protease activity. The enzyme activity of the control sample (without inhibitor or metal ions) was set as 100%.

Bioassay

After being thoroughly washed with double-distilled water, penicillin solution (400 000 IU), and streptomycin solution (320 000 IU), *P. redivivus* was suspended in 50 mmol sodium phosphate·L^{-1} buffer (pH 7.2). Fifteen microlitres of nematode solution (containing approximately 50–60 nematodes) was mixed with 100 µL of sample in a sterile 1.5 mL Eppendorf tube and incubated at room temperature. Control nematodes were treated in the same manner but with boiled sample.

The nematocidal activity of the liquid culture was tested every 2 days during the culture course. The bioassay was carried out with extracellular protein that was concentrated 10-fold (salting out with ammonium sulfate, dissolved, and then dialyzed against distilled water) from the liquid culture. After 48 h, the number of dead nematodes was determined under a light microscope. Mortality of nematodes was determined as described by Huang et al. (2005).

The effect of purified protease on nematodes was performed with the method described above. However, a few drops of the mixture were taken out after 24, 36, and 48 h, respectively, and placed on slides. The slides were observed in an Eclipse E 800 microscope (Nikon) to check the body damage of the tested nematodes. The protease concentration used for the bioassay was 4.36 µg·mL^{-1} and 2.576×10^{-2} PU·mL^{-1}, which is close to the activity concentration of the crude extract protein.

Preparation for N-terminal amino acid sequence

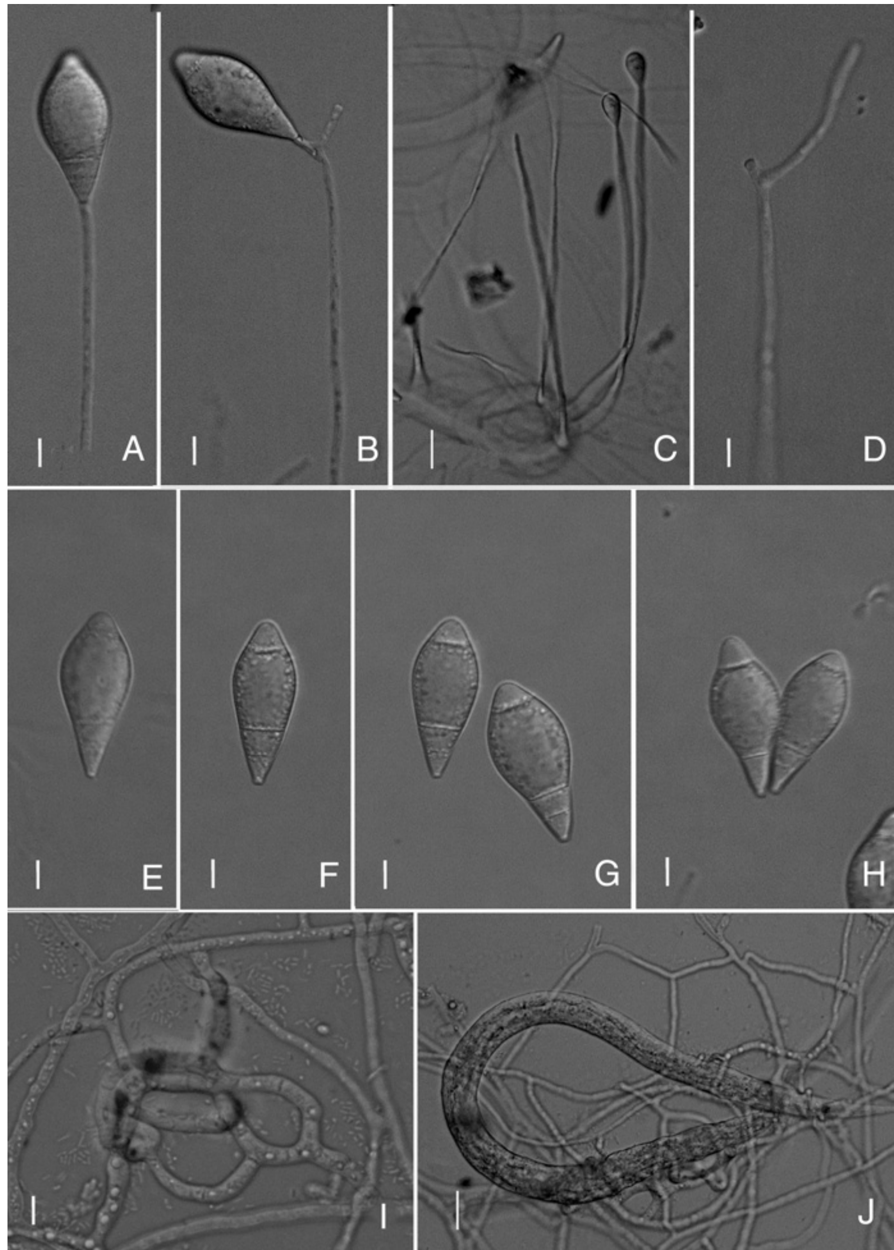
After electrophoresis, the purified protease was electroblotted onto a polyvinylidene difluoride membrane. The electroblotting buffer was 10 mmol 3-(cylohexylamino)-2-hydroxy-1-propanesulfonic acid·L^{-1} (CAPSO; pH 11) containing 10% methanol. After blotting, the protease bound to the polyvinylidene difluoride membrane was stained with Coomassie Brilliant Blue R-250, the membrane was washed with water and distain solution, and subsequently the protein was analyzed with a protein sequencer (PE/ABI PROCISE491) (American).

