Biotechnology Letters (2005) 27: 1123–1128
DOI 10.1007/s10529-005-8461-0

Isolation and characterization of a serine protease from the nematophagous fungus, *Lecanicillium psalliotae*, displaying nematicidal activity

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Received 23 March 2005; Revisions requested 14 April 2005; Revisions received 24 May 2005; Accepted 27 May 2005

Key words: Enzyme purification, *Lecanicillium psalliotae*, nematicidal activity, serine protease

Abstract

Lecanicillium psalliotae produced an extracellular protease (Ver112) which was purified to apparent homogeneity giving a single band on SDS-PAGE with a molecular mass of 32 kDa. The optimum activity of Ver112 was at pH 10 and 70 °C (over 5 min). The purified protease degraded a broad range of substrates including casein, gelatin, and nematode cuticle with 81% of a nematode (*Panagrellus redivivus*) being degraded after treating with Ver112 for 12 h. The protease was highly sensitive to PMSF (1 mM) indicating it to be a serine protease. The *N*-terminal amino acid residues of Ver112 shared a high degree of similarity with other cuticle-degrading proteases from nematophagous fungi which suggests a role in nematode infection.

Introduction

Verticillium comprises a heterogeneous group of asexual fungi and can be accommodated in the genera *Lecanicillium*, *Pochonia*, *Haptocillium*, and *Rotiferophthora* (Zare *et al.* 2000, Gams & Zare 2001), many of which are of considerable importance in agriculture as pathogens of insects and nematodes, and play an important role in biological control of insects and nematodes.

Serine proteases, chitinases and collagenases are virulence determinants of nematophagous and entomophagous fungi (Huang *et al.* 2004). Lopez-Llorca & Robertson (1992) isolated a serine protease P32 from *Pochonia suchlasporium* (syn. *Verticillium suchlasporium*) and found it was involved in penetration of nematode eggs. Segers *et al.* (1994) isolated a similar protease (VCP1) from *Pochonia chlamydosporium*. Tunlid *et al.* (1994) also isolated a serine protease (PII) from nematophagous fungus *Arthrobotrys oligospora*. Later, Åhman *et al.* (1996, 2002) cloned the gene of the PII, and constructed mutants containing additional copies of the *PII* gene. Moreover, collagenase was isolated from nematode-trapping fungi *Arthrobotrys amerospora* (Schenck *et al.* 1980), and chitinase from nematophagous fungi *P. chlamydosporium* and *P. suchlasporium* (Tikhonov *et al.* 2002).

Lecanicillium psalliotae (syn. *Verticillium psalliotae*) is an endoparasitic fungus, but little is known about its extracellular enzymes and other factors involved with infection. In this report, we describe the purification, biochemical characterization, nematicidal activity and *N*-terminal amino acid analysis of a cuticle-degrading serine protease, Ver112, from *L. psalliotae*.

Materials and methods

Organisms and growth conditions

Lecanicillium psalliotae was originally isolated from field soil in Yunnan Province and had been deposited in China General Microbiological Culture Collection Center (CGMCC1312). It was

grown in a medium containing (per litre): 1 g glucose, 1 g gelatin, 1 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4.7H_2O$, 2 g KH_2PO_4, and 0.001 g $FeSO_4$ $7H_2O$. The culture was carried out in 250 ml flask containing 60 ml medium at 26 °C and shaken at 200 rpm for 6 d on a rotary shaker.

The saprophytic nematode, *Panagrellus redivivus*, was maintained as described (Zhao *et al.* 2004). It was washed thoroughly with 50 mM sodium phosphate buffer (pH 7.0) before being used in the assays.

Infection and nematicidal activity analysis

A. *Lecanicillium psalliotae* was incubated on PDA at 26 °C for 5–6 d, then a block, 2 cm^2, in the center of plate was removed and 20–30 nematodes were added into the empty space. The fungus then grew into that space and the infection process was observed after incubating at 26 °C for another 2–5 d.

B. The effect of the protease on the nematode was investigated by adding approx. 50 nematodes to solutions of purified protease Ver112, boiled Ver112, crude culture filtrate, and 50 mM sodium phosphate buffer (pH 7.0). The mixture was incubated at 26 °C for 12–24 h, and numbers of dead nematodes were counted under a light microscope. The experiment was repeated three times.

