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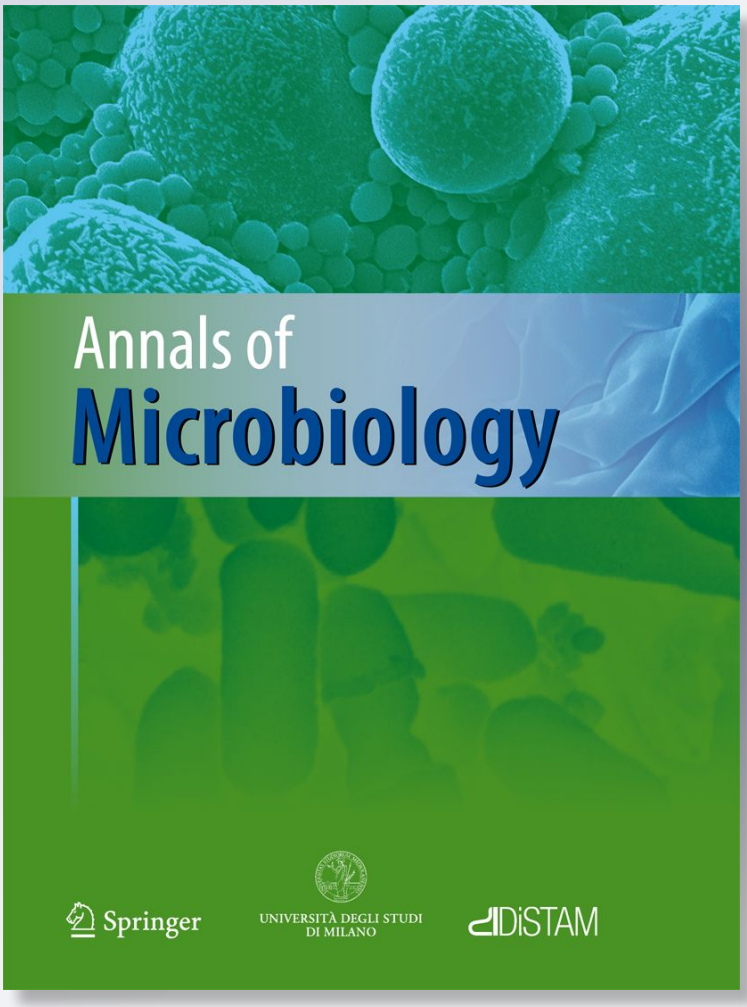

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ORIGINAL ARTICLE

Cloning and homology modeling of a serine protease gene (*PrC*) from the nematophagous fungus *Clonostachys rosea*

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Abstract The nematophagous fungus *Clonostachys rosea* can parasitize nematodes as well as insects and several fungi. Proteases play critical roles during infection and are considered important virulence factors in these fungi. In this study, a cuticle-degrading serine protease gene (*PrC*) was cloned for the first time from *C. rosea*. The gene contained three introns and four exons and encodes a polypeptide of 386 amino acid residues. The mature protein is 277 amino acid residues long and contains a conserved motif shared by peptidase S8 family members. Its N-terminal amino acid residues showed a high degree of sequence similarity with serine proteases from nematophagous and entomopathogenic fungi. Based on the PrC amino acid sequence, the three-dimensional structure has been predicted and compared with that of protease K. Our results provide a basis for further understanding the molecular mechanism of *C. rosea* infection of nematodes. Such knowledge could be explored for improving by genetic engineering the effectiveness of the use of fungal infections to control parasitic nematodes.

Keywords *Clonostachys rosea* · Cuticle-degrading protease · Gene cloning · Homology modeling · Sequence analysis

Lianming Liang and Jinkui Yang contributed equally to this work.

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Introduction

Plant-parasitic nematodes cause serious damages to crops, worth more than US $ 100 billion per year globally (Sasser and Freekman 1987). Methods for nematode control involve nematicides, crop rotation and biological agents. As the environment has been polluted by chemical pesticides, biological control methods using nematophagous fungi, the natural enemy of nematodes, are attracting increasing attention (Siddiqui and Mahmood 1996; Nordbring-Hertz et al. 2000).

