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Short communication

Purification and characterization of an extracellular serine protease from *Clonostachys rosea* and its potential as a pathogenic factor

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Abstract

An extracellular protease (PrC) was purified from an isolate of *Clonostachys rosea* (syn. *Gliocladium roseum*) to apparent homogeneity. The protease had a molecular mass of 33 kDa estimated by SDS-PAGE. The optimum activity of PrC was at pH 9–10 and 60 °C (over 10 min). The purified protease could degrade a broad range of substrates including casein, gelatin and nematode cuticle. 80 ± 5% of nematodes (*Panagrellus redivivus*) were immobilized and degraded after treating with PrC for 48 h. The protease was highly sensitive to PMSF (phenylmethyl sulfony fluoride) (5 mM) indicating it belonged to the serine protease family. The N-terminal amino acid residues of PrC are ATQSNAPWGL, which share a high degree of similarity with other cuticle-degrading proteases from nematophagous and entomopathogenic fungi suggesting PrC play a role in infection process.

Keywords: *Clonostachys rosea*; Serine protease; Biochemical characterization; Nematicidal activity

1. Introduction

Nematophagous fungi play important roles in reducing nematode populations by their antagonistic behaviour. Some of them have shown great potential as biocontrol agents. The development of biocontrol agents to control nematodes is of major importance because the traditional methods based on the use of nematicides and antihelminthic drugs are no longer effective and cause major environment and health concerns [1].

The nematode cuticle is a thin and flexible exoskeleton, composed primarily of proteins, including collagens [2,3]. It can therefore be assumed that the activity of proteases is important for the infection of nematodes by nematophagous fungi. Several extracellular serine proteases have been detected and partly characterized from *Arthrobotrys oligospora*, *Pochonia suchlasporia* (syn. *Verticillium suchlasporium*), *Pochonia chlamydosporia* (syn. *Verticillium chlamydosporium*), *Paecilomyces lilacinus* and *Lecanicillium psalliotae* (syn. *Verticillium psalliotae*). These studies suggested the extracellular serine protease is a key enzyme and virulence factor involved in the fungi penetration to nematodes [4–10].

The fungus *Clonostachys rosea* (syn. *Gliocladium roseum*) is a common saprophyte in the soil worldwide [11]. Morandi et al. [12] found that *C. rosea* could suppress sporulation of *Botrytis cinerea* and be used for control of botrytis blight. Dong et al. [13] reported that some toxin isolated from filtrate of *C. rosea* showed strong nematicidal activities against *Caenorhabditis elegans*, *Panagrellus redivivus* and *Bursaphelenchus xylophilus*. Zhao et al. [14] purified an extracellular protease (Lmz1) and found it involved in fungal pathogenicity. In this report, we described the purification, biochemical characterization, nematicidal activity and N-terminal amino acid analysis of an extracellular serine protease (PrC) from endoparasitic fungus *C. rosea*.

2. Materials and methods

2.1. Organisms and culture conditions

The isolate of nematophagous fungus *C. rosea* used in this study was originally isolated from field soil samples in Yunnan Province and had been deposited in China General Microbiological Culture Collection Center

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(CGMCC 0806). It was incubated on PDA (potato dextrose agar) medium. The protease production medium contains: 2 g l^{-1} glucose, 2 g l^{-1} gelatin, 2 g l^{-1} peptone, 1 g l^{-1} yeast extract, 1 g l^{-1} $(NH_4)_2SO_4$, 0.01 g l^{-1} $FeSO_4$, 0.5 g l^{-1}·$MgSO_4$, 2 g l^{-1} KH_2PO_4, 0.1 g l^{-1}·$CaCl_2$, and *Panagrellus redivivus* ($2–3 \times 10^5$ nematodes l^{-1}), pH 7.0–7.5 adjusted with 1 M NaOH. The culture was carried out in 250 ml shaking flask containing 60 ml medium at 26 °C and 200 rpm for 7 days on a rotary shaker.