Protein concentration determination and isolation of nematode cuticle

Protein concentration was determined by the method of Bradford using BSA as a standard.

Isolation of nematode cuticle was done, according to the method of Cox *et al.* (1981). All isolation steps except those involving SDS were performed on ice.

Protease activity analysis

A semi-quantitative casein-plate method (Zhao *et al.* 2004) was used to detect the protease activity of supernatant liquid and purification fractions. Proteolytic activity was measured using a modified Lowry assay. One unit (U) of protease activity was defined as the amount of enzyme that hydrolyzed the substrate and produced 1 mg tyrosine in 1 min under the assay conditions.

Effects of pH and temperature on enzyme activity

The optimum pH was determined by mixing the purified protease with the Britton Robinson universal buffer system at pH values between 3 and 12, and protease activity was quantitatively assayed. The pH stability was studied by mixing it with the same buffer and incubating at 26 °C for 1 h, then the pH of mixtures were adjusted to pH 10 and the residual activity were measured. The experiment was repeated three times.

The optimum temperature was determined by incubating protease and casein at different temperatures (4, 25, 30, 40, 50, 60, 70, 75 and 80 °C) for 5 min, and protease activity was quantitatively assayed. The temperature stability was determined by incubating the protease with 50 mM phosphate buffer (pH 10.0) for 10 min at various temperatures, and the residual activity was measured. The experiment was repeated three times.

SDS-PAGE and N-*terminal amino acid sequence analysis*

SDS-PAGE was performed with a Mini-PROTEAN III gel system (Bio-Rad), using slab gels, 0.5 mm thick, of 12% (v/v) polyacrylamide, according to the method of Laemmli, and the proteins were stained with Coomassie Blue G-250.

Chromatographic pure fractions of protease Ver112 were subjected to SDS-PAGE and electroblotted onto polyvinylidene fluoride membrane (Millipore). Cylohexylaminopropaneulfonic buffer, pH 11, containing 20% (v/v) methanol was used as electrophoretic buffer in a Mini-PROTEAN III gel system (Bio-Rad). Protein bands identified by Coomassie Blue were excised and subjected to Edman degradation using a Procise 491 Protein Sequencer (PE).

Results

The natural infection by nematophagous fungus, Lecanicillium psalliotae

Lecanicillium psalliotae grew on PDA solid medium at room temperature (23–25 °C) and produced white and compact mycelia and falcate

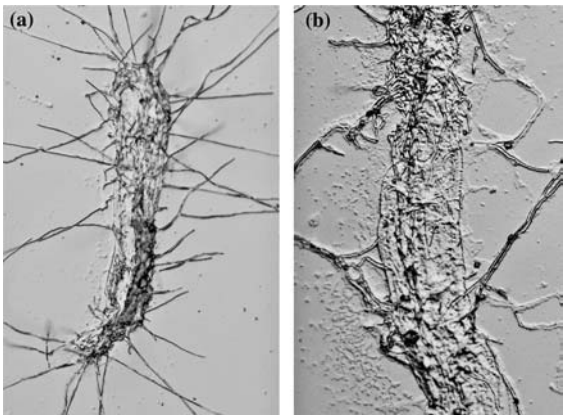

Fig. 1. The natural infection of the nematophagous fungus *L. psalliotae* (a). Mycelia penetrating through the cuticle of nematode. (b). Degrading nematode. Scale bar: 100 μm.

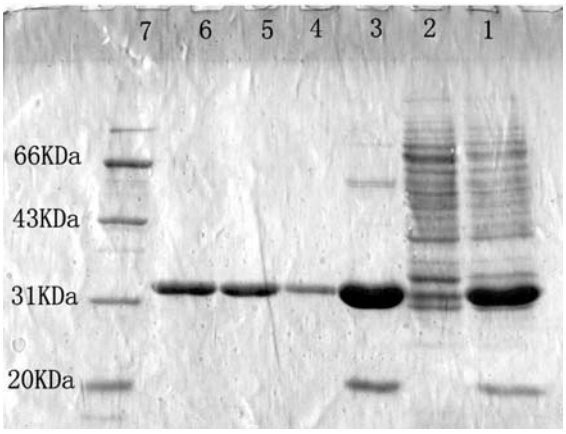

Fig. 2. SDS-PAGE electrophoresis gel Lane 1: Crude extract (ultrafiltration). Lane 2: Fraction flow through from the Hi-Trap SP FF column Lane 3: Fraction Ver112 from the Hi Trap SP FF column Lane 4,5 and 6: Fraction Ver112 from the HiPrep Phenyl FF column. Lane 7: Low molecular weight maker.

conidia. The mycelia of *L. psalliotae* penetrated through the cuticle of nematodes during the infection, and nematodes were degraded completely (Figure 1).

