Microbiological Research **164** (2009) 665—673

Available online at www.sciencedirect.com

ScienceDirect

Microbiological Research

www.elsevier.de/micres

Purification, characterization, and gene cloning of an alkaline serine protease from a highly virulent strain of the nematode-endoparasitic fungus *Hirsutella rhossiliensis*

Bin Wang[a,b], Xiaoying Liu[c], Wenping Wu[c], Xingzhong Liu[a,*], Shidong Li[d]

[a]*Key Laboratory of Systematic Mycology and Lichenology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China*
[b]*National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China*
[c]*Novozymes China, 14 Xinxi Lu, Shangdi Zone, Haidian District, Beijing 100085, China*
[d]*Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100081, China*

Received 5 December 2008; received in revised form 19 January 2009; accepted 31 January 2009

KEYWORDS
Hirsutella rhossiliensis;
Nematode-endoparasitic fungus;
Serine protease;
Virulence factor

Summary

Hirsutella rhossiliensis OWVT-1 has substantial potential as a biocontrol agent against plant-parasitic nematodes. Serine proteases have emerged as a potentially useful factor in the nematode–fungus interactions. When grown in liquid culture with the nematode *Panagrellus redivivus* as the sole nitrogen source, an extracellular alkaline protease (Hasp) was produced by the OWVT-1. The purified Hasp killed the juveniles of the soybean-cyst nematode (*Heterodera glycines*) and degraded proteins of the nematode cuticle. The molecular mass of Hasp was estimated to be 33 kDa. The optimum pH and temperature for enzyme activity were pH 9 and 75 °C. The amino acid sequence obtained by the N-terminal sequence analysis was applied for the primer design to isolate the *Hasp* cDNA gene, which consists of 1170 bp open reading frame. Analysis of the cDNA and corresponding genomic sequence revealed that *Hasp* included four exons (279, 186, 513, and 192 bp) divided by three introns (65, 99, and 93 bp). Southern blotting showed that *Hasp* was a single-copy gene in the genome. The deduced amino acid sequence was very similar to other serine proteases of endoparasitic and egg-parasitic fungi of nematodes and of entomopathogenic fungi but was less similar to the serine proteases of nematode-trapping fungi. In a phylogenetic analysis of the amino acid

*Corresponding author. Tel.: +86 10 64807512; fax: +86 10 64807505.
E-mail address: liuxz@sun.im.ac.cn (X. Liu).

0944-5013/$ - see front matter
doi:10.1016/j.micres.2009.01.003

sequences of serine proteases, the serine protease of *H. rhossiliensis* OWVT-1 clustered with the serine proteases of parasites of nematode eggs rather than with those of the trapping fungi.

Introduction

The nematode cuticle is a complex structure that represents a significant barrier to penetration and infection by fungi (Cox et al. 1981). To penetrate their host's cuticle, both nematophagous and entomopathogenic fungi depend on collagenases, chitinases, and proteases (Morton et al. 2004). Among these enzymes, proteases have received the most attention (Morton et al. 2004). Understanding how nematophagous fungi use these enzymes to infect nematodes should help us understand how to exploit these fungi for the biocontrol of nematodes.

Nematophagous fungi (including trapping, endoparasitic, and egg-parasitic fungi), are important natural enemies of nematode pests. The proteases from nematode-trapping fungi and egg parasites have been extensively investigated. A serine protease (PII) from the trapping fungus *Arthrobotrys oligospora* has been demonstrated to be a key enzyme in the infection process (Tunlid et al. 1994). The transformants that contained additional copies of the *PII* gene captured and killed nematodes more rapidly than the wild type (Ahman et al. 2002). Similar enzymes have also been purified and characterized from the trapping fungus *Monacrosporium microscaphoides* (Wang et al. 2006) and the egg-parasitic fungi *Paecilomyces lilacinus* (Bonants et al. 1995), *Pochonia chlamydosporia* (= *Verticillium chlamydosporium*) (Seger et al. 1994), and *Lecanicillium psalliotae* (Yang et al. 2005). Little is known, however, about the cuticle-degrading enzymes of the endoparasitic fungi.

Hirsutella rhossiliensis is a typical endoparasitic fungus of nematodes. It produces adhesive spores that attach to and penetrate the cuticle of passing nematodes (Jaffee and Zehir 1985). An isolate of *H. rhossiliensis*, OWVT-1, was selected from more than 80 isolates. It parasitized a higher percentage of nematodes than the other isolates (Liu and Chen 2000) and was highly effective in suppressing *Heterodera glycines*, the soybean-cyst nematode, in greenhouse experiments (Chen and Liu 2005).