Cloning and sequencing procedures

Two degenerate primers, PP4 (5′-(A/C)A(A/T) G(A/C)T (G/T)(A/T)(C/T) GAA C(G/C)(G/T) (C/T)CT-3′) and FF2 (5′-TTA AG(C/T) (G/A)(G/T)(A/T/C) (G/T)CC (G/A)TT G(A/T)A G-3′), were designed on the basis of the gene sequences of serine proteases from the nematophagous fungi *A. oligospora* (PII and Aoz1), *Monacrosporium megalosporum*, and *Lecanicillium psalliotae*. The GenBank accession Nos. of these serine protease genes are X94121,

© 2006 NRC Canada

Fig. 1. Description of *Monacrosporium microscaphoides*. (A–D) Conidiophores, (E–H) conidia with two or three septa, (I) three-dimension networks (traps), (J) nematode trapped by the traps. Scale bars: Figs. 1A, 1B, and 1D–1I = 10 µm; Fig. 1C = 20 µm; Fig. 1J = 50 µm.



AF516146, AB120125, and AY692148, respectively. PP4 and FF2 were used in combination with high-fidelity polymerase (Pfu DNA polymerase, 5 U·µL^{-1}) (BioBasic Inc., Toronto, Ontario) to amplify the partial gene from total DNA. Isolation of total fungal DNA was carried out according to the CTAB method (Zhang et al. 1996). The PCR conditions were predenaturation at 94 °C for 3 min before polymerase addition, followed by 30 cycles consisting of denaturation at 94 °C for 40 s, primer annealing at 50 °C for 40 s, and primer extension at 72 °C for 100 s. After cycling, the reaction mixture was kept at 72 °C for 10 min and cooled to 4 °C.

The PCR product was purified with a PCR fragment recovery kit (TaKaRa). Subsequently, the purified DNA fragment was cloned into the pGEM-T (Promega) vector and subjected to sequencing.

The corresponding cDNA fragment was obtained with the TaKaRa RNA PCR kit according to the manufacturer's protocol. Total RNA extraction was performed according to the manual of TRIzol (Invitrogen). Target mRNA was converted into cDNA using reverse transcriptase (AMV). cDNA was amplified by PCR using specific primers FF2′ (5′-TTAAGC GGCTCCGTTGTAG-3′) and PP4′ (5′-CAAGATGTTGAA CGGCCT-3′). The resulting amplified cDNA fragment was purified as described above.

The primers used for sequencing were the T7 (forward) and SP6 (reverse) universal primers and the specific primers PP4′ and FF2′, respectively.

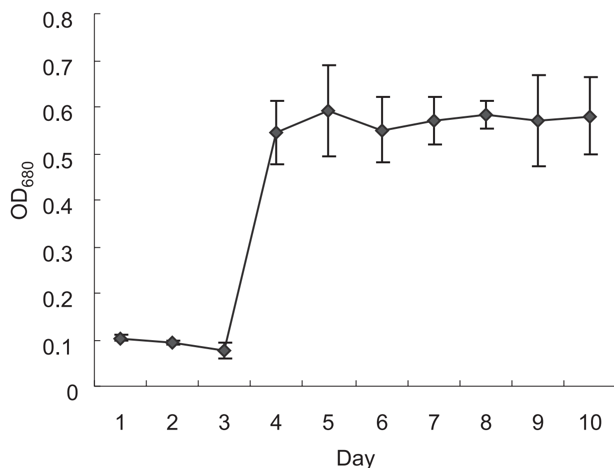
© 2006 NRC Canada

Table 1. Purification of the extracellular protease from *Monacrosporium microscaphoides*.