Extracellular protease production and purification

The protease was produced on the second day and reached the highest activity (5.7 U ml^{-1}) on the sixth day at 26 °C. Purification factors and yields at each step are summarized in Table 1. As can be seen in Figure 2, the final preparation migrated as a single band of protein on SDS-PAGE, indicating a homogeneous protein with an apparent molecular mass of 32 kDa.

Effects of the pH and temperature on enzyme activity

The protease was most stable between pH 9 and 10. It was stable below 30 °C but lost activity above 60 °C (over 10 min). The optimum activity of Ver112 was at pH 10 and 70 °C (over 5 min).

Protease inhibitors and hydrolysis of various protein substrates

Proteolytic activity was strongly inhibited by 1 mM PMSF (98% inhibition) (Table 2), indicating that Ver112 belongs to the serine-type

Table 1. Purification of the extracellular protease (Ver112) from *L. psalliotae*.

Purification procedure	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (units mg^{-1})	Recovery (%)	Purification factor
Culture filtrate	500	267	2845	11	100	1
Ultrafiltration	100	132	2094	16	74	1.5
HiTrap SP FF	10	10	400	40	14	3.6
HiPrep Phenyl FF	2	2	95	48	3	4.4

Culture filtrate was collected by vacuum filtration and then protease was concentrated by ultrafiltration (5 kDa cutoff membrane, Milipore). Buffer and sample were filtrated by filtration membrane (0.22 μm, Milipore) before being used. The sample was applied to a HiTrap SP FF column (Amersham) equilibrated with 10 mM sodium phosphate buffer (pH 6.0), the bound proteins were eluted with 10 mM sodium phosphate buffer (pH 6.0) containing 0.5 M NaCl at 1 ml min^{-1}. Fractions containing protease activity from the HiTrap SP FF were pooled and mixed with 3.4 M $(NH_4)_2SO_4$ in a proportion of 3:2 (v/v, sample: buffer). The sample was applied to a Hiprep 16/10 Phenyl FF (high sub) column (Amersham) equilibrated with 50 mM sodium phosphate buffer (pH 7.0) and 1 M ammonium sulfate, then eluted with 50 mM sodium phosphate buffer (pH 7.0) at 2 ml min^{-1}.

Table 2. Effects of various inhibitors on the protease activity of Ver112.

Inhibitor	Concentration	Relative activity (%)
Control	–	100
Leupeptin	0.01 mM	100
	0.1 mM	100
Aprotinin	1 μg ml^{-1}	96
	2 μg ml^{-1}	96
EDTA	1 mM	108
	10 mM	98
Pepstatin A	0.1 μM	104
	1 μM	98
PMSF	0.1 mM	5
	1 mM	2

The purified protease was incubated with the inhibitor at 26 °C for 10 min prior to the addition of substrate casein. Protease activity was assayed using the method described above. The proteolytic activity in control incubated without the inhibitors (corresponding to 100%) was 48 U, for three replicates.

Table 3. Hydrolysis of various protein substrates by the serine protease Ver112, purified from *L. psalliotae*.

Substrate	Relative activity (%)
Casein	100
BSA	29
Gelatin	20
Collagen	14
Nematode cuticle[a]	12

The purified protease was incubated with the substrate at pH 10 and 70 °C for 5 min, and protease activity was quantitatively assayed. The maximum activity corresponding to 100% was 48 U, for three replicates.
[a]Fragments of cuticle prepared from the nematode *P. redivivus*.

peptidase group. Another serine protease inhibitor (Aprotinin), metal chelator (EDTA), aspartic protease inhibitor (Pepstatin A) and Leupeptin only had a weak effect on the protease, with less than 10% inhibition. The purified protease can degrade a broad range of substrates, among them casein was the most easily degraded; BSA, gelatin, collagen and nematode cuticle were more difficult to hydrolyze (Table 3).

