A potential virulence factor involved in fungal pathogenicity: serine-like protease activity of nematophagous fungus *Clonostachys rosea*

M.L. Zhao[1, 2], J.S. Huang[3], M.H. Mo[1] and K.Q. Zhang[1*]

[1]Key Laboratory for Conservation and Utilization of Bio-Resource, Yunnan University, Kunming, 650091, Yunnan, P.R. of China
[2]Department of Biological Sciences and Biotechnology, Tsinghua University, Beijing 100084, P.R. of China
[3]Environment and plant protection institute, State Key Laboratory of Biotechnology for Tropical Crops, Chinese Academy of Tropical Agricultural Sciences, Haikou, 571101, Hainan, P.R. of China

Zhao, M.L., Huang, J.S., Mo, M.H. and Zhang, K.Q. (2005). A potential virulence factor involved in fungal pathogenicity: serine-like protease activity of nematophagous fungus *Clonostachys rosea*. Fungal Diversity 19: 217-234.

A serine-like protease (designated as Lmz1) was purified to homogeneity from *Clonostachys rosea*. It showed a molecular mass of approximately 33 kDa, pI 10.5, optimal activity of Lmz1 at 60°C and pH 11-12, and broad pH stability between pH 5-12. Lmz1 has a Km value for Suc-(Ala)2-Pro-Phe-pNA of 1.45 mM. The protease activity was completely inhibited by PMSF or streptomyces subtilisin inhibitor, and partially inhibited by collagenase inhibitor I; all indicated the presence of a serine residue in the active site and Lmz1 is thus most likely a member of the serine protease family. Its N-terminal amino acid sequence was directly sequenced by Edman degradation. The recombinant plasmid harbouring a cDNA gene encoding the mature protease was integrated into yeast chromosome DNA and the gene was successfully expressed as demonstrated via activity analysis, ELISA and Western blotting. The nematotoxic activity of the protease involving in fungal infection was characterised by nematode-immobilisation and cuticle degradation with the purified Lmz1 or recombinant protease, and further verified by immunodepletion using anti-Lmz1 antiserum prepared in mice. These observations suggest a research model for fungal infection mechanism of serine-like protease involvement.

Key words: *Clonostachys rosea*, functional analysis, nematotoxic activity, serine-like protease.

[*]Corresponding author: Zhang Keqin; e-mail: kqzhang1@yahoo.com.cn

Abbreviations: EDTA, ethylene diaminotetraacetic acid; PMSF, phenylmethylsulphonyl fluoride; SSI, streptomyces subtilisin inhibitor, IPTG, isopropyl-D-thiogalactopyranoside; YNB, yeast nitrogen base; X-Gal, 5-bromo-4-chloro-3-indolyl-beta-D-galactoside; Lmz1, alkaline serine protease from *Clonostachys rosea*, Suc-AAPF-pNA, chymotrypsin substrate N-Succinyl-(Ala)2-Pro-Phe-p-nitroanilide; FCA, Freund's Complete Adjuvant; FIA, Freund's Incomplete Adjuvant; DAB, 3,3'-diaminobenzidine tetrahydrochloride; OPD, O-Phenylenediamine Dihydrochloride; HRP, horseradish peroxidase.

Patent Status: We are applying for a patent in China (Application No. 03117932.0), based on the main contents of this article.

Introduction

Clonostachys rosea (syn. *Gliocladium roseum*, Hans *et al*., 1999), a mitosporic fungus, is widely used as a biocontrol agent on phytopathogenic fungi (Li *et al*., 2002). This mycoparasitic fungus is also a facultative parasite of pathogenic nematodes (Yang *et al*., 2000). During our continuing investigation on nematophagous fungi from China (Dong *et al*., 2004; Mo *et al*., 2005), an isolate of *Clonostachys rosea* (isolate 87), which was isolated from a soil sample in Yunnan Province, was shown to be able to parasitise and digest nematodes (Zhang and Zhao, 2004).

Proteases are virulence determinants of entomogenous fungi (St Leger *et al*., 1987; St Leger *et al*., 1988; St Leger *et al*., 1997; Gillespie *et al*., 1998), and human pathogenic fungi (Kwon-Chung *et al*., 1985; Frosco *et al*., 1992). Nematode cuticle being composed of proteins, including collagens, suggests that fungal extracellular proteases are likely involved in the infection of nematodes. It has been shown that culture supernatant and purified protease of fungi can immobilise nematodes and degrade cuticle of nematodes (Åhman *et al*., 2002; Zhao *et al*., 2004). Several extracellular proteases isolated from nematophagous fungi belong to serine proteases with high homology to members of the subtilase family (Segers *et al*., 1994; Tunlid *et al*., 1994; Bonants *et al*., 1995; Åhman *et al*., 2002; Zhao *et al*., 2004).

Little is known about the proteases secreted by the mycoparasitic fungus *Clonostachys rosea*, but extracellular proteases may be a key enzyme in nematode infection process. In this study we reported the purification, and the properties of a serine-like protease with nematotoxic activity from the nematophagous fungus, and the heterologous expression of this enzyme in yeast for functional analysis, including immunodepletion experiment. The unique properties and important role of the serine-like protease may be used in the selection of virulent strains and genetic improvement of nematophagous fungi for application in biocontrol programmes.