Purification step	Total protein (mg)	Total activity (PU[a])	Specific activity (PU·mg^{-1})	Purification (*n*-fold)	Yield (%)
Crude extract[b]	2.194	2.465	1.124	1.000	100.00
Source 15Q	0.752	1.705	2.267	2.016	69.17
Phenyl superose[c]	0.109	0.644	5.908	5.256	26.12

[a]PU, proteolytic unit. Protease activity was assayed using the substrate casein. One PU is defined as described in the Materials and methods.
[b]Crude extract was obtained by ultrafiltration of the culture filtrate through a 5 kDa membrane to remove unwanted small molecules.
[c]The purified enzyme.

Fig. 2. Activity of extracellular protease in culture filtrate from *Monacrosporium microscaphoides* at various time points. The maximum protease activity was at day 5 during cultivation. Each point represents the average of triplicate assays, and the bars represent the standard deviation values.



Results

Strain selection

Of the five nematode-trapping fungi examined, *M. microscaphoides* produced the highest amount of protease in liquid culture (data not shown). Immobilization of nematodes by the mycelia of *M. microscaphoides* was also observed (Fig. 1). On basis of these abilities, this strain was used for further study. The morphological characteristics of *M. microscaphoides* are presented in Fig. 1.

Proteolytic activities and nematocidal activities of the culture filtrate of *M. microscaphoides*

Maximum proteolytic activity of the culture filtrate was found on the fifth day after inoculation and remained level during the following 5 days (Fig. 2). The mortality rates of the nematodes in the Eppendorf tubes with concentrated culture filtrate were 15%, 40%, 60%, and 65% on second, fourth, sixth, and 10th day, respectively. In the negative controls, the mortality rate was below 15%. The maximum nematocidal activity, i.e., 80% nematodes immobilized after 48 h of incubation with the aliquot, was found on the eighth day after inoculation. From this point on, we decided to harvest the culture filtrate after 8 days of growth for further purification.

Purification of the *M. microscaphoides* extracellular protease Mlx

Protease Mlx was purified from culture filtrates of *M. microscaphoides* by anion-exchange chromatography and hydrophobic interaction chromatography. Protease activity and total protein were monitored during the purification process and are presented in Table 1.

Characterization of purified protease

The purified protease was found to be almost homogeneous on the SDS–PAGE gel. Based on the electrophoretic mobility, the molecular mass of this purified protein was determined as approximately 39 kDa (Fig. 3). The pI was about 6.8 on the polyacrylamide gel.

The protein was not heat stable; incubation at 70 °C for 10 min resulted in 90% reduction of the proteolytic activity. The optimal temperature for the protease activity was observed at 60 °C; at higher temperature, the activity dropped probably owing to thermal inactivation. The optimum pH for Mlx has a value of 9; the activity declined above and below this value.

Mlx has relatively broad substrate specificity. It showed strong hydrolytic activity against casein, BSA, and skimmed milk (Table 2). It could also moderately hydrolyze gelatin and denatured collagen. But native collagen and cuticle prepared from nematode *P. redivivus* were hydrolyzed by the protease at a considerably less efficient rate (Table 2).

Metallic ions Cu^{2+}, Zn^{2+}, and Al^{3+} significantly reduced the enzyme activity, whereas Ca^{2+} and Mg^{2+} inhibited the enzyme activity only moderately (Fig. 4). The effects of the different Mlx inhibitors selected are summarized in Table 3. Most inhibitors have minor effects on the protease activity but phenylmethanesulfonyl fluoride (PMSF) completely inhibited the activity. Conversely, addition of the reducing agent dithiothreitol (DTT; 1.0 mmol·L^{-1}) significantly enhanced the proteolytic activity (Table 3).

The properties of the purified protease are summarized in Table 4 and compared with other serine proteases from nematophagous fungi.

Bioassay of purified protease

Penagrellus redivivus nematodes treated with the purified protease were immobilized after 24 h of incubation. In the following 24 h, the cuticle of the nematode was gradually

© 2006 NRC Canada

Table 2. Hydrolysis of various protein substrates by the purified protease.

Substrate	Enzyme activity as % of control (SD)
Casein	100.0 (0.005)
BSA	65.6 (0.060)
Skimmed milk	79.8 (0.004)
Gelatin	52.6 (0.002)
Collagen	15.9 (0.001)
Collagen (denatured)[a]	48.1 (0.024)
Nematode cuticle	20.6 (0.004)

Note: The purified enzyme sample was incubated with the substrate at 65 °C for 40 min.
[a]Denatured by heating at 100 °C for 15 min.

Table 3. Inhibition profile of the purified protease.