Nematicidal analysis

From the results of Table 4, the crude enzyme and purified protease had better nematicidal effects than culture filtrate. The majority of the tested nematodes (81–100%) were dead and degraded after being treated with crude enzyme and purified protease for 12 h, and the mortalities of *P. redivivus* were paralleled to the proteolytic activities of protease.

Table 4. Mortality of the nematodes *P. redivivus* by protease extracts from *L. psalliotae*.

Sample	Protease activity (U ml^{-1})	Mortality[a] (%)
Control[b]	0	6 ± 2
Culture filtrate	6	28 ± 3
Ultrafiltration	21	78 ± 2
HiTrap SP FF	40	100
HiTrap SP FF and PMSF[c]	1	12 ± 4
HiPrep Phenyl FF	32	81 ± 2
HiPrep Phenyl FF (heating)[d]	0	6.5 ± 1
HiPrep Phenyl FF and PMSF[c]	0	10 ± 2

Extracts were incubated with nematodes in microcentrifuge tube (for three replicates) at 26 °C for 12 h. The numbers of dead and total nematodes were counted in a light microscope.
[a]The proportion of dead nematodes to total nematodes.
[b]50 mM sodium phosphate buffer (pH 7.0).
[c]Protease treated with serine protease inhibitor (PMSF).
[d]Protease heated at 100 °C for 10 min.

Nematode cuticle became rough after being treated for 8–10 h (Figure 3-B, C), and was degraded completely after 12 h (Figure 3-D), but the cuticle of control nematode was intact (Figure 3-A).

N-*Terminal amino acid sequence analysis*

The N-terminal amino acid residues of the purified protease Ver112 were AITQQQGAPW, and alignment result (Figure 4) showed that it was similar to other serine proteases from other nematophagous and entomopathogenic fungi.

Discussion

The nematophagous and entomophagous fungi are important for biological control and they secrete several extracellular enzymes. Serine proteases are important during the infection of

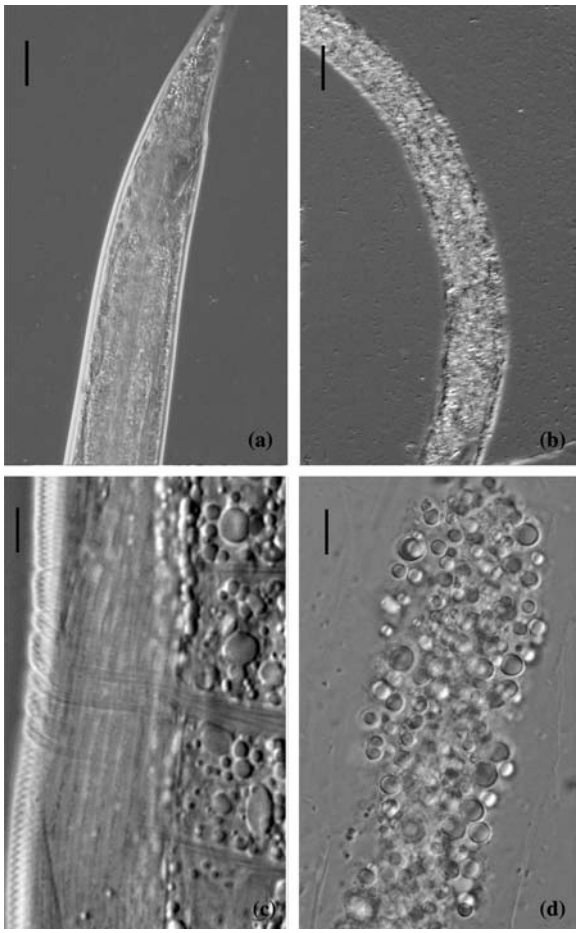

Fig. 3. Nematode treated with purified protease Ver112 (a). Control nematode. (b) and (c). Nematode treated with Ver112 for 8–10 h. (d). Nematode treated with Ver112 after 12 h. Scale bar: 100 μm (a, b and d), 10 μm (c).