The saprophytic nematode *P. redivivus* was grown in an oatmeal medium at 26 °C. *P. redivivus* was washed thoroughly with 50 mM sodium phosphate buffer (pH 7.0) before being used in the assays.

2.2. Infection and nematicidal activity analysis

C. rosea was incubated on PDA at 26 °C for 5–6 days, then a block of 2 cm^2 medium in the center of plate was removed and 20–30 nematodes were added to the empty block [15]. The fungus then grew into the space and the infection process was observed after incubating at 26 °C for another 2–5 days.

The effect of protease on nematode was investigated according to the method of Yang et al. [10].

2.3. Protein concentration and protease activity analysis

Protein concentration was determined by the method of Bradford [16] using BSA (bovine serum albumin) as a standard.

Protease activity was assayed by a modified caseinolytic method [17]. One unit (U) of protease activity was defined as the amount of enzyme that hydrolyzed the substrate and produced 1 μg tyrosine in 1 min under the assay conditions.

2.4. Purification of the protease

Three L filtrate broth supernatant containing the extracellular enzyme was subjected to ultrafiltration in a CENTRAMATE™ and CENTRAMATE™ PE tangential-flow systems (PALL FILTRON, USA). The molecular weight cutoff of the used CENTRAMATE™ cassette was 100 and 5 K, respectively. The enzyme was precipitated by adding ammonium sulfate slowly to 90% saturation after the culture filtrate was concentrated by ultrafiltration (300 ml). The saturated solution was left overnight at 4 °C, centrifuged (10,000 rpm, 15 min) and the precipitate was further dissolved in minimum amount of 20 mM Tris–HCl (pH 9.0), containing 1 M $(NH_4)_2SO_4$, and insoluble materials were removed by centrifugation. The crude enzyme preparation was loaded on a HiLoad 16/10 Phenyl Sepharose column (Amersham, Sweden), connected to a AKTA Explorer100 system (Amersham, Sweden), equilibrated with 20 mM Tris–HCl (pH 9.0), containing 1M $(NH_4)_2SO_4$. The column was eluted with a linear salt gradient (1–0 M in 20 mM Tris–HCl buffer, pH 9.0). Fractions containing protease activity from the HiLoad 16/10 Phenyl Sepharose column were pooled and loaded on a SOURCE 15Q 4.6/100 PE column (Amersham, Sweden), equilibrated with 20 mM Tris–HCl (pH 9.0). The protease was eluted out from the column by using a continuous NaCl gradient (0–0.5 M). Fractions of 3 ml were collected at a flow rate of 1 ml min^{-1}. All fractions with absorbance at 280 nm were assayed for protease activity using a caseinolytic method [17]. The active protease fractions were pooled, desalted and stored in aliquots at −20 °C until needed.

2.5. Characterization of the purified enzyme

2.5.1. Molecular weight and isoelectric point determination

SDS–PAGE was performed according to the method of Laemmli [18] under reducing conditions on a 14% polyacrylamide gel.

Isoelectric focusing was performed with Bio-Rad Mini-Protean II Gel System using carrier ampholyte from Amersham Biosciences (pH range from 5.2 to 10.3) at a constant of 3000 V for 1.5 h [19].

2.5.2. Effect of temperature and pH on enzyme activity

Optimal temperature was determined by performing a standard activity assay in a temperature range from 25 to 80 °C. In order to determine optimal pH, the enzymic assay was carried out at different pH values, at 60 °C. Buffer used as follows: 0.1 M sodium acetate (pH 3.0–5.5); 0.1 M sodium phosphate (pH 6.0–7.5); 0.1 M Tris–HCl (pH 8.0–9.0); 0.1 M glycine–NaOH (pH 9.5–11.0); and 0.1 M sodium carbonate (pH 11.5–12.0).