Inhibitor	Concn.	Enzyme activity as % of control (SD)
None	—	100.0 (0.03)
PMSF	1.0 mmol·L^{-1}	0.5 (0.01)
PMSF	0.1 mmol·L^{-1}	1.1 (0.01)
EDTA	1.0 mmol·L^{-1}	97.4 (0.05)
Pepstain A	10.0 μmol·L^{-1}	127.0 (0.08)
Leupeptin	100.0 μmol·L^{-1}	99.7 (0.07)
DTT	1.0 mmol·L^{-1}	179.0 (0.16)
Aprotinin	1.0 μg·mL^{-1}	92.7 (0.06)

Note: The purified enzyme sample was preincubated with various inhibitors at room temperature for 5 min. PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

Fig. 3. Protein analysis by SDS–PAGE. Lanes: 1, marker; 2, 7 μg of total protein extract loaded; 3 and 4, 0.6 μg of purified protease loaded.

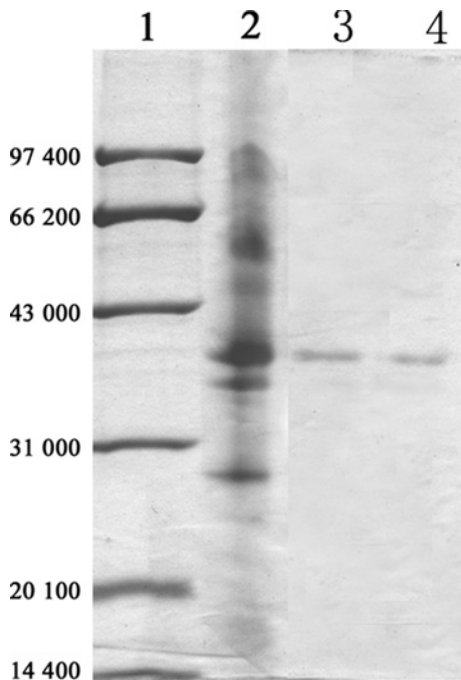
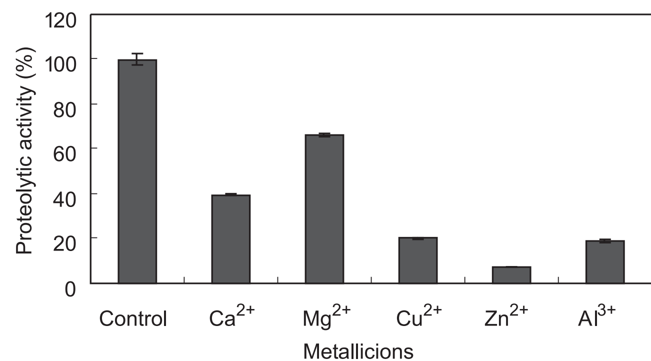


Fig. 4. Effects of various metallic ions on the proteolytic activity of the purified protease. The purified enzyme was mixed with equal volumes of different metallic ions (50 mmol·L^{-1}). The control was mixed with distilled water. After preincubation at 37 °C for 2 min, quantitative assay of the proteolytic activity was carried out as described in the Materials and methods.



degraded by the purified protease. As shown in Fig. 5, control nematodes lived freely and microscopic examination revealed no evidence of cuticle degradation.

Sequencing

Direct protein sequencing of the blotted protease resulted in a sequence of AEQLDSTWGL. This sequence is only one amino acid different from the N-terminal region of the *A. oligospora* serine proteases PII and Aoz1 (AEQTDSTWGL). Under the conditions described in the Materials and methods, a 1271-bp PCR fragment was amplified repeatedly using degenerate primers. The 1271-bp fragment was cloned into the pGEM-T vector and 10 white colonies were selected and sequenced. The nucleotide sequence has been deposited in GenBank under accession No. AY841167. The nucleotide sequence is 1271 bp in length, covers the whole open reading frame, and consists of two exons and one intron (509–560). Alignment results showed that this fragment has 79% homology with the *Aoz1* and *PII* genes (data not shown).

The gene encodes a polypeptide of 405 amino acid residues. The N-terminal amino acids that we sequenced are located at positions 121–131 of the polypeptide (Fig. 6). We therefore consider the beginning site of the mature peptide at position 121.

Alignment of the amino acid sequences of serine proteases from nematophagous fungi shows that all of these serine proteases have the conserved catalytic triad of aspartic acid – histidine – serine (Fig. 6). The deduced amino acid sequence of the purified protease shows 84% homology with the proteases (PII and Aoz1) from other nematode-trapping fungi. PII and Aoz1 are 96% identical. The sequence of the purified protease, together with the sequences of PII and Aoz1, shows relatively low homology (less than 45%) with the proteases VCP1 and pSP-3, which were purified from the egg- and female-parasitic fungi *Pochonia chlamydosporia* and *Paecilomyces lilacinus*, respectively. VCP1 and pSP-3 are 60% identical.