Fig. 4. Alignment of *N*-terminal amino acid sequences from protease PII, Aoz1, CDEP1, PL, Pr1, LePR, VCP1 and Ver112. They were isolated from *A. oligospora* (*PII* and *Aozl*), *Beauveria bassiana*, *P. lilacinus*, *Metarhizium anisopliae*, *L. lecanii*, *P. chlamydosporium* and *L. psalliotae*, respectively.

nematodes (Lopez-Llorca & Robertson 1992, Bonants *et al*. 1995), and several cuticle-degrading serine proteases (P32, VCP1, PII, PL, and Aozl) have been isolated from different fungi (Lopez-Llorca & Robertson, 1992, Segers *et al*. 1994, Tunlid *et al*. 1994, Bonants *et al*. 1995, Zhao *et al*. 2004). These proteases share similar characteristics of a low molecular mass and broad substrate spectrum.

There is increasing evidence that proteases play an important role in infection of nematode eggs. Segers *et al*. (1994) found that eggs of *Meloidogue incognita* and *Globodera rostochiensis* were more susceptible to infection by *Pochonia chlamydosporium* after pre-treatment with purified VCP1. Lopez-Llorca *et al*. (2002) also found reduction in the infection of *Meloidogue javanica* eggs by three species of fungal parasites, *Pochonia rubescens* (syn. *V. suchlasporium*), *P. chlamydosporium* and *Lecanicillium lecanii* upon addition of PMSF or other serine protease inhibitors.

The biochemical characterization of Ver112 is similar to VCP1, P32 and PL, which were isolated from egg-parasitic or nematode-parasitic fungi, *P. chlamydosporium*, *P. suchlasporium* and *Paecilomyces lilacinus*, respectively: they all have similar molecular masses (32–33 kDa) (Lopez-Llorca & Robertson 1992, Segers *et al*. 1994, Bonants *et al*. 1995), and a high *p*I value (10.2), but the optimum reaction temperature of Ver112 (70 °C) is higher than that of PL (60 °C). These proteases are inhibited by PMSF. However, another two serine proteases PII and Aoz1, isolated from nematode-trapping fungus, *A. oligosporia*, both of them have lower *p*I value (4.6 and 4.9) (Tunlid *et al*. 1994, Zhao *et al*. 2004) and higher molecular mass (35 and 38 kDa), which suggests that the biochemical characterization of protease from nematode-trapping and egg-parasitic or nematode-parasitic fungi are different. Whether the differences between them are connected with their mode of infecting nematodes is not known but some data indicate that the higher *p*I value is important for the hydrolytic activity and binding of the enzyme to fragments of insect cuticle (St Leger *et al*. 1992).

Like other serine proteases, purified Ver112 also can degrade various protein substrates (Table 3), but it has more proteolytic activity than PII (relative activity of casein, BSA, gelatin, collagen and nematode cuticle is 100, 6, 11, 0.4 and 10%, respectively) (Tunlid *et al*. 1994). From the results of nematicidal analysis (Table 4, Figure 3) and hydrolysis of various protein substrates

(Table 3) it can be concluded that serine protease Ver112, from *L. psalliotae* can degrade the nematode cuticle effectively and can be inhibited by serine protease inhibitor (PMSF) or heating. The majority of *P. redivivus* individuals (81%) were degraded after treating with Ver112 for 12 h, however, only 77% of nematodes were immobilized after treating with PII for 20–22 h (Tunlid *et al*. 1994). The N-terminal amino acid residues analysis also showed that these proteases shared a high degree of similarity (Figure 4), which also suggests that these serine proteases may play the same role in infection process of nematode.

Although the purified protease has obvious nematicidal activity (Table 4), the crude enzyme degrades the nematodes more effectively (Table 4) which suggests that other enzymes and factors may play a role in the infection process. In fact, chitinase has been isolated from some nematophagous and entomophagous fungi which can hydrolyze the eggshell of nematode (Tikhonov *et al*. 2002). Additionally, chitinolytic and collagenolytic activity also was determined in crude enzyme from *L. psalliotae* (data not shown). Therefore, further studies are necessary to ascertain the role of extracellular enzymes and other factors in infection of nematode.

Acknowledgements

We are grateful to Dr. Dilantha Fernando in the University of Manitoba, Canada, and Dr. Li Haipeng in the Ludwig-Maximilians-Universität München, Germany, for their invaluable discussions and assistance in preparing this manuscript. We also express our gratitude to Zhou Wei for her invaluable help in facilitating the work. The work was funded by the projects from Ministry of Science and Technology of China (approved No.2003CB415102, 2002BA901A21) and Department of Science and Technology of Yunan Province (approved No. 2004C0001Z, No.2003C0003Q).

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