2.5.3. Effect of metal ions on purified enzyme activity

Purified protease was pre-incubated for 1 h with different metal ions including Ca^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, Hg^{2+}, at 5 mM final concentration, Protease activity was measured as described above.

2.5.4. Effect of oxidizing agents, detergents and inhibitors on purified enzyme activity

To investigate the effect of oxidizing agents, detergents and protease inhibitors on enzyme activity, H_2O_2, Triton X-100, SDS, Tween-20, PMSF and EDTA was added to the enzyme, respectively, and the mixture was allowed to stand at 26 °C for 1 h. Protease activity was measured as described above.

2.5.5. Hydrolysis of protein substrates and nematode cuticle assay

The purified protease was incubated with substrates (casein, skimmed milk, BSA, gelatin, collagen and nematode cuticle) at pH 9 and 60 °C for 10 min, and protease activity was quantitatively assayed. Nematode cuticle was extracted according to the method of Cox et al. [2].

2.6. N-terminal amino acid sequence analysis

The N-terminal amino acid sequence of the purified protease sample was determined on an ABI Procise 491 protein sequencer (Applied Biosystems, USA).

3. Results

3.1. The natural infection by nematophagous fungus C. rosea

C. rosea was grown on PDA solid medium at room temperature (23–25 °C). The morphological characters were similar to the *C. rosea* described by Schroers et al. [11]. The strain showed a strong ability to immobilize, infect and degrade the nematodes (Fig. 1), and over 80% nematodes were penetrated by the mycelia after 48 h.

3.2. Protease production

C. rosea was inocubated at 26 °C in the protease production medium under shaking (200 rpm) for 15 days. Protease activity was assayed every 24 h after inoculation. Protease activity was steadily increased, and maximum protease activity (30 U ml^{-1}) was found at 7th day after inoculation and then slowly decreased.

3.3. Protease purification

Purification factors and recoveries at each step were summarized in Table 1. The protease activity of crude enzyme extract was concentrated by ultrafiltration and ammonium sulfate precipitation. About 62% protease recovery was achieved with 2.48-fold purification (Table 1). Following ammonium sulfate precipitation the crude enzyme solution was applied to a column of a HiLoad 16/10 Phenyl Sepharose FF column. Most protease activity was observed in the peak of the eluted fractions with 7.94-fold purification. The active fraction from the previous step was applied to the anion exchange column (SOURCE 15Q 4.6/100 PE column) and resulted in

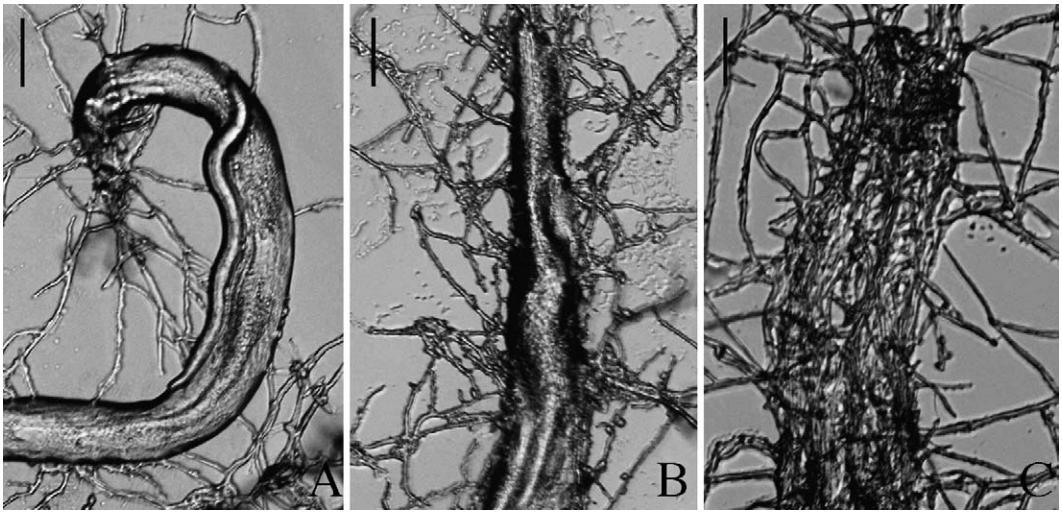

Fig. 1. Nematode (*Panagrellus redivivus*) was infected by *Clonostachys rosea*. (A) Mycelia and nematode. (B) Mycelia penetrating through the cuticle of nematode. (C) Degrading nematode. Scale bar: 50 μm.