Materials and methods

Microorganisms and culture conditions

Clonostachys rosea (isolate 87, CGMCC 0806) was isolated from soil sample in Western suburb of Kunming, Yunnan Province and identified by the Institute of Microbiology, Academic Sinica, Beijing, P.R. of China. It was grown on PDA plates and maintained on PDA slants with transfer every 3-4 months. A single-spore isolate of *Clonostachys rosea* was chosen to be

examined for protease production and was activated on PDA plate for 4 days at 28°C prior to inoculation into LMZ liquid medium (Zhao *et al*., 2004). *Escherichia coli* JM109 and *Pichia pastoris* GS115 were cultured using standard growth conditions (LB medium and 37°C for *E. coli*, YEPD medium and 30°C for *P. pastoris*). The nematode *Panagrellus redivivus* L. (Goodey) was grown axenically in semiliquid oat medium at 28°C for 5 days and then stored at 4°C until use. Nematodes were washed extensively with autoclaved distilled water, penicillin solution (400 000 IU), and streptomycin solution (320 000 IU) before being used in bioassays.

Chemicals and substrates

All the chemical reagents were of analytical grade. Azocoll (a substrate for non-specific protease, which releases red dye when degraded), PMSF, EDTA, N-Suc-AAPF-pNA, ponceau S, O-phenylenediamine dihydrochloride, and electrophoresis reagents were purchased from Sigma-Aldrich. Low Molecular Weight Calibration Kit for SDS Electrophoresis, Phenyl-Sepharose (high sub), Sephacryl S-200 High Resolution, and isoelectric point Calibration Kit for IEF were obtained from Amersham Biosciences. SSI inhibitor was kindly provided by Novozymes. Collagenase Inhibitor I was from Calbiochem. Restriction endonucleases, pfu DNA polymerase, pMD 18-T vector, and CIAP were from TaKaRa. YNB was purchased from Difco. *Pichia pastoris* GS115, pPIC9K and G418 were from Invitrogen. FCA and FIA were from Pierce. HRP-IgG was obtained from Dako (Denmark).

Buffers

The following buffers were used in purification steps: buffer A, 50 mM sodium phosphate containing 1.5 M ammonium sulfate; buffer B, 50 mM sodium phosphate, pH 7.0; buffer C, 50 mM sodium phosphate containing 0.15 M NaCl. Britton-Robinson universal buffers (pH 2-12) were used to determine pH profile.

Protease production and ammonium sulphate precipitation

LMZ liquid medium (Zhao *et al*., 2004) was used for protease production of *Clonostachys rosea*. One litre supernatant was subjected to concentration of crude enzymes by ammonium sulphate precipitation of 80% saturation in an ice bath.

Purification of protease

All steps were performed on ice or at 4^{o}C. Protease activity of the culture filtrate and purification fractions were determined using the chromogenic substrate azocoll at 45^{o}C and measured in duplicate, averaged and expressed as A_{520} units ml^{-1} min^{-1}. Variation between duplicates was less than 10%. The above precipitate were collected by centrifugation (8000 rpm, 20 min, Heraeus), dissolved in 50mM sodium phosphate buffer (pH 7.0). A sample (15ml) filtered through a 0.45 μm filter was applied to a HiPrep™ 16/10 Phenyl (high sub) column (20ml) with ÄKTA explorer system, equilibrated with 50mM sodium phosphate buffer containing 1.5 M ammonium sulphate. Bound proteins were eluted with a linear gradient of decreasing ammonium sulphate (0-100% Buffer B), and 4-ml fractions were collected. All fractions with absorbance at 280nm were assayed for protease activity using the azocoll substrate.

Gel electrophoresis

All fractions with a protease activity from hydrophobic runs of crude protease were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis using a 12% resolving and 5% stacking gel (Bio-Rad MiniProtean III gel device) following standard methods (Sambrook *et al*., 1989). Gels were stained with Gelcode® Blue Stain Reagent (Coomassie G-250, Pierce) following gel electrophoresis.

Gel filtration for fine purification

Fractions with the same protein band pattern were pooled, concentrated by ultrafiltration (Millipore, cut-off 3 kDa), and exchanged in 50mM sodium phosphate buffer containing 0.15 M NaCl (pH 7.0). The final concentrated sample (2 ml) was loaded onto a Sephacryl S-200 High Resolution gel filtration column (180 ml) equilibrated with 50mM sodium phosphate buffer containing 0.15 M NaCl (pH 7.0). The column was eluted with the equilibration buffer and 3-ml fractions were collected. Fractions with single band pattern by gel assay were pooled, desalted by small desalting column, frozen, and lyophilised.

Protein determination

The amount of protein was determined by the method of Bicinchoninic Acid with a BCA Protein Assay Reagent Kit according to the manufacturer (Pierce), using bovine serum albumin (BSA) as the standard.

Isoelectric focusing for pI assay

Isoelectric focusing was performed at 10°C using Immobiline DryPlate in a Multiphor II Electrophoresis Unit according to the manufacturer's instructions (Pharmacia). The isoelectric point was estimated using a broad pI calibration kit (pI 3.5-9.3). The IEF gel was stained with Coomassie Brilliant Blue.