Although a number of serine proteases have been reported from nematophagous fungi (Morton et al. 2004), only one neutral serine protease has been reported from a highly virulent strain (Wang et al. 2007). The present study describes a novel extracellular alkaline protease (Hasp) from strain OWVT-1 of *H. rhossiliensis*. The enzyme was purified, its gene was cloned, and its activity against nematodes was examined in a bioassay.

Materials and methods

Organisms and cultural conditions

The bacterivorous nematode *Panagrellus redivivus* was cultured on oatmeal substrate (water/oatmeal = 2/5, v/w). Second-stage juveniles (J2) of the soybean-cyst nematode were obtained by hatching eggs in 4 mM $ZnCl_2$ (Liu and Chen 2000).

H. rhossiliensis OWVT-1 was maintained on potato dextrose agar and cultured at 25 °C in the dark in a liquid medium containing 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% NaCl, 0.005% $ZnSO_4$, 0.005% $FeSO_4$, 2% sucrose, and 1% peptone. To determine which nitrogen sources induced proteolytic enzymes, the peptone in liquid medium was replaced by one of the following: 0.4% yeast extract, 1% tryptone, 1% gelatin, or 5% sterilized *P. redivivus* culture.

Purification of protease

H. rhossiliensis OWVT-1 was cultured in the liquid medium with the 5% sterilized *P. redivivus* culture as the nitrogen source. After 12 d, the mycelium was collected by centrifugation at 14,000*g* for 20 min. The culture supernatant was brought to 80% saturation with solid ammonium sulfate, resuspended in 25 mM Tris–HCl buffer (pH 7.4), and then dialyzed against the same buffer. The crude preparation was loaded onto a Q Sepharose Fast Flow column, equilibrated with 25 mM Tris–HCl buffer (pH 7.4), followed by a linear gradient of NaCl (0–0.8 M) at a flow rate of 4 ml/min. The fractions with protease activity (tubes 14–16) were pooled, concentrated by ultrafiltration with an Aminicon YM-3 membrane, and then applied onto a Superdex^{TM} 75 HR10/30 column against 25 mM Tris–HCl buffer (pH 7.4) at a flow rate of 0.5 ml/min. Fractions with protease activity were

pooled and concentrated by ultrafiltration with the same membrane.

Characterization of the purified enzyme

Proteolytic activity was calculated by measuring the hydrolysis of insoluble (cross-linked) chromogenic substrate AZCL-Casein (Megazyme). An appropriate amount of enzyme (20 μl) was incubated with 0.2% AZCL-Casein solution in 25 mM Tris–HCl buffer (pH 7.4) at 45 °C for 20 min. The solution was then centrifuged at 600 rpm and 4 °C to stop the reaction, and the supernatant was transferred to a microplate and its absorbance measured at 595 nm using a microplate reader (Bio-Rad). One unit (U) was defined as the amount of enzyme giving an absorbance change of 0.2 OD in 20 min.

Protein content was determined according to Bradford using bovine serum albumin (BSA) as the standard (Bradford 1976). The molecular weight of the purified enzyme was determined by SDS-PAGE on a 15% polyacrylamide gel. The gels were stained with GelCode Blue Stain Reagent (Pierce). The following were used as molecular weight standards: phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

The pH profiles of the purified enzyme were measured using the universal buffer (100 mM succinic acid, 100 mM HEPES, 100 mM CHES, 100 mM CAPSO, 1 mM $CaCl_2$, 150 mM KCl, and 0.01% Triton X-100). The pH stability was determined by incubating the purified enzyme at different pH values at 40 °C for 2 h before the activity assay was performed. The temperature profiles were measured at temperatures ranging from 20 to 95 °C at 5 °C intervals. Thermal stability was investigated by incubating the purified enzyme at 40, 65, 70, 75, 80, 85, 90, and 95 °C for 10 min before the activity was assayed. Enzyme solution (20 μl) was pre-incubated at 25 °C for 5 min with 5 μl of each of the following inhibitors: 10 mM PMSF and 0.5 M EDTA (pH 8.0). Residual enzyme activity was assayed as described previously in this section.

Substrate utilization by Hasp was examined using several chromogenic substrates: BA (BA-*p*NA), FGL (*N*-Succinyl-Phe-Gly-Leu-*p*NA), AAPF (*N*-Succinyl-Ala-Ala-Pro-Phe-*p*NA), and GGF (*N*-Succinyl-Gly-Gly-Phe-*p*NA). Each substrate was prepared at 2 mg/ml in 25 mM Tris–HCl buffer (pH 7.4). After incubation with 40 μl enzyme solution at 25 °C for 30 min, the absorbance was measured at 405 nm. The kinetic constants for AAPF were determined using an iterative least-square fit to the Michaelis–Menten equation; five AAPF concentrations were applied (500, 250, 200, 150, 100, 75, 50, and 25 mg/l).