Table 1
Purification of the extracellular protease (PrC) from *C. rosea*

Purification procedure	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification factor	Recovery (%)
Culture filtrate	707.6	12029	17	1.0	100
$(NH_4)_2SO_4$ precipitation	176.9	7465	42.2	2.5	62
HiPrep Phenyl FF	48.3	6520	135	7.9	54.2
SOURCE 15Q	6.7	1615	241	14.2	13.4

13.4% protease recovery (14.2-fold purification) with a specific activity of 241 U mg^{-1} of protein (Table 1).

3.4. Characterization of purified PrC

3.4.1. Molecular weight and pI determination

The purified enzyme showed a single protein band on the 14% Coomassie Brilliant Blue R-250 stained gel, and the molecular weight of the purified PrC was estimated to be 33 kDa by SDS-PAGE (Fig. 2). The isoelectric point of the pure PrC from *C. rosea* was above 10.0 (data not shown).

3.4.2. Effect of pH and temperature on enzyme activity

The optimum pH of the enzyme was determined to be 9.0–10.0 (Fig. 3). The enzyme was stable at pH between 4 and 10 and more than 60% activity was retained after incubation for 1 h at 26 °C.

Temperature optimization studies at pH 9, showed that 60 °C was the optimum temperature (Fig. 4). The study on thermal stability of the purified enzyme showed the enzyme activity was stable at 20–40 °C for 30 min incubation, but inactivated at temperatures over 70 °C after 30 min incubation.

3.4.3. Effect of metal ions on enzyme activity

The purified protease showed it did not require Ca^{2+} for activity. Ca^{2+}, Mg^{2+}, Fe^{2+} had almost no effect on protease activity. Zn^{2+}, Cu^{2+}, Hg^{2+} inhibited the enzyme activity (>60%). Mn^{2+} enhanced enzyme activity by 38%.

3.4.4. Effect of oxidizing agents, detergents and inhibitors on enzyme activity

The protease activity was stimulated up to 21% in the presence of H_2O_2. Various surfactants, such as Tween-20, also increased protease activity up to 16%, but SDS and Triton X-100 inhibited protease activity up to 45.6% and 36.5%,

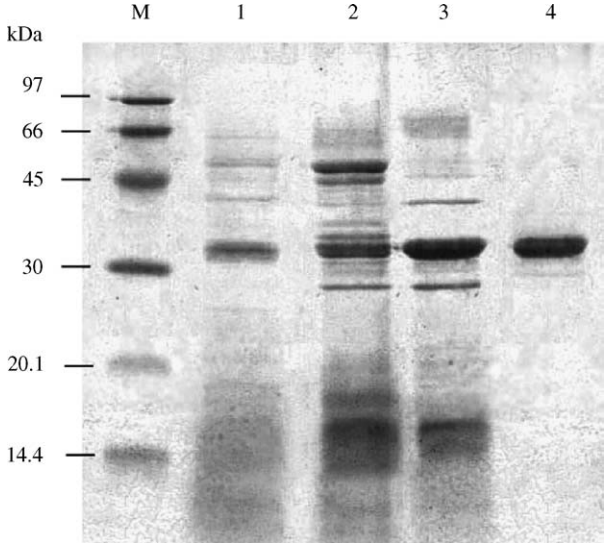

Fig. 2. 14% SDS-PAGE of protease from *C. rosea*. Lane M, standard protein makers of different molecular weights; Lane 1, ultrafitration protease; Lane 2, ammonium sulfate precipitated and dialyzed protease; Lane 3, HiPrep Phenyl FF purified protease; Lane 4, SOURCE 15Q purified protease.