Inhibition of the purified protease

Twenty µl of protease sample and 5 µl PMSF (10 µM), collagenase inhibitor I (10 µM), EDTA (0.5 M, pH 8.0) and SSI (10 µM) were added to microcentrifuge tubes and incubated for 5 min at room temperature. Protease activity was assayed using the azocoll method as previously described (Zhao *et al*., 2004).

Temperature and pH effects

To study the effect of temperature on the enzyme reaction rate, reaction mixture (5mg azocoll in 1 mL buffer with different pH value) was pre-incubated for 5 min in a range from 4°C to 75°C, then added the purified protease, the reaction time was 10 min. To determine thermal inactivation of the protease at various temperature, samples were incubated for various time and then assayed. For pH studies, Britton-Robinson universal buffers (pH 2-12) was used and reaction mixture was incubated for 30 min at 55°C.

Determination of K_m value

Kinetic parameter of the purified protease (designated as Lmz1) was determined at 55°C on a UV/Vis spectrophotometer with a SHIFT II software using a model peptidyl-p-nitroanilides (N-suc-AAPF-pNA). One unit (U) of enzyme activity is defined as the amount of enzyme required for the hydrolysis of one µmol of substrate per min.

Immobilisation of nematodes in bioassays

Suspensions containing 30-50 washed nematodes in 150 µl sterile water were transferred to microtiter wells and incubated with 15 µl purified Lmz1 for 24 h. An untreated nematode sample served as a control. The mobility of treated nematodes were investigated by microscopic examination.

N-terminal sequence determination

After electrophoresis, the purified protease was electroblotted onto a PVDF transfer membrane (Millipore) and stained with Gelcode® Blue Stain Reagent for protein microsequencing. Protein sample containing 30 picomole of purified protease was subjected to Edman degradation using a PE/ABI 491 (Applied Biosystems) protein sequenator. PTH amino acid peaks were identified in the HPLC elution profile with an Applied Biosystems Procise sequencer.

cDNA cloning

Agar pieces from colony edges of *Clonostachys rosea* (isolate 87) were inoculated into LMZ and cultured for 4 d at 28^{o}C by shaking at 150 rpm. The *Clonostachys rosea* mycelia were harvested, and samples were immediately flash-frozen in liquid nitrogen and stored at -70^{o}C. Fungal total RNA was isolated using an RNeasy Plant Mini Kit™ (Qiagen) according to the manufacturer's instruction for the target cDNA amplification by degenerate primer. The RACE (Rapid Amplification of cDNA Ends) method [3'RACE System for Rapid Amplification of cDNA Ends (Invitrogen)] was employed to obtain a partial cDNA encoding mature protease. Molecular construction was performed according to standard procedure (Sambrook *et al*., 1989). Two degenerate primers deduced from a portion of the N-terminal amino acid sequence (ATQSNA) were designed [17bp, primer Cr3-1: 5' > GC(AGCT) AC(AGCT) CA(AG) AG(CT) AA(CT) GC < 3'; primer Cr3-2: 5' > GC(AGCT) AC(AGCT) CA(AG) TCI AA(CT) GC < 3'] for the 5' end of the mature protease gene. PCR conditions were as follows: pre-denaturation at 94^{o}C for 3 min prior to polymerase addition, followed by 30 cycles consisting of denaturation at 94^{o}C for 1 min, primer annealing at 58^{o}C for 1 min, and primer extension at 72^{o}C for 1 min. After cycling, the reaction mixture was kept at 72^{o}C for 10 min and then cooled to 4^{o}C. The amplified cDNA target fragment (approximately 0.9 kb) was separated by electrophoresis in a 1.0% agarose gel in 0.5X Tris-borate-EDTA and visualised with EtBr and UV irradiation using standard procedures (Sambrook *et al*., 1989). A slice of agarose containing the DNA band was excised using a clean razor blade and purified using Waters Gel Extraction Kit according to the manufacturer instructions (TaKaRa). The purified cDNA fragment was cloned into the pMD 18-T vector (TaKaRa) and then transformed into *E. coli* JM109. Recombinant plasmids were extracted from bacterial cultures using E.Z.N.A. Plasmid Miniprep Kit I (Omega Bio-tek).

DNA sequencing

Plasmid DNA containing insert fragment from respective ten colonies were sequenced using the Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (Version 3.0, Perkin Elmer) and an ABI PRISM™ 3700 DNA Analyser. The seqencing primers were M13 F (forward) and M13 R (reverse) universal primers.

Construction of recombinant expression vector

High fidelity pfu DNA polymerase was applied in the amplification of target region encoding mature protease by two specific primers (pfu-F: 5'> CAC GGA ATT CGC GAC TCA AAG TAA CG < 3'; pfu-R: 5'>CAT GGA ATT CTT ACA CGC CGC AAC CC < 3'), with *Eco*R I recognition site added additional four base pairs. PCR conditions using pfu DNA polymerase were as follows: pre-denaturation at 94^{o}C for 3min, followed by 35 cycles consisting of denaturation at 94^{o}C for 1 min, primer annealing at 55^{o}C for 1 min, and primer extension at 72^{o}C for 2 min. After cycling, the reaction mixture was kept at 72^{o}C for 10 min and then cooled to 4^{o}C. PCR products with specific single band were recovered by DNA Fragment Purification Kit (Takara) according to the instruction of manufacture. pPIC9K was digested with *Eco*R I at 37^{o}C, and then treated by CIAP. Purified target cDNA fragment also digested with *Eco*R I at 37^{o}C, and then was ligated to pPIC9K.