Nematicidal activity

The effect of Hasp on nematodes was investigated by incubating 50–100 healthy soybean-cyst nematode J2 in 300 μl of 25 mM Tris–HCl buffer (pH 7.4, control), crude enzyme, or the purified Hasp. After 12 h at 25 °C, numbers of dead and living juveniles were determined using an inverted microscope. For observing how the enzyme affected the surface of the J2 cuticle, J2 were kept in enzyme for 24 h at 25 °C, fixed, sputter-coated with gold, and observed with a scanning electron microscope (Day and Scott 1973).

N-terminal amino acid sequencing and cloning of the full-length *Hasp* cDNA

The purified Hasp was electroblotted onto a PVDF membrane (Bio-Rad) after SDS-PAGE and stained with GelCode Blue Stain Reagent (Pierce). Protein bands were subjected to Edman degradation and sequenced on an Applied Biosystems (ABI) at Peking University (Beijing, China).

H. rhossiliensis OWVT-1 mycelia for RNA extraction were grown in the same enzyme-inducing liquid medium as described in Sections 2.1 and 2.2. The mycelia were collected by filtration and then total RNA was extracted with TRizol reagent (Invitrogen). Reverse transcription was carried out by using SuperScript II RT enzyme (Invitrogen) and the supplied oligo (dT) primer. Based on the partly sequenced amino acid sequence, degenerate primer 5′-GA(A/G) GCN TC(C/G) CA(C/T) CCN GA (A/G) TT-3′ was designed and used to conduct 3′ rapid amplification of cDNA ends (RACE). Two gene-specific primers were derived from the 3′-RACE product: 5′-GGA GAA TGA TGA CCT CTG A-3′ and 5′-GCG AGT CTT GGA CAG CAA A-3′ for 5′-RACE. The gene-specific primer 5′-CTC AAA ATG AAG CTG TCC CTC A-3′ based on the 5′-RACE product and the oligo (dT) primer were used to amplify the full-length cDNA.

Amplification of the *Hasp* gene from genomic DNA and sequence analysis

Genomic DNA was isolated from *H. rhossiliensis* OWVT-1 mycelia with the Wizard Plus Minipreps DNA Purification System (Promega). Primers 5′-CTC AAA ATG AAG CTG TCC CTC A-3′ and 5′-TAG ACT ATC GAG CCC GCC CC-3′, designed from the full-length

cDNA sequence, were used to amplify the *Hasp* gene from the genomic DNA. The 5′-flanking region of *Hasp* was obtained by the genomic walking method using the DNA Walking SpeedUp Kit (Seegene). The gene-specific primers (TSP-1 and TSP-2) used for genomic walking were 5′-ATG CCT CGA CAC CGG TAT CAA TG-3′ and 5′-CCC TGG CCG GCA CTG CTG TCG TA-3′.

The DNA sequence analysis, amino acid sequence alignment, and isoelectric points (pI) determination were performed with NCBI BLAST, DNAMAN, and DNASTAR programs. Signal peptide prediction was determined using the SignalP program (http://www.cbs.dtu.dk/services/SignalP). Phylogenetic analysis on the aligned sequences was carried out by the neighbor-joining method using the PAUP*4.0b10 program. To assess confidence in the resulting tree topologies, bootstrap tests were performed with 1000 bootstrap replications.

Southern blotting

Fungal DNA was extracted by the CTAB method (Raeder and Broda 1985). Genomic DNA was digested with *Apa*I, *Hind*III, *Sac*I, and *Xho*I at 37 °C overnight and subsequently separated by electrophoresis on 0.8% agarose gel. The DNA was transferred onto a nylon membrane (HyBond-N+, Amersham) by capillary transfer. Following prehybridization for 2 h at 65 °C in Church buffer (7% SDS, 1% BSA, 1 mM EDTA, 250 mM phosphate buffer pH 7.2), the blots were hybridized with radioactive probe synthesized by random priming of the *Hasp* fragments with [α-32P] dCTP at 65 °C for 16 h, and then were washed a few times at 65 °C. The membrane was exposed to X-ray film (Kodak) at −70 °C for autoradiographic analysis.

Results

Production and purification of an alkaline serine protease (Hasp)

Although all of the tested nitrogen sources supported abundant growth of *H. rhossiliensis* OWVT-1, only nematodes induced proteolytic activity in the culture filtrate. Maximum proteolytic activity occurred about 11 d after incubation at 25 °C. Purification of the Hasp was summarized in Table 1. An yield of 12.3% and 18.1 purification folds was achieved. The purified enzyme showed a single protein band upon SDS-PAGE (Figure 1) with a molecular mass of 33 kDa.