© 2006 NRC Canada

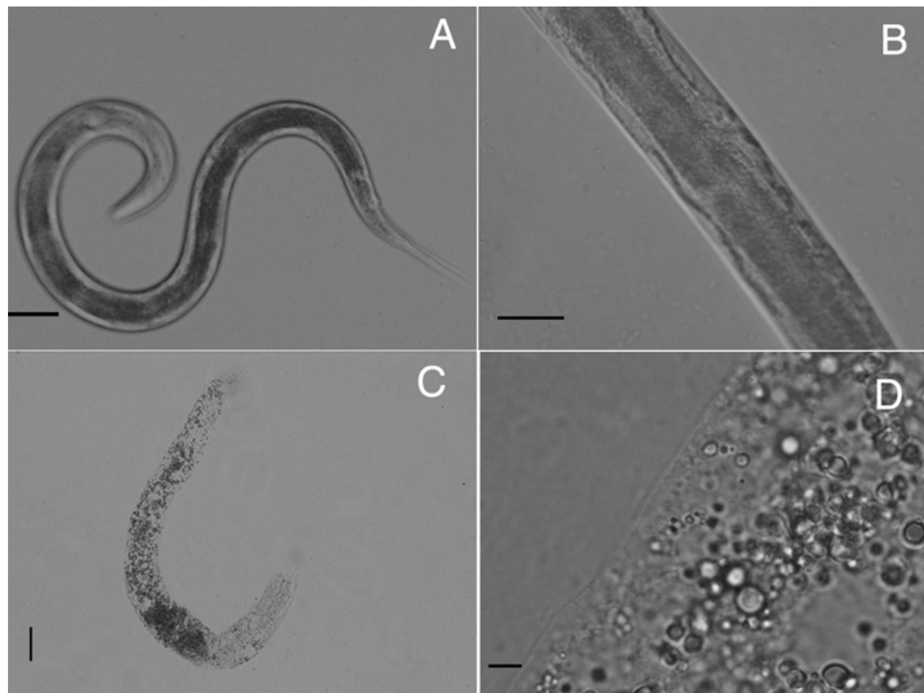
Table 4. Properties of infectious serine proteases isolated from nematophagous fungi.

Nematophagous fungi	Group	Serine protease	Molecular mass (kDa)	pI	Inhibitors of protease	Optimum pH	Substrates
Arthrobotrys oligospora	Nematode-trapping fungi	PII	35	4.6	PMSF, pPCMB, chymostatin, antipain	7–9	Casein, BSA, gelatin, denatured collagen, cuticle of nematode
Arthrobotrys oligospora	Nematode-trapping fungi	Aoz1	38	4.9	PMSF, SSI	6–8	Casein, gelatin, azocoll, cuticle of nematode
Monacrosporium microscaphodies	Nematode-trapping fungi	Mlx	39	6.8	PMSF	9	Casein, BSA, gelatin, skimmed milk, collagen, cuticle of nematode
Pochonia suchlasporia	Egg- and female-parasitic fungi	P32	32	—	PMSF, pCMB	8.5	Proteins from mature eggs
Pochonia chlamydosporia	Egg- and female-parasitic fungi	VCP1	33	10.2	PMSF	—	Casein, BSA, azocoll, eggshells of *Meloidogyne incognita*
Paecilomyces lilacinus	Egg- and female-parasitic fungi	pSP-3	33.5	>10.2	PMSF	10.3	Vitellin, eggshells of immature eggs
Lecanicillium psalliotae	Endoparasitic fungi	Ver112	32	—	PMSF	10	Casein, BSA, gelatin, skimmed milk, collagen, cuticle of nematode

Note: PMSF, phenylmethanesulfonyl fluoride; pCMB, *p*-chloromercuribenzoate; SSI, *Streptomyces subtilisin* inhibitor; BSA, bovine serum albumin.

© 2006 NRC Canada

Fig. 5. The action of purified protease against the nematode *Penagrellus redivivus* observed under a light microscope. (A) Background control (= heat-denatured enzyme) of free-living *P. redivivus* after 48 h of incubation; (B) *P. redivivus* immobilized by purified serine protease after 24 h of incubation; (C and D) cuticle of *P. redivivus* treated with purified protease was degraded after 36 and 48 h of incubation, respectively. Scale bars: Figs. 1A–1C = 50 µm; Fig. 1D = 5 µm.



Discussion

Although the application of some nematophagous fungi as a nonchemical means against plant-parasitic nematodes has given some encouraging results, few of them are reliable as good biological control agents, as described by Kim and Riggs (1992). This has prompted wide resource investigation for more effective strains, and further understanding of virulence factors is important to improve the pathogenicity of these fungi. Our novel results show that both crude protein extract and purified protease isolated from *M. microscaphoides* have nematocidal activity. Previously reported infectious proteases from nematode-trapping fungi, PII and Aoz1, are products of orthologous genes from *A. oligospora* and show strong similarity (Zhao et al. 2004). Thus, we describe here for the first time the characterization of a virulence factor from another nematode-trapping genus *Monacrosporium*.