Characterisation of recombinant pPIC9K and electroporation into yeast host

After resistance screening, shuttle plasmid pPIC9K was extracted from transformed *E. coli* JM109 culture and digested with *Eco*R I for insert validation. DNA sequencing by 5'AOX1 primer was also performed for insert direction identification. After linearisation of pPIC9K with *Sac* I, recombinant DNA was introduced into host cell *Pichia pastoris* GS115 by Bio-Rad Micropulser Electroporation Apparatus according to the manufacture's instruction.

Screening of transformant harbouring coding region for mature protease

After G418 screening (1mg/ml or 2 mg/ml resistance level), small-scale expression experiments were carried out with positive colonies. Genomic DNA of yeast positive transformants with extracellular protease activity were extracted by yeast genomic DNA extraction kit (V-gene) for further PCR characterisation.

Preparation of polyclonal antibody against the purified protease Lmz1

Normal sera of mice were extracted as background before immunisation. Two BALB/C female mice were immunised by celiac injection with the mixture of purified protease and FCA for initial or FIA for enhancement injection.

Measurement of polyclonal antibody

The antiserum titer of anti-Lmz1 antibodies from immunised mice after third enhance injection were determined by the enzyme-linked immunosorbent assay (ELISA). O-ohenylenediamine dihydrochloride (OPD) were used as the substrate of HRP.

Western blotting

After SDS-PAGE, gels containing recombinant protein were soaked in Tris/Glycine transfer buffer for 2 min and electroblotted onto nitrocellulose using the electroblotted apparatus (Bio-Rad) according to the manufacturer's instructions. Non-specific binding sites on the NC membrane were blocked with 1% defatted milk powder (calcium free, 0.01 M phosphate buffer pH 7.4). Normal serum and anti-Lmz1 serum were respectively diluted 1/400 and incubated with the antigen (recombinant protease) from yeast for 45 min at room temperature by slow shaking. The membrane slices were washed three times with PBS buffer (pH 7.4), prior to incubation with HRP-IgG, each for 10 min. After that, the membranes were incubated with rabbit anti-mouse peroxidase conjugate (1/2000 dilution) for 45 min at room temperature. Before adding buffered 3'-diaminobenzidine tetrahydrochloride (DAB, 0.6mg/ml), substrate for peroxidase, the excess secondary antibody was removed by washing the membranes three times with PBS buffer, each for 10 min. Subsequently, the NC membranes were stained with DAB substrate and H_2O_2.

Immunodepletion for confirmation of alkaline protease with nematotoxicity

Portions of the *Panagrellus redivivus* nematode suspension (100µl) containing 80-100 nematodes were transferred to different wells in six-well plate, then 50µl of crude protease and purified protease preparation (Lmz1) were respectively added as sample groups. Meanwhile, 50µl of polyclonal antisera were accordingly added to control groups. After static incubation of the wells at room temperature for 24 h, the numbers of mobile and immobile nematodes were counted in a light microscope. The experiments were performed with duplicate and repeated twice. Controls were incubated without

protease extracts or polyclonal antisera. The statistical significance of the differences in frequency of mobile and immobile nematodes in experimental groups versus control (background or polyclonal antibodies control) was tested by analysis of variance (F test).

Results

Identification of Clonostachys rosea

The single-spore isolate of *Clonostachys rosea* reported here produced two kinds of conidiophores. All oval conidia have no septation, same as characteristics described by Hans *et al*. (1999). In *C. rosea*, the dimorphy consisted of (i) early-formed *Verticillium*-like conidiophores (primary conidiophores) with few long divergent phialides, and with conidia adhering in small round masses on each phialide, and (ii) later-formed penicillate conidiophores (secondary conidiophores) with smaller compressed phialides and imbricate conidial columns. In our study we observed the parasitism of *Clonostachys rosea* to *Panagrellus redivivus* nematodes on PDA plate. This fungus killed preys and grew out of the infected nematodes (Fig. 1).

Purification of nematocidal protease and electrophoretic analyses

We were able to maximise protease production by growing the fungal mycelium in LMZ medium for six days prior to harvest for purification (Table 1). The purification procedure included ammonium sulfate fractionation, hydrophobic interaction, and gel filtration chromatographies. Protease activity was monitored during the course of purification by quantitative assay using azocoll. A fungal protease was purified to apparent homogeneity from culture filtrate of *Clonostachys rosea*. The mobility of the target protein band using SDS-PAGE corresponded to a molecular mass of 33 kDa (Fig. 2). Analytical IEF of the purified protease showed a pI of approximate 10.5.

Determination of K_m value

The Km value for the purified protease was estimated to be of 1.45 mM against Suc-(Ala)2-Pro-Phe-pNA.

Effects of temperature and pH on enzymatic activity

Temperature profile of the purified protease were illustrated and the optimum of temperature for hydrolysis of azocoll was 60^{o}C (Fig. 3). pH profile

Table 1. Purification of Lmz from fungal supernatant of *Clonostachys rosea*.