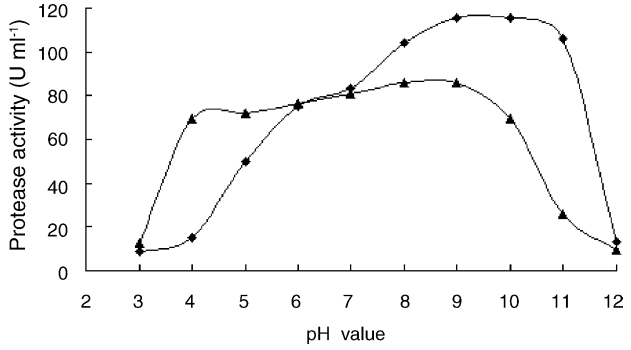

Fig. 3. Effect of pH on protease activity: (◆) optimum pH; (▲) pH stability. The value is the average of three replication.

respectively. The activity of purified enzyme was completely inhibited in the presence of 5 mM PMSF, indicating that the protease is a serine protease. In contrast, almost no inhibition was observed with 5 mM EDTA.

3.4.5. Hydrolysis of protein substrates and nematode cuticle

The protease showed high hydrolytic activity against skimmed milk and casein, moderate hydrolysis of BSA, gelatin and nematode cuticle. The activity against native collagen was very low.

3.5. Nematicidal analysis

The results of Table 2 showed the crude enzyme and purified protease could immobilize the nematode *P. redivivus*. Majority of nematodes (70–100%) were immobilized after being treated with crude enzyme and purified protease for 48 h, and crude enzyme had stronger nematicidal activity than purified protease.

3.6. N-terminal amino acid sequence analysis

The N-terminal sequence of first 10 amino acid residues of the purified protease was ATQSNAPWGL, which had a high degree homology (32–80%) with other serine proteases from nematophagous and entomopathogenic fungi *Verticillium lecanii*, *Trichoderma harizianum*, *Metarhizium anisopliae*, *A. oligospora*, *P. chlamydosporia*, *L. psalliotae* and *P. lilacinus*

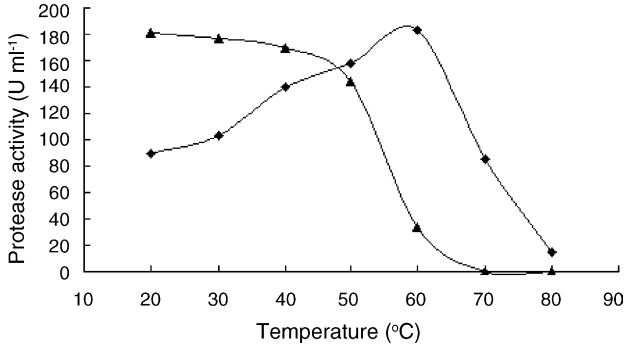

Fig. 4. Effect of temperature on protease activity: (◆) optimum temperature; (▲) temperature stability. The value is the average of three replication.

Table 2
Mortality of the nematodes *Panagrellus redivivus* by protease extracts from *C. rosea*

Samples	Protease activity (U ml^{-1})	*P. redivivus* mortality (%)[a]			
		12 h	24 h	36 h	48 h
Crude enzyme	149.3	40 ± 5	75 ± 5	90 ± 5	100
Boiled crude enzyme[b]	0	5 ± 2	10 ± 2	20 ± 5	20 ± 5
PrC	161.5	25 ± 5	50 ± 5	60 ± 5	80 ± 5
Boiled PrC[b]	0	5 ± 2	5 ± 2	10 ± 5	10 ± 5

[a] The proportion of dead nematodes to total nematodes.
[b] Protease heated at 100 °C for 10 min.