Biochemical characterization of Hasp

Maximum activity of Hasp was at pH 9 and 75 °C (Figure 2). The enzyme retained 70% of its maximum activity at a pH range of 4–11 at 40 °C for 120 min (Figure 2A). The proteolytic activity was stable below 70 °C and declined quickly above 85 °C (Figure 2B), indicating that Hasp has a wider temperature range and more thermal stability than other serine proteases from the nematophagous fungi (Tunlid et al. 1994; Wang et al. 2006; Bonants et al. 1995; Seger et al. 1994; Yang et al. 2005).

Enzyme activity of Hasp was completely suppressed by the serine protease inhibitors PMSF

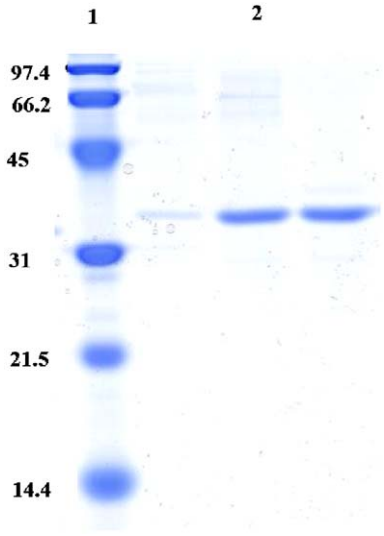

Figure 1. Purity and molecular weight of the protease Hasp from *H. rhossiliensis* OWVT-1 determined via SDS-PAGE. Lane 1, molecular weight markers; lane 2, the purified Hasp.

Table 1. Purification of the alkaline serine protease Hasp from *Hirsutella rhossiliensis* OWVT-1.

Purification procedure	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude extract	2259.6	294.4	7.7	100.0	1.0
Ammonium sulfate	1151.7	48.8	23.6	51.0	3.1
Q-Sepharose FF	376.8	4.4	85.6	16.7	11.2
Superdex 75	278.6	2.0	139.3	12.3	18.1

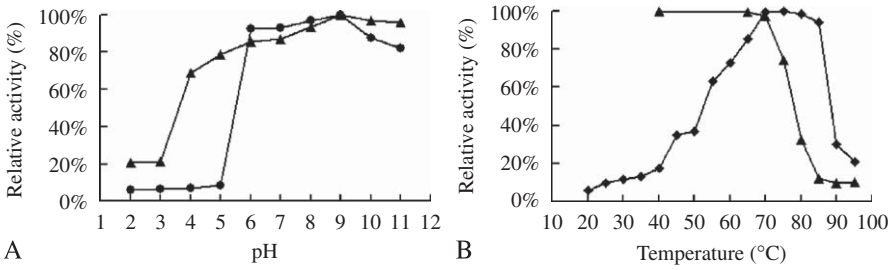

Figure 2. Effect of pH and temperature on Hasp activity. Relative activity is expressed as the percentage of the maximum activity (14 U/ml) under standard assay conditions: (A) (●) pH profile, (▲) pH stability; (B) (●) thermal profile, (▲) thermal stability.

(9.4%) but not by Metallo protease inhibitor EDTA (112.1%). Moreover, the chymotrypsin-like property of Hasp was also demonstrated by its highly active cleaving of AAPF (100%) but not GGF (43.8%), BA(0.80%), or FGL (0.41%). These characteristics demonstrated that Hasp was a serine protease. As determined from the Lineweaver–Burk plot, the Michaelis–Menten constant (K_m) of Hasp for AAPF was 2.96×10^{-4} M.

Nematicidal activity and degradation of nematode cuticle

The mortality of soybean-cyst nematode J2 was significantly higher ($P < 0.05$) in purified Hasp solutions ($43 \pm 5\%$ mortality at 4 U/ml and $53 \pm 4\%$ mortality at 8 U/ml) than in the buffer control ($22 \pm 2\%$ mortality). The furrows in the nematode cuticle were more obvious when the J2 were treated with the purified enzyme than with buffer, indicating that Hasp degraded some cuticle protein. In contrast to the purified enzyme, the crude enzyme killed all of the tested soybean-cyst nematode J2 and caused greater degradation of the cuticle, indicating that some factors in the crude enzyme but not in the purified enzyme may contribute to cuticle degradation.

N-terminal amino acid sequencing and gene cloning

The N-terminal of Hasp was sequenced and the first 20 amino acid residues were ALKQQPGAPWGLGRISHREP. An internal part of Hasp was also sequenced as VIDTGVEASHPEF due to the cleavage of the peptide during preservation at −20 °C. Based on the amino acid sequence, the 3′-RACE product, the 5′-RACE product, and the full-length cDNA were obtained in turn with the same RNA sample as template. The genomic DNA sequence was amplified by PCR and genomic walking. The complete nucleotide sequence and deduced amino acid sequence of *Hasp* have been deposited at the GenBank database (accession no. DQ422145).