Purification step	Total volume (ml)	Total protein (mg)	Total activity (PU) [b]	Specific activity (PU/mg)	Yield (%)	Purification (fold)
Crude supernatant	1000	671.25	60800.5	90.58	100	1.0
$(NH_4)_2SO_4$ fractionation [a]	80	74.484	38995.3	523.54	64.14	5.78
Phenyl-Sepharose	120	19.818	29424.6	1484.74	48.40	16.39
Sephacryl S-200	25	6.2	22786.1	3675.18	37.48	40.57

a: The 40-80% saturation precipitate.
b: Protease activity was assayed using the substrate azocoll and expressed as A_{520}, proteolytic units (PU) defined as the increase of A_{520} $ml^{-1}min^{-1}$ (1 cm path length).

Table 2. Effects of several inhibitors on *Clonostachys rosea* protease.

Inhibitor compound, final concentration in assay buffer	Remaining activity, %
None	100
EDTA (0.5 M, pH 8.0)	98
PMSF (10mM)	0.8
SSI	1.3
Collagenase Inhibitor I	39

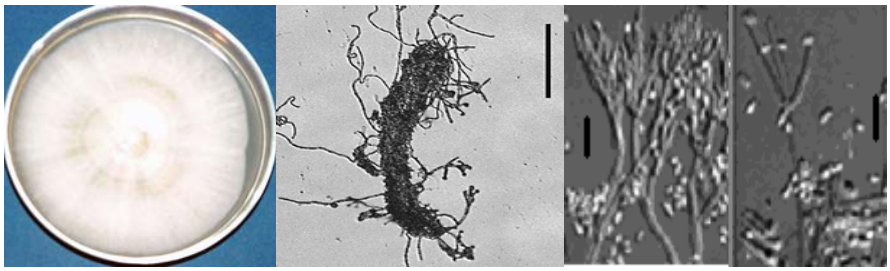

Fig. 1. Characteristics of *Clonostachys rosea*. The fungus produces verticillate and penicillate conidiophores. **a.** Colony shape on the PDA plate; **b.** The nematode *Panagrellus redivivus* was infected by *Clonostachys rosea* and fungal mycelia grew out of the infected nematode. Bar=50 μm **c.** Penicillate secondary conidiophores, phialides and phialomeristem spores were shown. Bar = 10 μm.

(pH 2-12) showed pH stability of Lmz1 was between 5 and 12. The pH optimum for hydrolysis of azocoll by Lmz1 was found to be pH 11-12 (Fig. 4).

Protease inhibition

The enzymatic activity of the purified protease Lmz1 was not inhibited measurably by EDTA, but it was completely inhibited by PMSF or SSI, and partially inhibited by collagenase inhibitor I (Table 2).

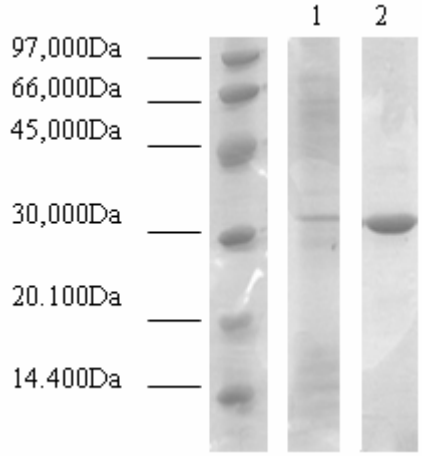

Fig. 2. SDS-PAGE pattern of crude and purified protease preparations. Lower molecular mass marker with values are indicated on the left. Lanes 1 and 2 respectively received 40 µl of crude and purified protease samples.

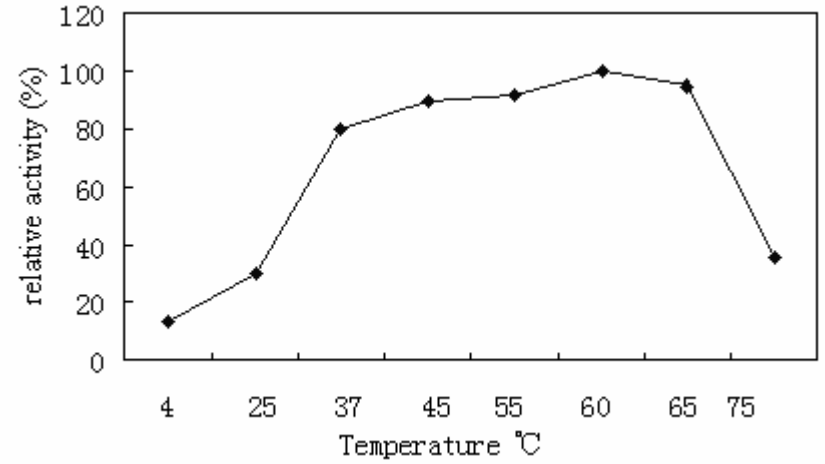

Fig. 3. Effect of temperature on the activity of the purified protease. Each point represents the average of duplicate assays.