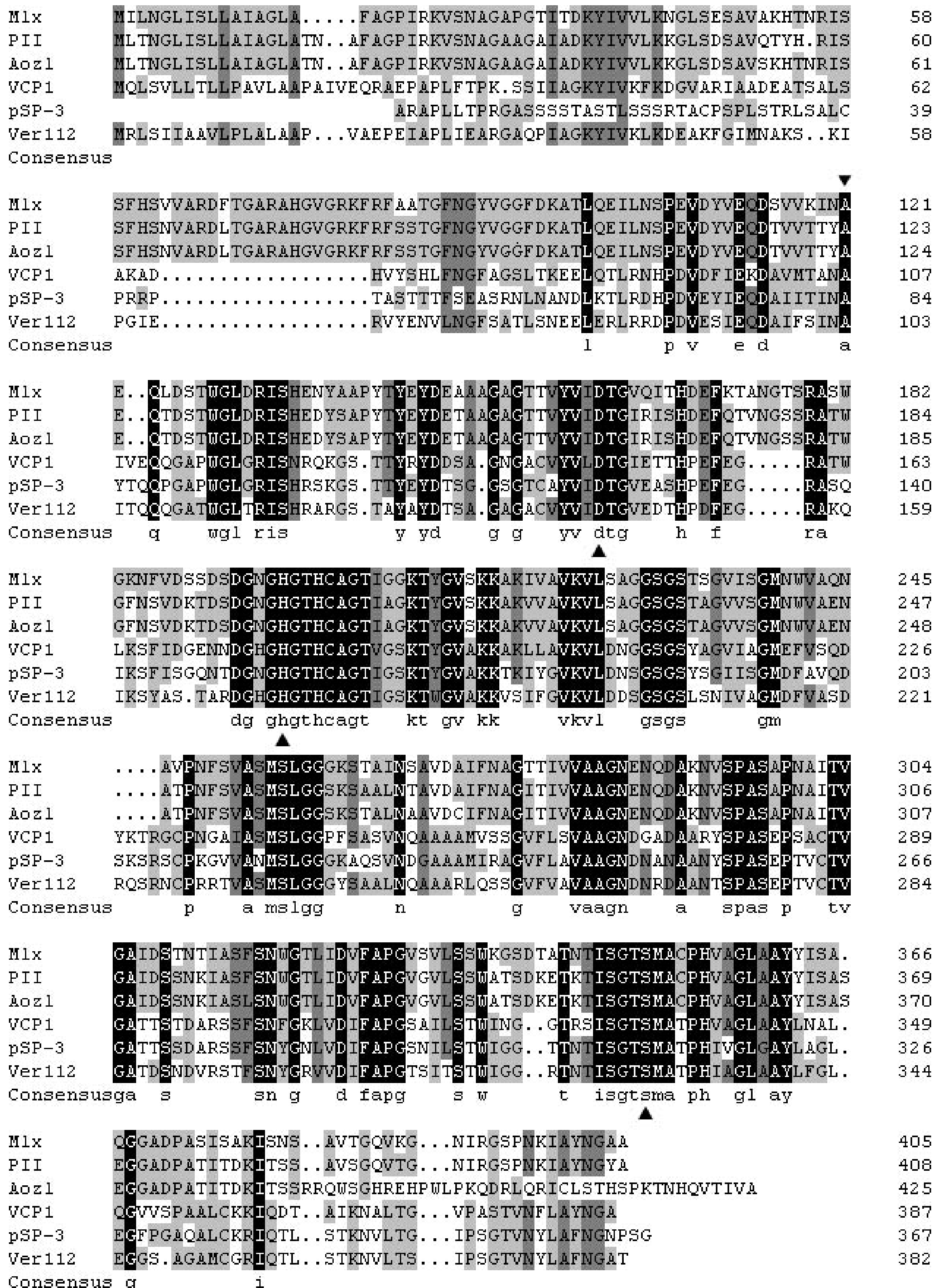
The cuticle of nematodes is rigid and composed of proteins and chitin (Tunlid et al. 1994) and acts as a barrier to prevent nematodes from fungal infection (Huang et al. 2004). Hydrolytic enzymes, especially proteases, chitinases, and collagenases, are believed to be involved in the penetration process, and several hydrolytic enzymes of these types have been identified as a virulence factor. In our study, one infectious protease was purified. However, as the maximum nematocidal activity was observed 3 days later than the maximum proteolytic activity, we cannot rule out that other enzymes and factors were present in the culture filtrate. The purified protease showed stronger nematocidal activity than the extracellular protein extract. This may be due to the presence of nonnematocidal proteases or to the fact that the purified protease, like PII (Åhman et al. 2002), might have some other nematocidal activity apart form its hydrolytic activity. Thus, more studies such as immunolocalization of the purified protease are necessary to verify this hypothesis.