Sequence analysis of *Hasp*

The open reading frame of *Hasp* was 1170 bp. The nucleotide sequence surrounding the start codon matched the consensus for initiation of translation for fungal genes (ANNATG). *Hasp* contained four exons (279, 186, 513, and 192 bp) and three introns (65, 99, and 93 bp). The introns were demarcated by the highly conserved dinucleotides GT and AG on the 5′ and 3′ ends, respectively.

Hasp encoded a polypeptide of 389 amino acid residues, with a predicted isoelectric point of 8.0. A computer analysis of the coding region revealed the presence of a signal peptide of 15 amino acids (MKLSLILAILPAVLA) enriched with hydrophobic ones (87%). The pro-peptide and mature peptide of Hasp were composed of 91 and 283 amino acid residues, respectively, and shared conserved motifs with subtilisin_N and peptidase_S8. Multiple sequence alignment showed that the deduced amino acid sequence of *Hasp* was homologous to serine proteases from other nematophagous fungi (Figure 3). However, the putative protein sequence was more similar to serine protease sequences from the egg-parasitic fungi *P. lilacinus* (PLP 65.3%) and *P. chlamydosporia* (VCP1 61.6%) than to the sequences from trapping fungi such as *A. oligospora* (PII 39.3%), *A. oligospora* (Aoz1 36.0%), and *M. microscaphoides* (Mix 41.0%).

The copy number of the *Hasp*

Only single hybridization signal was detected when the genomic DNA was digested with the restriction enzymes *Apa*I and *Hin*dIII (which did not cut in the probe region) whereas two bands were detected when the DNA was digested with restriction enzymes *Sac*I and *Xho*I (which did cut once in the probe region) (Figure 4). The results indicated