The nematode cuticle is a thin and flexible exoskeleton, composed primarily of proteins, including collagens (Cox et al. 1981; Maizels et al. 1993). At the early stage of nematode infection by nematophagous fungi, penetration of the nematode cuticle through combined mechanical activity and hydrolytic enzymes has been proposed to be crucial (Huang et al. 2004). Extracellular serine proteases secreted by fungi can degrade the nematode cuticle and help the penetration process. During the past several years, several cuticle-degrading proteases have been purified and characterized from different nematophagous fungi, including *Arthrobotrys oligospora* (Tunlid et al. 1994; Zhao et al. 2004), *Metacordyceps chlamydosporia* (syn. *Verticillium chlamydosporium*) (Segers et al. 1994), and *Lecanicillium psalliotae* (St Leger et al. 1987).

Clonostachys rosea (syn. *Gliocladium roseum*) belongs to the fungal family Bionectriaceae (Blaxter et al. 1992). It colonizes living plants as an endophyte, digests materials in the soil as a saprophyte, and is also known to be a parasite of other fungi and nematodes (Toledo et al. 2006). It produces a wide range of volatile organic compounds that are toxic to other microbes (Stinson et al. 2003). As a result, it is of great interest as a biological control agent. *C. rosea* infects nematodes via conidia that are capable of

attaching to nematode cuticle, and producing germ tubes that penetrate the host cuticle and kill the host (Zhang et al. 2008). In our previous reports, a protease called PrC was isolated from *C. rosea*, and its biochemical properties were characterized (Li et al. 2006). PrC was found to be highly sensitive to phenylmethanesulfonyl fluoride (PMSF) and could degrade a broad range of substrates including casein, gelatin and nematode cuticle (Li et al. 2006). However, its encoding DNA sequence information was unknown and the putative protein structure was not investigated. In this study, the gene encoding PrC was cloned and compared to other serine proteases from nematophagous and entomopathogenic fungi. Furthermore, we modeled the three-dimensional (3D) structure of PrC and compared it with that of protease K.

Materials and methods

Strains

The isolate (YMF 1.00611) of *Clonostachys rosea* used in this study was isolated originally from field soil samples in Yunnan Province and has been deposited in the China General Microbiological Culture Collection Center (CGMCC 0806). It is maintained on potato dextrose agar (PDA) medium at 26°C for routine culture.

Escherichia coli DH 5α was used in all DNA manipulations and grown in Luria-Bertani (LB) medium.

Amplification of the nucleotide sequence

The fungus *C. rosea* was cultured in PL-4 liquid medium (Yang et al. 2005a) on a rotary shaker (150 rpm) at 28°C for 6 days. Genomic DNA was extracted from the mycelium using an E.Z.N.A.© Fungal DNA Mini Kit (Omega Bio-Tek, Norcross, GA) according to the manufacturer's protocol.

A pair of degenerate primers (p245 and p248) was designed to amplify the conserved sequence of protease PrC from *C. rosea*, according to the conserved sequences of serine proteases from nematophagous and entomopathogenic fungi (Wang et al. 2006). The genomic DNA was used as template, and PCR conditions followed those described in a previous report (Yang et al. 2005b). After obtaining the conserved sequence of the PrC gene, the 5′ and 3′ unknown sequences of the protease gene were amplified using a DNA walking SpeedUp™ kit (Seegene, Seoul, Korea). Two groups of three primers were designed according to the conserved sequence of *PrC*: primers 5sp1, 5sp2, and 5sp3 were used to amplify the sequence upstream of the 5′ region and primers 3sp1, 3sp2, and 3sp3 for the sequence downstream of the 3′ region. The PCR amplifications were carried out following the manual of the commercial kit. The primers are listed in Table 1.

Cloning and sequencing

The PCR products were purified from a 1% agarose gel using DNA fragment purification kit ver. 2.0 (Takara, Shiga, Japan) and subcloned into pMD18-T Vector (Takara). The plasmid DNA was sequenced using an ABI 3730 automated DNA sequencer (Perkin-Elmer, Fremont, CA) with four fluorescent dyes. Raw sequences were assembled using seqman of the DNAStar (Lasergene, Madison, WI) software package.

Sequence analysis

Database searches were performed using BlastX (http://www.ncbi.nlm.nih.gov/BLAST). Signal sequence prediction was performed using Signal P (http://www.cbs.dtu.dk/services/signalP). Potential post-translational modification sites of the protease were identified through comparisons with the database PROSITE (http://www.expasy.ch/prosite/). Fifteen reported cuticle-degrading protease sequences were aligned with PrC using ClustalX2.0. A phylogenetic tree was constructed by the neighbor joining (NJ) method using the MEGA program 4.1 (Tamura et al. 2007). Bootstrap analysis with 1,000 replicates was used to estimate the relative support for clades produced by the NJ analysis.