Mlx is a subtilisin-like serine protease characterized by its aspartic acid – histidine – serine catalytic triad and high sensitivity to PMSF. Serine proteases are the most common type of fungal proteases. The six infectious proteases from nematophagous fungi described here are all medium-sized subtilisin-like serine proteases with broad substrate specificity and have effects on degradation of nematode cuticles and eggshells (Jansson et al. 1997). However, proteases from nematode-trapping fungi have comparatively higher molecular mass and lower pI values than proteases from egg- and female-parasitic fungi. Mlx shares these biochemical traits (see Table 4). These broad-spectrum proteases might play a role during the saprophytic growth of these fungi and in the infection process as well (Segers et al. 1994; St. Leger 1993; Tunlid et al. 1994). Among these proteases, the function of PII was best studied. Experiments using various PII mutants and detailed expression studies suggested that PII unlikely has a role in adhesion but may become involved in initiation and development of traps in *A. oligospora* and as such could facilitate the infection of nematodes (Åhman et al. 2002).

Extensive study and careful analysis of the fungal proteases (important virulence factors in the infectious process) are considered to be helpful in the explanation of various aspects of the infection process. In particular, analysis of the sequence data and nematophagous activity of the infectious protease might reveal which amino acid and (or) domain of the sequence is essential for the fungal pathogenicity. Cor-

© 2006 NRC Canada

Fig. 6. Alignment of amino acid sequences of serine proteases from nematophagous fungi. The dark shading areas are conserved regions and the light-gray shading areas are regions of variability between the various proteases. ▼, proregion cleavage site; ▲, conserved catalytic triad of aspartic acid – histidine – serine. The GenBank acc. Nos. of PII, Aoz1, Mlx, VCP1, pSP-3, and Ver112 are CAA63841, AAM93666, AAW21809, CAD20578, AAA91584, and AAU01968, respectively.



© 2006 NRC Canada

rect analyses, however, are dependent on data obtained from nematophagous fungi of different groups, different species and (or) genera, and different sources of origin. Our study serves as a foundation for improving the pathogenicity of nematophagous fungi and provides a basis for further studies.

Acknowledgements

We thank Hong Luo, Jun Li, and Yu'e Hao for their helpful advice on this study. We are also grateful to Dr. Xiaowei Huang, Lemin Zhang, and Dilantha Fernando for critical reading and assistance in revising this manuscript. Finally, we thank members of the laboratory for fruitful discussions. The work was funded by the projects from Ministry of Science and Technology of China (approved Nos. 2003CB415102 and 2002BA901A21), by Natural Science Foundation of China (grant 30570059), and the Department of Science and Technology of Yunnan Province (approved Nos. 20040001Z and 2005NG05).

References

Åhman, J., Johanson, T., Olsson, M., Punt, P.J., van den Hondel, C.A.M.J.J., and Tunlid, A.S. 2002. Improving the pathogenicity of a nematode-trapping fungus by genetic engineering of a subtilisin with nematotoxic activity. Appl. Environ. Microbiol. **689**: 3408–3415.

Barker, K.R. 1998. Introduction and synopsis of advancements in nematology. *In* Plant and nematode interactions. American Society of Agronomy, Inc., Crop Science Society of America Inc., and Soil Science Society of America, Inc., Madison, Wisconsin.

Bonants, P.J.M., Fitters, P.F.L., Thijs, H., Den-Belder, E., Waalwijk, C., and Henfling, J.W.D.M. 1995. A basic serine protease from *Paecilomyces lilacinus* with biological activity against *Meloidogyne hapla* eggs. Microbiology (Reading, UK), **141**: 775–784.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**: 248–254.

Butt, T.M. 1990. Fungi infection processes — a mini-review. Vtb International Colloquium for Invertebrate Pathology Adelaide. Society for Invertebrate Pathology, Knoxville, Tennessee, USA. pp. 121–124.

Cox, G.N., Kush, M., and Edgr, R.S. 1981. Cuticle of *Caenorhabditis elegans*: its isolation and partial characterization. J. Cell Biol. **90**: 7–17.

Dackman, C., Chet, I., and Nordbring-Hertz, B. 1989. Fungal parasitism of the cyst nematode *Heterodera scbacbtii*: infection and enzymatic activity. FEMS Microbiol. Lett. **62**: 201–208.

Huang, X.W., Zhao, N.H., and Zhang, K.Q. 2004. Extracellular enzymes serving as virulence factors in nematophagous fungi involved in infection of the host. Res. Microbiol. **115**: 811–816.