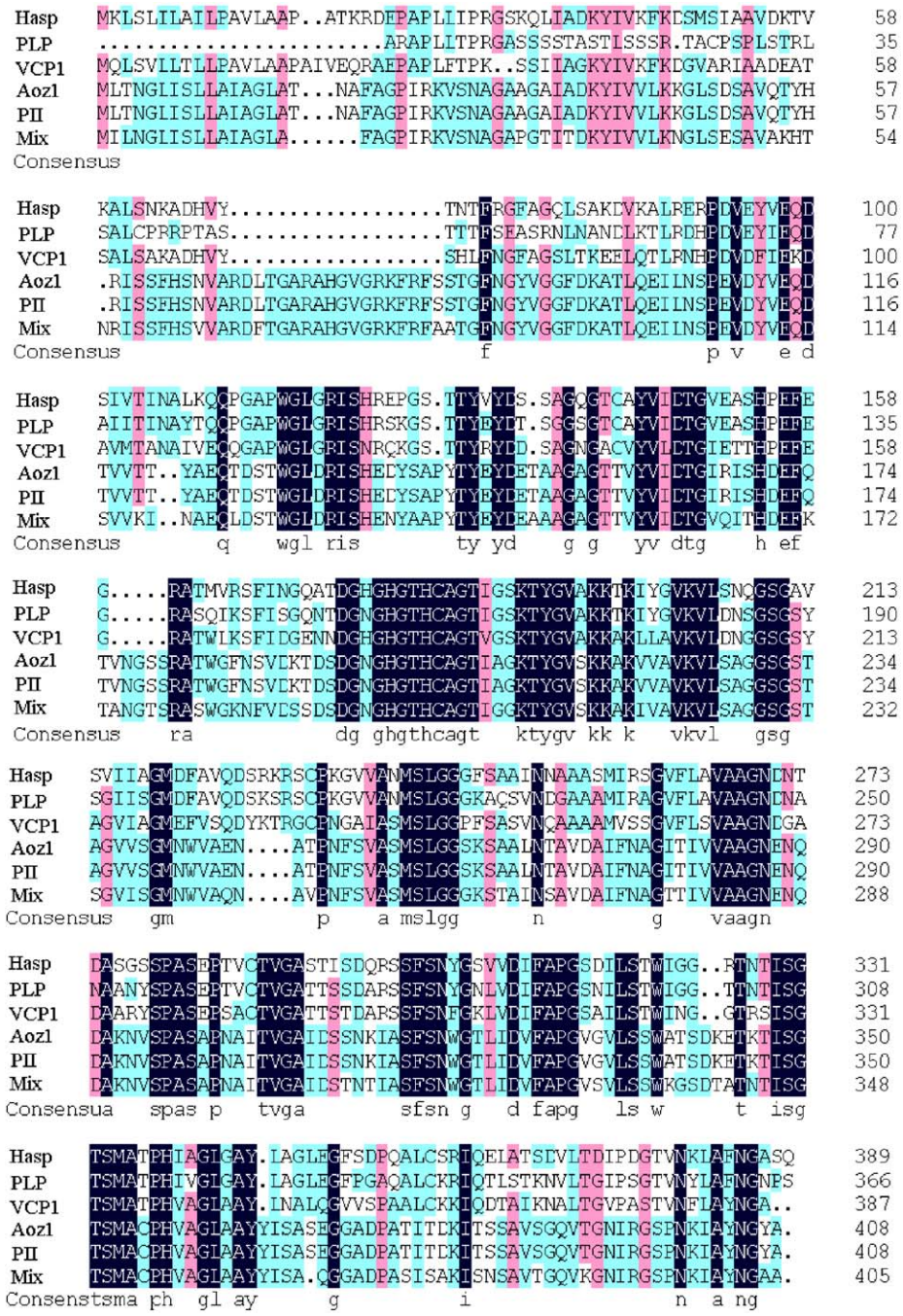

Figure 3. Alignment of serine proteases from nematophagous fungi. Multiple amino acid sequence alignment was performed with *H. rhossiliensis* Hasp (DQ422145), *M. microscaphoides* Mix (AY841167), *P. lilacinus* PLP (L29262), *P. chlamydosporium* VCP1 (AJ427454), *A. oligospora* PII (X94121), and *A. oligospora* Aoz1 (AF516146).

that the genome of *H. rhossiliensis* OWVT-1 contains a single copy of *Hasp*.

Discussion

Extracellular serine proteases play an important role in the infection of nematodes by the nematode-trapping and egg-parasitic fungi (Tunlid et al. 1994; Lopez-Llorca et al. 2002), but only one neutral protease has been purified from the highly virulent nematode-endoparasitic fungi (Wang et al. 2007). Here, we describe the detection, purification, and characteristics of a new alkaline serine protease Hasp from the nematode-endoparasitic fungus *H. rhossiliensis* OWVT-1.

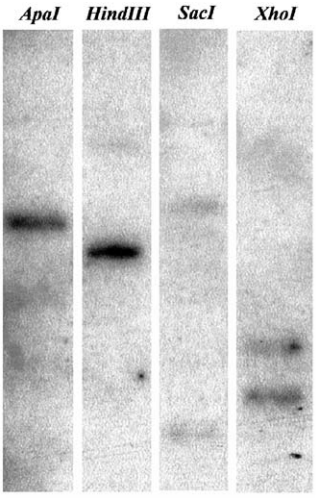

Figure 4. Copy number of Hasp. *H. rhossiliensis* OWVT-1 genomic DNA was digested with *Apa*I, *Hind*III, *Sac*I, or *Xho*I. *Apa*I and *Hind*III did not cut in the probe region, whereas *Sac*I and *Xho*I cut once in the probe.

Production of Hasp

In liquid culture, *H. rhossiliensis* OWVT-1 produced proteases only when nematodes were used as the single source of nitrogen. Other proteinous substrates such as yeast extract, tryptone, peptone, or gelatin, which have been commonly used as nitrogen sources for inducing serine protease production (Tunlid et al. 1994; Bonants et al. 1995; Seger et al. 1994; Yang et al. 2005), did not induce protease expression of this fungus. Enzyme production by *H. rhossiliensis* OWVT-1 was possibly repressed by simple nutrient sources while induced by the specific substrate.

In the higher eukaryotes, the CAAT-box and the TATA-box are important for the initiation of transcription (McKnight and Kingsbury 1982). A TATA-box and two CAAT elements were found −180, −240, and −360 bp upstream of the start codon ATG in the *Hasp* gene. The fungal protease genes possess TATA-box upstream of the start codon as well as GATA and CREA elements. It has been proved that GATA elements mediate N-repression of enzyme induction (Dhawale and Lane 1993) and that CREA elements cause carbon-catabolite repression in ascomycete fungi (Screen et al. 1997). The two kinds of regulatory elements in *Hasp* might represent the genetic basis for the repression of Hasp production by simple nutrient sources.

Characteristics of Hasp

The molecular weight of Hasp was similar to that of serine proteases from egg-parasitic fungi *P. lilacinus* PLP (Bonants et al. 1995) and *P. chlamydosporium* VCP1 (Seger et al. 1994) (32–33 kDa) but was less than that of serine proteases from the nematode-trapping fungi *A. oligospora* PII (Tunlid et al. 1994), *A. oligospora* Aoz1 (Zhao et al. 2004), and *M. microscaphoides* Mix (Wang et al. 2006) (35–39 kDa). The optimum pH and temperature for Hasp activity were pH 9 and 75 °C, which were similar to the optima for other alkaline serine protease from nematophagous fungi (Tunlid et al. 1994; Wang et al. 2006; Bonants et al. 1995; Seger et al. 1994; Yang et al. 2005).