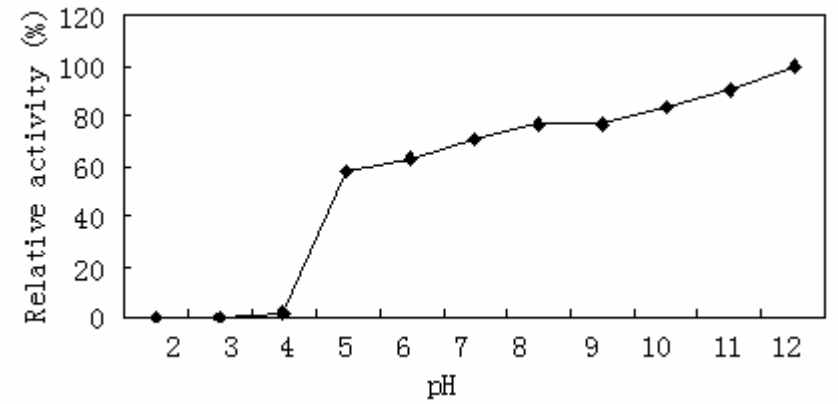

Fig. 4. Effect of pH on the activity of purified Lmz1. Each point represents the average of duplicate assays.

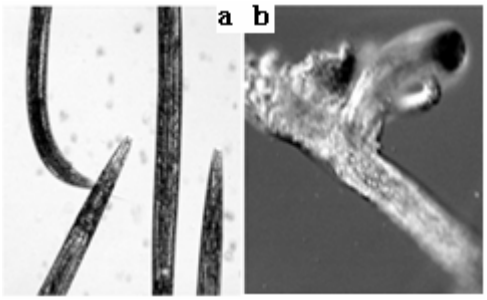

Fig. 5. Immobilisation of nematodes and cuticle degradation in bioassay. **a.** *Panagrellus redivivus* nematodes was immobilised by the purified alkaline serine-like protease. **b.** Evidence of partial cuticle degradation by the Lmz1 was clearly shown (The internal content of nematode leaks out.

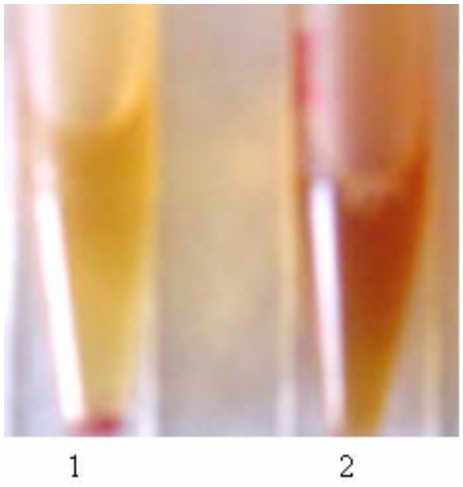

Fig. 6. Protease activity of culture filtrate from target engineered yeast against azocoll, which release a red dye when degraded. 1, wild *Pichia pastoris* GS115 as control; 2, the target engineered yeast. 22

Fig. 7. Western blotting of the recombinant protease and other proteins from flow-through peak. M, Lower molecular mass marker (kDa); 1A, 4A, Supernatant sample containing protease from engineered yeasts; 2A, 3A, *Clonostachys rosea* protein sample from flow-through peak by HIC. 1B, 2B, 3B, 4B: control, normal serum from healthy mouse as primary antibody.

N-terminal region

The purified Lmz1 protein was electroblotted to a PVDF membrane for direct microsequencing. PTH amino acid peaks in the HPLC elution profile

revealed an unique N-terminus of NH_2-A-T-Q-S-N-A-Q-?-?-? (? Indicating unidentified accurately amino acid residues). The sequence has been deposited in the SWISS-PROT protein database under the accession number P83492.

Substrate specificity

The specificity of Lmz1 was tested using various substrates (suspended or dissolved in 50 mM sodium phosphate buffer, pH 7.0) at 55^{o}C. The protease digested casein, gelatin, azocoll, suc-AAPF-pNA, and nematode cuticle.

Immobilisation of nematodes in bioassays and degrading of nematode cuticle

Most nematodes exposed to the purified protease Lmz1 were immobilised after 24 h, and microscopic examination revealed cuticle degradation (Fig. 5). Control nematodes treated in the parallel manner, but without protease, were not immobilised and showed no evidence of cuticle degradation.

cDNA sequence

The cDNA sequence encoding the mature protease has been deposited in GenBank under the accession number AY207377. No significant similarity of the cDNA sequence to other alkaline serine proteases were found by BLASTN against public database. Very interestingly, a BLASTN search of the NCBI/GenBank database using the partial cDNA sequence (AY207377) reveals partial 92% identity to a ribosomal-like protein from *Neurospora crassa* (XM_330092).

Characterisation of recombinant pPIC9K

Target insert in recombinant plasmid pPIC9K was verified by digestion with *Eco*R I. DNA sequencing data also showed the correct insert ORF direction.

Extracellular protease assay of positive transformants

Two chromogenic substrates (azocoll and suc-AAPF-pNA) were respectively used for recombinant protease assay. Both substrates were degraded by culture supernatant from *Pichia pastoris* GS115 positive transformants. Protease activity was assayed as average value of 120 (the increase of A_{520} ml^{-1} min^{-1}) using azocoll method (Fig. 6). The recombinant

protease were extracellular and biologically active. Protein content of supernatant from transformed yeast was determined by BCA method as 9 mg/ml of total protein.

Western blotting

After four injections, antisera titer reached 1/6400 by ELISA test. Using a polyclonal antibody anti-Lmz1, the successful expression of recombinant protease in yeast was further verified by immunoblot analysis (Fig. 7). Western blot showed the recombinant protease had a molecular mass of 42 kDa (Fig. 7) due to protein glycosylation modification in yeast host.