(Fig. 5). Moreover, the N-terminal amino acid residues of PrC shared 60% homology with Lmz1.

4. Discussion

Serine proteases are widely distributed among fungi and almost exclusively extracellular enzymes. There are growing evidences showing that these proteases are important determinants of pathogenicity [4–10]. The biochemical characterization of PrC is very similar to VCP1, P32, pSP3 and Ver112, which were isolated from egg-parasitic or endoparasitic fungi *P. chlamydosporia*, *P. suchlasporia* and *P. lilacinus* and *L. psalliotae*, respectively. They all had similar molecular mass (32–33 kDa) [7,8,10,20], and higher p*I* value (10.2). However, another two serine proteases PII and Aoz1, isolated from nematode-trapping fungus *A. oligospora*, had lower p*I* value (4.6 and 4.9) and higher molecular mass (35 and 38 kDa) [5,9], suggesting that the biochemical characterizations of proteases from nematode-trapping and egg-parasitic or endoparasitic fungi are different.PrC and Lmz1 were isolated from the same fungus (*C. rosea*) under different growing condition, they shared same molecular weight (33 kDa) and optimum reaction temperature (60 °C), but they shared different p*I* and optimum reaction pH.

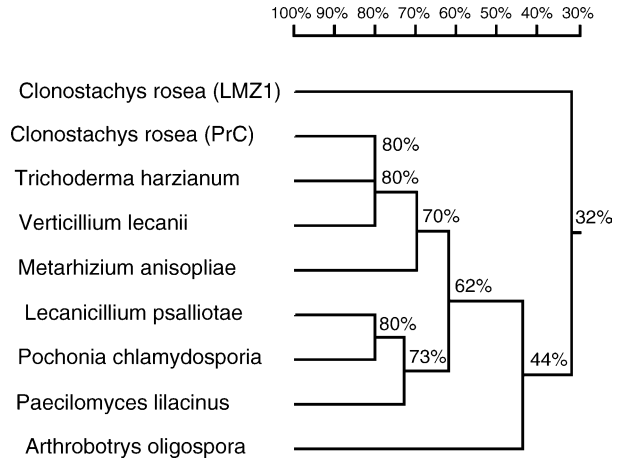

Fig. 5. Alignment of N-terminal amino acid sequences from proteases Lmz1, PrC, PrB, LePR, PrA, Ver112, VCP1, PIP, PII and Aoz1. They were isolated from *C. rosea* (Lmz1 and PrC), *Trichoderma harizianum*, *Verticillium lecanii*, *Metarhizium anisopliae*, *Lecanicillium psalliotae*, *Pochonia chlamydosporia*, *Paecilomyces lilacinus* and *Arthrobotrys oligospora* (PII and Aozl), respectively. The N-terminal amino acid residues of PII are same as Aozl.

Furthermore, the N-terminal amino acid residues of PrC shared higher homology with other serine proteases from nematophagous and entomopathogenic fungi than Lmz1 (Fig. 5).

Like other serine proteases, purified PrC can also degrade various protein substrates. Moreover, PrC have more proteolytic activity than PII (relative activity of casein, BSA, gelatin, collagen and nematode cuticle is 100%, 5.99%, 10.5%, 0.42% and 9.84%, respectively) [5]. The protease was stable in the presence of some oxidizing agents (H_2O_2), detergents and surfactants (Tween-20, SDS and Triton X-100). These properties implied the protease can be useful in the industry applications, such as detergents.

The N-terminal amino acid residues analysis also showed that these proteases shared a high degree of similarity (Fig. 5), which also suggested that these serine proteases might play the same role in infection process of nematode. The purified PrC exhibited obvious nematicidal activity (Table 2). However the crude enzyme could degrade the nematode more effectively than purified protease (Table 2), suggesting that other enzymes including Lmz1 and factors involve in the infection process. Therefore, further studies are necessary to ascertain the role of extracellular enzymes and other factors in infection of nematode.

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