Homology modeling

The sequence of mature PrC was used in homology modeling. Homology modeling was performed using the SWISS-PROT program (http://swissmodel.expasy.org//SWISS-MODEL.html; (Guex and Peitsch 1997; Schwede et al. 2003; Arnold et al. 2006) with the 2.5 Å proteinase K coordinate (1pfgA) (Saxena et al. 1996) as a template. Pymol Ver. 0.99 (DeLano 2002) was used in the visualization and analysis of the structure. All residues but the last three were used in the modeling.

Table 1 Primers used for PCR amplification

Primer	Sequences (5′-3′)
p245	AARTAYATYGTCAAGYWSAAG
p248	AYRTGDGGRSWRGCCATSGA
5sp1	GCCACCCTCGTACTCAAAGTC
5sp2	GTCGTCGTAGGTGTAGGTGGTG
5sp3	ATGGACGAGATGGTGGAGGA
3sp1	CCAACGCTGCCAACTACTC
3sp2	TACTCCAACTACGGCAGCA
3sp3	GTCCTCTCCGCCTACAACAA

Results and discussion

Cloning of the cuticle-degrading serine protease PrC

A 1,074-bp PCR product was successfully amplified using primers p245 and p248. In silico analysis indicated that the sequence does not contain the full open reading frame (ORF) of the protease when aligned with other serine protease. Consequently, DNA walking was performed based on the sequence obtained. A 710 bp fragment from the 5′ upstream region and a 784 bp fragment from the 3′ downstream region of the conserved sequence were amplified. Finally, the three fragments were assembled into a sequence of 2,568 bp containing the complete ORF of the PrC gene. The full length nucleotide sequence of *PrC* has been submitted to GenBank, under accession number GQ149467.

Sequence analysis

The sequence of the gene *PrC* comprises three introns and four exons (Supplementary Fig. 1). The cDNA sequence (1,161 bp) encodes a polypeptide of 386 amino acid residues, including a conserved signature motif of the peptidase S8 family. Alignment with other fungal protease sequences revealed that PrC is a typical secreted fungal serine protease, with a pre-pro sequence. The whole polypeptide of PrC contains a signal peptide of 15 amino acid residues and a pro-peptide of 94 amino acid residues, indicating that this protease is a secreted protease, similar to several other reported serine proteases from nematophagous fungi. The mature protease contains 277 amino acids, with a predicted molecular weight of 27 kDa, and p*I* of 6.82. The molecular weight of the purified PrC is 33 kDa (Li et al. 2006)—higher than the predicted value. The molecular weight difference suggests that PrC is likely modified after translation. The first ten amino acids of PrC are ATQTGAPWGI, differing by two amino acids from the N-terminal sequence of a previously obtained protease PrC (ATQSNAPWGL) (Zhao et al. 2004; Li et al. 2006). This indicates genetic polymorphisms at the N-terminal end of PrC and that more than one PrC gene likely exists in the genome. The PCR conditions used amplified only one PrC gene. This is consistent with findings with the proteases from another nematode-trapping fungus, *A. oligospora*. Disruption of the gene *PII* in *A. oligospora* has only a limited effect on the pathogenicity of *A. oligospora*. However, mutants containing additional copies of the *PII* gene developed more infection structures and had a greater speed of capturing and killing nematodes than wild type strains (Åhman et al. 2002). These observations suggest that duplications of protease genes contributing to fungal infection might be common in fungal pathogens.

When scanning the sequence of mature PrC in the prosite database, the three active site motifs of serine proteases were found at positions 35–46 (AFIIDTGIytsH, subtilase_asp), 70–80 (HGThVAGtVGG, subtilase_his) and 223–233 (GTSmAsPhVAG, subtilase_ser). Asp39, His69 and Ser225 make up the catalytic triad of PrC. An asn glycosylation motif at 131–134 (NMSL) and an alkaline phosphatase active site motif at 96–104 (VlDSSGSGT) were also found in the sequence of PrC.