Immunodepletion effect of antisera

Based on polyclonal antibodies (anti-Lmz1) raised from BALB/C mice, immunodepletion was carried out for validation of immobilisation effect by the alkaline serine protease. Experimental groups included polyclonal antibodies (I), crude proteases (II), and purified protease (III). Addition of polyclonal antibodies to II and III served as depletion influence. The detection index of nematotoxic activity was immobilisation effect to nematodes. The result showed both crude and purified proteases could immobilise nematodes, but the results were opposite by groups of polyclonal antibodies addition into II and III. All indicated that polyclonal antibodies (anti-Lmz1) from BALB/C mice selectively depleted the serine protease (Lmz1). Crude proteases added with anti-Lmz1 illustrated a decrease in activity compared to control preparations and showed dosage-related inhibition effect of anti-Lmz1. Polyclonal antibodies without immobilisation effect to nematodes served as negative control. The immunodepletion experiment further confirmed the deleterious effect of *Clonostachys rosea* alkaline serine protease.

Discussion

Infection mode of Clonostachys rosea

The nematophagous fungus *Clonostachys rosea*, also a mycoparasitic fungus, can produce a large number of adhesive spores that adhere to passing nematodes and infect or kill the host. Here, infection of model nematodes (*Panagrellus redivivus*) by *Clonostachys rosea* was first observed by a light microscopy. The action mode is similar to other fungal endoparasites, such as *Hirsutella rhossiliensis* (Chen, 1997), *Verticillium chlamydosporium* and

Verticillium suchlasporium (Lopez-Llorca and Robertson, 1992; Vladimir *et al*., 2002).

Protease production

The conditions for protease production by *Clonostachys rosea* used in this study (0.2% gelatin and LMZ liquid culture) were same as those used with *Arthrobotrys oligospora* (Zhao *et al*., 2004). The choice of gelatin as the extracellular protease inducer contributed to maximal production of the alkaline serine-like protease.

The serine-like protease

Protein seems to be the most important barrier of invertebrates to fungal infection because it is a major component of the exoskeleton of nematodes (Cox *et al*., 1981; Maizels *et al*., 1993). The extracellular proteases of *Clonostachys rosea* may be responsible for cuticle penetration of proteacious barriers presented by host nematodes. This is supported by evidences of immobilisation bioassay and nematode cuticle degradation by the natural fungal alkaline serine-like protease or recombinant protease. Blotting analysis of fungal proteases from flow-through peak in hydrophobic interaction chromatography showed several bands. This suggests that *Clonostachys rosea* produced several serologically Lmz1-related extracellular proteases. Further study is required to examine the action mode of these proteases during infection on nematodes.

Purification of the serine-like protease

Ion exchange chromatography on Q-Sepharose Fast Flow or SP-Sepharose FF failed to separate target protease. And several ion purification attempts with different pH buffer were unsuccessful (data not shown). Only hydrophobic interaction chromatography can easily work. The alkaline protease (Lmz1) shared several biochemical characteristics with VCP1, a major alkaline protease secreted by *Pochonia chlamydosporia* (Lopez-Llorca, 1990) in soya peptone medium (syn. *Verticillium chlamydosporium*, Gams and Zare, 2001), with Pr1, a major protease secreted by the entomopathogen *Metarhizium anisopliae* (Segers *et al*., 1994), and with PL from egg-parasite *Paecilomyces lilacinus* (St Leger *et al*., 1987). These common features included similar charge (approximate pI 10), size (approximate 33 kDa), sensitivity to PMSF and the ability to hydrolyse chymotrypsin substrate suc-AAPF-pNA. These observations suggest that extracellular proteases involved in fungal infection

process from different fungi are similar but not identical to each other. This study provides some evidences of a chymotrypsin-like enzyme from the mycoparasitic fungus *Clonostachys rosea* involved in fungal infection. The 33 kDa alkaline serine-like protease produced by *Clonostachys rosea* is a good candidate for the cuticle degradation, but different isoenzymes of the protease may be produced depending on the growth medium. Therefore, further studies are necessary to characterise the role of the serine-like protease in nematode cuticle penetration and digestion of internal tissues. Future work, immuno-localising the 33 kDa protease in nematodes infected by the fungus *Clonostachys rosea* and the use of specific inhibitor and antibodies against the purified protease as negative controls, will further clarify the involvement of the fungal protease in the infection process. The results of treatment of saprophytic nematode (*Panagrellus redivivus*) with the fungal native alkaline serine protease and recombinant protease clearly suggested a crucial role of the extracellular protease Lmz1 in fungal pathogenesis. Furthermore, heterologous expression and immunodepletion of the serine protease showed that the serine-like protease was involved in fungal infection to host nematodes. This study confirms the existence of a serine-like proteinase in *Clonostachys rosea*. In addition, this proteinase is able to cleave efficiently nematode cuticle for hyphal penetration.

Acknowledgements

Research performed in this study were supported by the Natural Science Foundation of Yunnan Province (No. 2004C0001Z, 2003RC03). We thank Prof. Huang Zhunxi at Yunan Normal University for kindly providing expression vector and host cell. Thanks also to Dr. Wu Wenping in the R & D center of Novozymes (China Headquarters) for his valuable discussions, to Prof. Chen Guoqiang, Dr. Lun Yun, Dr. Huang Wei at Tsinghua University, Dr. Liu Xuefeng, Luo Hong, Tian Baoyu and Long Yunlei at Yunnan University for their kind help.