The amino acid sequence alignment of nematophagous fungi clearly showed that serine proteases could be divided into two groups: group I was from nematode-endoparasitic/egg-parasitic fungi (Hasp, PLP, and VCP1); group II was from nematode-trapping fungi (PII, Aoz1, and Mix) (Figure 3). The similarity between Hasp and the most similar proteases from nematophagous fungi is about 65.3%. Purified Hasp killed nematodes and degraded some cuticle proteins, but it did not destroy the nematode cuticle like other proteases from nematode-trapping and egg-parasitic fungi (Wang et al. 2006; Yang et al. 2005).

Phylogenetic analysis of the subtilisin superfamily

The complete amino acid sequences of 14 serine proteases (including six serine proteases from nematophagous fungi, eight from bacteria, and one each from a vertebrate, an insect, a nematode, and a cyanobacterium) were aligned with the software clustal X (Bagga et al. 2004). A phylogenetic tree was developed based on the full amino acid sequences of 14 serine proteases. The tree showed that serine proteases from nematode-trapping fungi (PII, Aoz1, and Mix) clustered into one clade, while those from endoparasites or egg parasites (Hasp, PLP, and VCP1) clustered into another clade (Figure 5). Hasp was highly homologous to the serine proteases from nematode/egg parasites and entomopathogenic fungi. It also showed evolutionary divergence of the proteases from parasitic fungi (nematodes and eggs parasites) and nematode-trapping fungi. All the proteases from parasitic fungi were far from those from bacteria, the vertebrate, and nematode.

Role of the subtilisin from nematophagous fungi

The proteases from nematode-endoparasitic and egg-parasitic fungi were detected early during

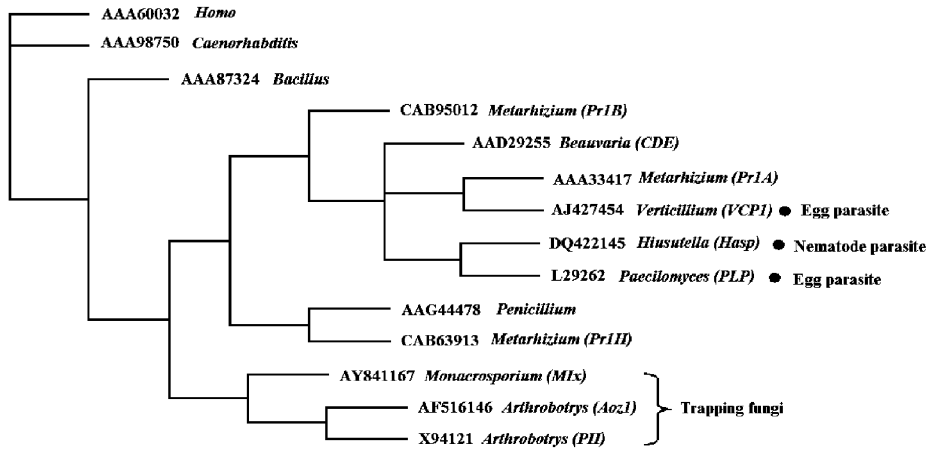

Figure 5. Neighbor-joining tree of 59 subtilisin protein sequences. Bootstrap support and accession numbers are provided in the figure. Nematophagous fungi are labeled in bold.

infection of eggs or free-living nematodes (Lopez-Llorca and Robertson 1992). In contrast, the proteases from the nematode-trapping fungi were not detected early during infection but were highly expressed later, when the fungus was killing and colonizing the nematode (Ahman et al. 2002). Therefore, the proteases of nematode-endoparasitic/egg-parasitic fungi and nematode-trapping fungi might differ not only in amino acid sequence but also in gene expression and function during infection.

The present results showed that Hasp could kill the J2 of the soybean-cyst nematode and degrade proteins of the nematode cuticle. Furthermore, sequence analysis showed that Hasp was similar to the proteases from the nematode-endoparasitic/egg-parasitic fungi. Whether Hasp might assist in penetrating the cuticle is not known yet. Further studies of gene over-expression and knock out in the wild-type strain will increase our understanding of the exact function of *Hasp* in the infection of nematodes.

Acknowledgments

This research was jointly supported by the National Natural Science Foundations of China (No. 30370953) and the Hi-Tech Program of China (2003AA246010). We thank R&D Novozymes China for providing research facilities. We would also thank Dr. Hanying Yu and Ying Yang for their valuable suggestion in the manuscript revision and Mr. Ence Yang for phylogenetic analysis.