Comparison of PrC with other serine proteases isolated from nematophagous and entomopathogenic fungi

PrC showed high amino acid sequence identities (41–52%) with cuticle-degrading proteases from nematophagous and

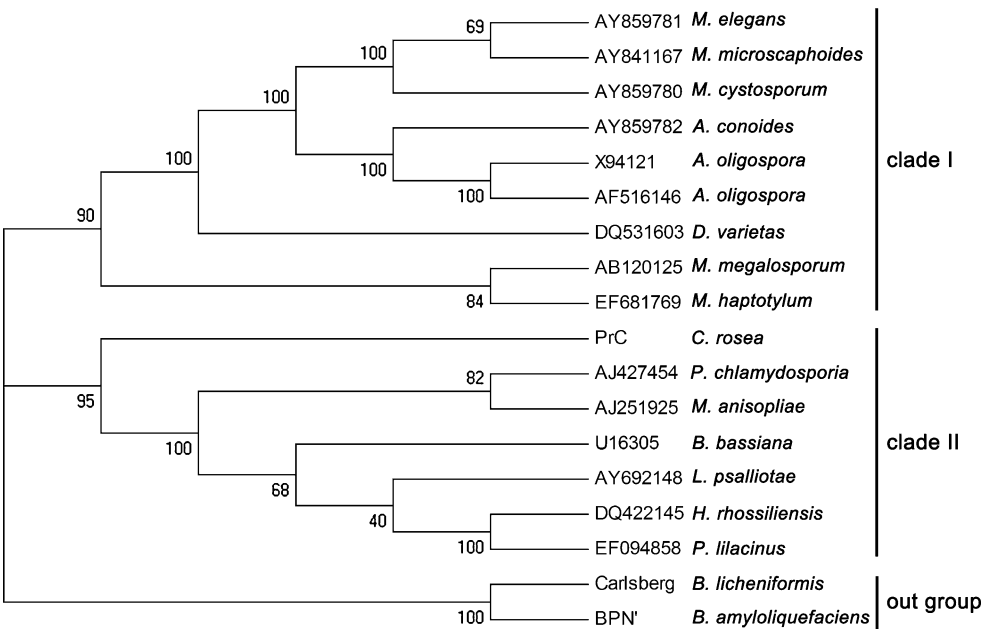

Fig. 1 Phylogenetic tree showing the relationship between a cuticle-degrading serine protease (PrC) and other cuticle-degrading proteases from nematophagous fungi. The two bacterial serine proteases, subtilisin BPN' from *Bacillus amyloliquefaciens* and subtilisin Carlsberg from *Bacillus licheniformis*, were used as outgroup taxa. Confidence values were assessed from 1,000 bootstrap replicates of the original sequence data

entomopathogenic fungi (Supplementary Fig. 2). The alignment suggests that PrC is a typical serine protease. To standardize the description, all sequences in the alignment are numbered according to the reference sequence AB120125, with gaps in AB120125 numbered +1, +2 etc. The analysis shows that the catalytic triads (Asp187, His224 and Ser379), as well as the S1 (residues 283–286, 309–313, 377–381) and S4 (residues 255–259, 262, 283–287, 292) substrate-binding sites are highly conserved.

Based on the alignment of only the mature proteases from nematophagous and entomopathogenic fungi, a phylogenetic tree (Fig. 1) was constructed using Mega 4.1 (Tamura et al. 2007). Two bacterial serine proteases, subtilisin BPN' from *Bacillus amyloliquefaciens* and subtilisin Carlsberg from *B. licheniformis*, were used as outgroup. The 16 proteases were clustered into two clades. Clade I was composed of nine proteases from nematode-trapping fungi, and clade II was composed of six proteases from parasitic fungi. As expected, the aligned sequences revealed that the two clusters of proteases were more conserved within each clade than between the two clades. In clade II, all the signal peptides were 15 amino acids long, and the signal peptides in clade I had 2–6 additional amino acids. Some conserved indels between the two clades were found in both the pro-peptide region and in the mature proteins. One common clade I insertion was at position 274 (+1)–274 (+4). Deletions were observed at positions 57–80, and 200–204. Two disulfide bridges (182–274 [+4] and 329–410) were conserved within clade II (except for U16305 for the second disulfide bridge), but no cysteine existed in the corresponding position in clade I. A conserved cysteine was also found in clade I at position 365, which was identified as free cysteine and was conserved in all the proteases except PrC. This is an unusual difference between PrC and other cuticle proteases. Another obvious difference between PrC and other cuticle proteases was an insertion at 103 (+1)–103 (+7) in the propeptide region, although the amino acids near the insertion (99–105) were quite different (Supplementary Fig. 2). This result is very similar to our previous report (Yang et al. 2007). Because these two groups of fungi have different mechanisms of killing nematodes, these proteases likely represent different evolutionary adaptation to facilitate fungal virulence against nematodes.