References

Åhman, J., Johansson, T., Olsson, M., Punt, P.J., Van den Hondel, C.A.M.J.J. and Tunlid, A. (2002). Improving the pathogenicity of a nematode-trapping fungus by genetic engineering of a subtilisin with nematotoxic activity. Applied and Environmental Microbiology 68: 3408-3415.

Bonants, P.J.M., Fitters, P.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 141: 775-784.

Chen, S. (1997). Infection of *Heterodera glycines* by *Hirsutella rhossiliensis* in a Minnesota soybean field. Journal of Nematology 29: 573-578.

Cox, G.N., Kusch, M. and Edgar, R.S. (1981). Cuticle of *Caenorhabditis elegans*: its isolation and partial characterization. Journal of Cell Biology. 90: 7-17.

Dong, J.Y., Zhao, Z.X., Cai, L., Liu, S.Q., Zhang, H.R., Duan, M. and Zhang, K.Q. (2004). Nematicidal effect of freshwater fungal cultures against the pine-wood nematode, *Bursaphelenchus xylophilus*. Fungal Diversity 15 : 125-135.

Frosco, M., Chase, T.Jr. and Macmillan, J.D. (1992). Purification and properties of the elastase from *Aspergillus fumigatus*. Infection and Immunity 60: 728-734.

Gams, W. and Zare, R. (2001). A revision of *Verticillium* sect.Prostrata. III. Generic classification. Nova Hedwigia 72: 329-337.

Gillespie, J.P., Bateman, R. and Charnley, A.K. (1998). Role of cuticle-degrading protease in the virulence of *Metarhizium* spp. for the desert locust, *Schistocerca gregaria*. Journal of Invertebrate Pathology 71: 128-137.

Hans, J.S., Gary, J.S., Keith, A.S. and Walter, G. (1999). Classification of the mycoparasite *Gliocladium roseum* in *Clonostachys* as *C. rosea*, its relationship to *Bionectria ochroleuca*, and notes on other *Gliocladium*-like fungus. Mycologia 91: 365-385.

Kwon-Chung, K.J., Lehman, D., Good, C. and Magee, P.T. (1985). Genetic evidence for role of extracellular proteinase in virulence of *Candida albicans*. Infection and Immunity 49: 571-575.

Li, G.Q., Huang, H.C., Kokko, E.G. and Acharya, S.N. (2002). Ultrastructural study of mycoparasitism of *Gliocladium roseum* on *Botrytis cinerea*. Botanical Bulletin of Academia Sinica 43: 211-218.

Lopez-Llorca, L.V. (1990). Purification and properties of extracellular proteases produced by the nematophagous fungus *Verticillium suchlasporium*. Canadian Journal of Microbiology. 36: 530-537.

Lopez-Llorca, L.V. and Robertson, W.M. (1992). Ultrastructure of infection of cyst nematode eggs by the nematophagous fungus *Verticillium suchlasporium*. Nematologica 39: 65-74.

Maizels, R.M., Blaxter, M.L. and Selkirk, M.E. (1993). Forms and functions of nematode surfaces. Experimental Parasitology 77: 380-384.

Mo, M.H., Huang, X.W., Zhou, W., Huang, Y., Hao, Y.E. and Zhang, K.Q. (2005). *Arthrobotrys yunnanensis* sp. nov., the fourth anamorph of *Orbilia auricolor*. Fungal Diversity 18: 107-115.

Sambrook, J., Fritsch, E.F. and Maniatis, T.M. (1989). Molecular Cloning: A Laboratory Manual. 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

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

St Leger, R.J., Charnley, A.K. and Cooper, R.M. (1987). Characterization of cuticle-degrading proteases produced by the entomopathogen *Metarhizium anisopliae*. Archives of Biochemistry and Biophysics 253: 221-232.

St Leger, R.J., Durrands, P.K., Charnley, A.K. and Cooper, R.M. (1988). The role of extracellular chymoelastase in the virulence of *Metarhizium anisopliae* for *Manduca sexta*. Journal of Invertebrate Pathology 52: 285-294.

St Leger, R.J., Joshi, L. and Roberts, D.W. (1997). Adaptation of proteases and carbohydrates of saprophytic, phytopathogenic and entomopathogenic fungi to the requirements of their ecological niches. Microbiology 143: 1983-1992.

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

Vladimir, E.T., Luis, V.L., Jesús, S. and Hans-Börje, J. (2002). Purification and Characterization of Chitinases from the Nematophagous Fungi *Verticillium chlamydosporium* and *V. suchlasporium*. Fungal Genetics and Biology 35: 67-78.

Yang, X.J., He, Y.X., Chen, F.R. and Zheng, L. (2000). Separating and selection of egg-mass fungi of Meloidogyne sp in Fujian province. Fujian Journal of Agricultural Sciences 15: 12-15.

Zhang, K.Q. and Zhao, M.L. (2004). Recent advance of nematophagous fungi from China and with a special case on *Clonostachys rosea* (*Gliocladium roseum*). Journal of Zhejiang University (Agriculture & Life Sciences) 30: 389-389.

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.

(Received 28 September 2004; accepted 16 April 2005)