Huang, X.W., Tian, B.Y., Niu, Q.H, Yang, J.K., Zhang, L.M., and Zhang, K.Q. 2005. An extracellular protease from *Brevibacillus laterosporus* G4 without parasporal crystals can serve as a pathogenic factor in infection of nematodes. Res. Microbiol. **156**: 719–727.

Jansson, H.B., and Nordbring-Hertz, B. 1988. Diseases of nematodes: infection mechanisms in the fungus–nematode system. Vol. 2. CRC Press, Boca Raton, Florida. pp. 59–72.

Jansson, H.B., Tunlid, A., and Nordbring-Hertz, B. 1997. Biological control: nematodes. *In* Fungal biotechnology. *Edited by* T. Anke. Chapman and Hall, Weinheim. pp. 38–50.

Kim, D.G., and Riggs, R.D. 1992. Biological control. American Phytopathological Society, Washington, D.C. pp. 133–142.

Liu, X.F., and Zhang, K.Q. 2003. *Dactylella shizishanna* sp. nov., from Shizi Mountain, China. Fungal Diversity, **14**: 103–107.

Lopez-Llorca, L.V., and Robertson, W.M. 1992. Immunocytochemical localization of a 32-kDa protease from the nematophagous fungus *Verticillium suchlasporium* in infected nematode eggs. Exp. Mycol. **16**: 261–267.

Luo, H., Mo, M.H., Huang, X.W., Li, X., and Zhang, K.Q. 2004. *Coprinus comatus*: a basidiomycete fungus forms novel structures and infects nematode. Mycologia, **96**: 1218–1225.

Schenck, S., Chase, T., Jr., Rosenzweig, W.D., and Pramer, D. 1980. Collagenase production by nematode-trapping fungi. Appl. Environ. Microbiol. **40**: 567–570.

Segers, R., Butt, T.M., Kerry, B.R., and Peberdy, J.F. 1994. The nematophagous fungus *Verticillium chlamydosporium* produces a chymoelastase-like protease which hydrolyses host nematode proteins in situ. Microbiology (Reading, UK), **140**: 2715–2723.

Sharon, E., Bar-Eyal, I., Chet, I., Herrera-Estrella, A., Kleifeld, O., and Spiegel, Y. 2001. Biological control of root-knot nematode *Meloidogyne javanica* by *Trichoderma harzianum*. Phytopathology, **91**: 687–693.

Siddiqui, Z.A., and Mahmood, I. 1996. Biological control of plant parasitic nematodes by fungi: a review. Bioresour. Technol. **58**: 229–236.

St. Leger, R.J. 1993. Parasites and pathogens of insects: biology and mechanisms of insect-cuticle invasion by Deuteromycete fungal pathogens. Vol. 2. Academic Press, London, UK. pp. 211–229.

Suarez, B., Rey, M., Castillo, P., Monte, E., and Llobell, A. 2004. Isolation and characterization of PRA1, a trypsin-like protease from the biocontrol agent *Trichoderma harzianum* CECT 2413 displaying nematocidal activity. Appl. Microbiol. Biotechnol. **65**: 46–55.

Tunlid, A., and Jansson, S. 1991. Proteases and their involvement in the nematophagous fungus *Arthrobotrys oligospora*. Appl. Environ. Microbiol. **57**: 2868–2872.

Tunlid, A., Rosen, S., Ek, B., and Rask, L. 1994. Purification and characterization of an extracellular serine protease from the nematode-trapping fungus *Arthrobotrys oligospora*. Microbiology (Reading, UK), **140**: 1687–1695.

Walter, H.E. 1984. Proteinases: methods with haemoglobin, casein, and azocoll as substrates. *In* Methods of enzymatic analysis. Vol. 5. *Edited by* H.V. Bergmeyer. Verlag Chemie, Weinheim, Germany. pp. 270–277.

Yang, J.K., Huang, X.W., Tian, B.Y., Wang, M., Niu, Q.H., and Zhang, K.Q. 2005. Isolation and characterization of a serine protease from the nematophagous fungus, *Lecanicillium psalliotae*, displaying nematicidal activity. Biotechnol. Lett. **27**: 1123–1128.

Zhang, D., Yang, Y., Castlebury, L.A., and Cerniglia, C.E. 1996. A method for the large scale isolation of high transformation efficiency fungal genomic DNA. FEMS Microbiol. Lett. **145**: 261–265.

Zhao, M.L., Mo, M.H., and Zhang, K.Q. 2004. Characterization of a neutral serine protease and its full-length cDNA from the nematode-trapping fungus *Arthrobotrys oligospora*. Mycologia, **96**: 16–22.

© 2006 NRC Canada