Three-dimensional structure of PrC

The 3D structure of PrC was predicted by homology modeling using the 3D structure of proteinase K as a template sharing 56% sequence identity. The structure of PrC showed the typical folding of a subtilisin-like serine protease (Fig. 2). It is composed of seven alpha helices, a

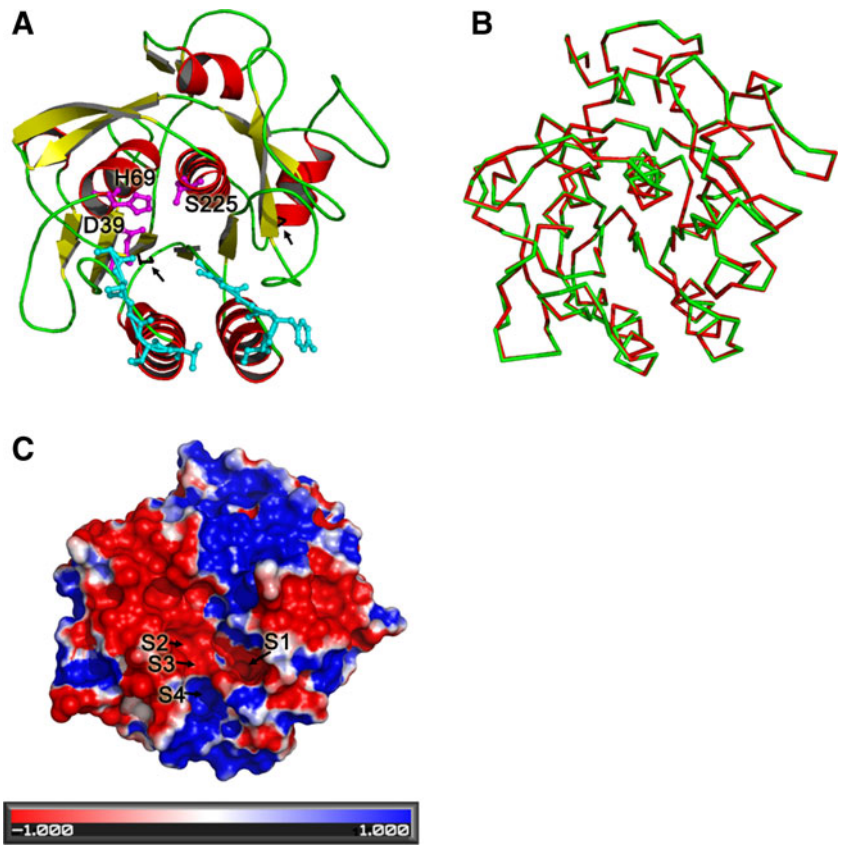

Fig. 2 **a**–**c** The predicted structure of PrC. **a** Ribbon representation of the PrC structure. The catalytic triad amino acids, D39–H69–S225, are represented as *magenta sticks*, and residues 101–105 and 135–139 involved in substrate binding site are represented as *cyan sticks*. The two disulfide bridges are shown as *black lines* indicated by *black arrows*. **b** Superimposition of the two proteases, PrC (*green*) and proteinase K (*red*). **c** The surface contour based on electrostatic potential. The four substrate binding pockets are shown

seven-strand parallel beta sheet, and two two-strand antiparallel beta sheets. The substrate binding site of PrC includes two peptide segments containing residues 101–105 and 135–139, respectively. The catalytic triad of PrC is composed of residues Asp39, His69 and Ser225. There are two disulfide bridges formed by residues Cys34–Cys124 and Cys179–Cys250 (Fig. 2a). Figure 2b shows the structural superimposition of PrC and proteinase K. Their overall folds are very similar to each other, the root mean square deviation (RMSD) of the two proteases is 0.23 Å when computed using the backbone atoms. The similarities in the overall 3D coordinate, sequence, and structural identity of the catalytic triad between PrC and proteinase K suggest that they have similar catalytic mechanisms. The polar residues located at the surface of PrC render the protease polarized overall. Meanwhile, the catalytic center of PrC is negatively charged (Fig. 2c). It has been observed that the anionic character can increase flexibility of an enzyme (Pasternak et al. 1999), and in particular increase the flexibility around the active site region (Kumar and Nussinov 2004). Large parts of PrC surface are positively charged, which is similar to VCP1 and Pr1, which were modeled recently (Liu et al. 2007), suggesting that the polarized surface may enhance the attraction between the protease and nematodes.

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