References

Ahman J, Johansson T, Olsson M, Punt PJ, Van Den Hondel C, Tunlid A. Improving the pathogenicity of a nematode-trapping fungus by genetic engineering of a subtilisin with nematotoxic activity. Appl Environ Microb 2002;68:3408–15.

Bagga S, Hu G, Screen SE, St. Leger RJ. Reconstructing the diversification of subtilisins in the pathogenic fungus *Metarhizium anisopliae*. Gene 2004;324:159–69.

Bonants PJM, Fitters PFL, Thijs H, Den Belder B, Waalwijk C, Henfling JWDM. A basic serine protease from *Paecilomyces lilacinus* with biological activity against *Meloidogyne hapla* eggs. Microbiology (UK) 1995;141:775–84.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.

Chen SY, Liu XZ. Control of the soybean cyst nematode by the fungi *Hirsutella rhossiliensis* and *Hirsutella minnesotensis* in greenhouse studies. Biol Control 2005;32:208–19.

Cox GN, Kusch NM, Edgar RS. Cuticle of *Caenorhabditis elegans*: its isolation and partial characterization. J Cell Biol 1981;90:7–17.

Day PR, Scott KJ. Scanning electron microscopy of fresh material of *Erysiphe graminis* f. sp. *hordei*. Physiol Plant Pathol 1973;3:433–5.

Dhawale SS, Lane AC. Compilation of sequence-specific DNA-binding proteins implicated in transcriptional control in fungi. Nucleic Acids Res 1993;21:5537–46.

Jaffee BA, Zehir EJ. Parasitic and saprophytic abilities of the nematode-attacking fungus *Hirsutella rhossiliensis*. J Nematol 1985;17:341–5.

Liu XZ, Chen SY. Parasitism of *Heterodera glycines* by *Hirsutella* spp. in Minnesota soybean fields. Biol Control 2000;19:161–6.

Lopez-Llorca LV, Robertson WM. Immunocytochemical localization of a 32-kDa protease from the nematophagous fungus *Verticillium suchlasporium* in infected nematode eggs. Exp Mycol 1992;16:261–7.

Lopez-Llorca LV, Olivares-Bernabeu C, Salinas J, Jansson HB, Kolattukudy PE. Prepenetration events in fungal

parasitism of nematode eggs. Mycol Res 2002;106: 499–506.

McKnight SL, Kingsbury R. Transcriptional control signals of a eukaryotic protein-coding gene. Science 1982; 217:316–24.

Morton CO, Hirsch PR, Kerry BR. Infection of plant-parasitic nematodes by nematophagous fungi – a review of the application of molecular biology to understand infection processes and to improve biological control. Nematology 2004;6: 161–70.

Raeder U, Broda P. Rapid preparation of DNA from filamentous fungi. Lett Appl Microbiol 1985;1: 17–20.

Screen S, Bailey A, Charnley AK, Cooper RM, Clarkson JM. Carbon regulation of the cuticle degrading enzyme PR1 from *Metarhizium anisopliae* may involve a trans-acting DNA-binding protein CRR1, a functional equivalent of the *Aspergillus* nidulans CREA protein. Curr Genet 1997;31:511–8.

Seger R, Butt TM, Kerry BR, Peberdy JF. The nematophagous fungus *Verticillium chlamydosporium* produces a chymoeslastase-like protease which hydrolyses host nematode proteins *in situ*. Microbiology (UK) 1994; 140:2715–23.

Tunlid A, Rosen S, Ek B, Rask R. Purification and characterization of an extracellular serine protease from the nematode-trapping fungus *Arthrobotrys oligospora*. Microbiology (UK) 1994;140:1687–95.

Wang M, Yang JK, Zhang KQ. Characterization of an extracellular protease and its cDNA from the nematode-trapping fungus *Monacrosporium microscaphoides*. Can J Microbiol 2006;52:130–9.

Wang B, Wu WP, Liu XZ. Purification and characterization of a neutral serine protease with nematicidal activity from *Hirsutella rhossiliensis*. Mycopathologia 2007; 163:169–76.

Yang JK, Huang XW, Tian BY, Wang M, Niu QH, Zhan KQ. Isolation and characterization of a serine protease from the nematophagous fungus, *Lecanicillium psalliotae*, displaying nematicidal activity. Biotechnol Lett 2005;27:1123–8.

Zhao ML, Mo MH, Zhang KQ. Characterization of a neutral serine protease and its full-length cDNA from the nematode-trapping fungus *Arthrobotrys oligospora*. Mycologia 2004;96:16–22.