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Preliminary crystallographic study of two cuticle-degrading proteases from the nematophagous fungi *Lecanicillium psalliotae* and *Paecilomyces lilacinus*

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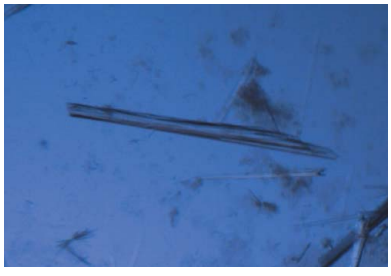


Cuticle-degrading proteases are extracellular subtilisin-like serine proteases that are secreted by entomopathogenic and nematophagous fungi. These proteases can digest the host cuticle during invasion of an insect or nematode and serve as a group of important virulence factors during the infection of nematodes by nematophagous fungi. To elucidate the mechanism of interaction between the proteases and the nematode cuticle, two cuticle-degrading proteases, Ver112 from *Lecanicillium psalliotae* (syn. *Verticillium psalliotae*) and PL646 from *Paecilomyces lilacinus*, were studied. The Ver112 protein and the complex between PL646 and the substrate-like tetrapeptide inhibitor methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone (MSU-AAPV) were crystallized using the hanging-drop vapour-diffusion method at 289 K. The crystals were analyzed by X-ray diffraction to resolutions of 1.65 and 2.2 Å, respectively. These analyses identified that crystals of Ver112 belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 43.7$, $b = 67.8$, $c = 76.3$ Å, $\alpha = \beta = \gamma = 90^\circ$. In contrast, crystals of the PL646–MSU-AAPV complex belonged to space group $P2_1$, with unit-cell parameters $a = 65.1$, $b = 62.5$, $c = 67.6$ Å, $\beta = 92.8^\circ$.

1. Introduction

Plant-parasitic nematodes can cause great damage in agriculture and forestry. Nematophagous fungi, the natural enemy of plant-parasitic nematodes, have the unique ability to capture and infect nematodes. Such abilities make them attractive biological agents to control the harmful nematodes. During the process of infecting nematodes, these fungi have to penetrate the cuticle or eggshell of the nematode and eventually invade and digest the host cells. The cuticle of nematodes consists mainly of proteins, including collagens (Cox *et al.*, 1981). The nematode eggshell consists of three layers: an outer vitelline layer, a middle chitinous layer and an inner lipid layer (Bird & McClure, 1976). During the early infection process, the mechanical pressure of hyphae may play a role. However, experimental results have shown that the degradation of cuticles (eggshells) by hydrolytic enzymes is likely to play a pivotal role. These enzymes include proteases, chitinases and collagenases which digest the main chemical constituents of nematode cuticles and eggshells.

Several proteases secreted by nematophagous fungi have been identified, cloned and homologously or heterologously expressed. The first pathogenicity-related serine protease P32 was isolated from *Verticillium suchlasporium* and was shown to be capable of degrading eggshells and cleaving eggshell proteins (Lopez-Llorca, 1990). Further immunocytochemical analysis showed that P32 was secreted during the infection of nematode eggs (Lopez-Llorca & Robertson, 1992). Two serine proteases PII and Aoz1 from the nematode-trapping fungus *Arthrobotrys oligospora* immobilized nematodes and hydrolysed the proteins of purified cuticle, resulting in structural changes in the nematode cuticle (Tunlid *et al.*, 1994; Zhao *et al.*, 2004). The VCP1 protease from *V. chlamydosporium* hydrolysed proteins located on the outer layer of the eggshells and exposed the chitin layer underneath (Segers *et al.*, 1994). Incubation of nematode eggs with pSP-3 from *Paecilomyces lilacinus* resulted in binding of the protease to the eggs (Bonants *et al.*, 1995). Other identified hydrolytic enzymes include Ver112 from *Lecanicillium psalliotae* (Yang, Huang, Tian, Wang *et al.*, 2005), Mlx from *Monacrosporium microscaphoides*

(Wang, Yang & Zhang, 2006), DS1 from *Dactylella shizishanna* (Wang, Yang, Lin *et al.*, 2006) and PrC from *Clonostachys rosea* (syn. *Gliocladium roseum*; Li *et al.*, 2006). All of these enzymes showed hydrolytic activity against nematode cuticle and resulted in the immobilization of nematodes.

While a growing list of extracellular enzymes have been identified as being important during the infection of nematodes, details of how these fungi penetrate the nematodes remain unclear. A thorough understanding of the three-dimensional structures of these enzymes and of their enzyme–substrate complexes would provide greater insight into the mechanism and specificity of enzyme catalysis and the interaction between proteases and the proteinous components of nematode cuticles. Structural models of PR1, Ver112 and VCP1 generated by homology modelling showed variable residues within the substrate-binding site and different conformational flexibilities in the S2 loop, which might affect the substrate specificity and catalytic activity of cuticle-degrading proteases (Liu *et al.*, 2007). However, no experimental determination of the three-dimensional structure of a cuticle-degrading protease has been made to date.

Peptide-derived chloromethyl ketones irreversibly inhibit serine proteases and form stable covalent complexes which are well suited to crystallographic studies (Stein & Trainor, 1986). Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone (MSU-AAPV) is the most effective chloromethyl ketone inhibitor for human leukocyte elastases (Wei *et al.*, 1988). Here, we report the crystallization of Ver112 and the cocrystallization of another cuticle-degrading protease PL646 with the tetrapeptide chloromethyl ketone inhibitor MSU-AAPV.

2. Materials and methods

2.1. Purification and inhibition tests

For the purification of protease PL646, the nematophagous fungus *P. lilacinus* strain YMF1.00646 isolated from the root-knot nematode *Meloidogyne* sp. in Yunnan Province (maintained in the Laboratory for Conservation and Utilization of Bio-resources, Yunnan University, People's Republic of China) was transferred from potato dextrose agar (PDA) medium to PL-4 liquid culture to induce protease production according to Yang, Huang, Tian, Wang *et al.* (2005). After 7 d incubation, 10 l culture broth was collected, centrifuged at 10 000*g* for 20 min and filtered through Waterman No. 1 filter paper to remove mycelia and insoluble residues. Ammonium sulfate was added to the supernatant to 80% saturation and precipitated by centrifugation at 10 000*g* for 30 min at 277 K. The pellet was resuspended in a minimum volume of 10 m*M* sodium phosphate buffer (PBS; pH 6.0) and dialyzed against the same buffer. The dialyzed fraction was loaded onto a Resource 15S column (20 ml) equilibrated with 10 m*M* PBS buffer pH 6.0. The column was washed with equilibration buffer to remove unbound proteins; the bound proteins were eluted with a linear gradient of NaCl (0–1.0 *M*) in the same buffer at a flow rate of 2 ml min^{-1}. Fractions with protease activity were pooled and loaded onto a Superdex 75 column pre-equilibrated with 50 m*M* PBS buffer pH 7.5 containing 0.15 *M* NaCl and eluted with the same buffer. Fractions possessing protease activity were run on an SDS–PAGE gel and appeared as a single band of molecular weight 33.5 kDa (Fig. 1). The fractions were pooled, dialyzed and concentrated by ultrafiltration. Protease activity and concentration were determined as described previously (Li *et al.*, 2006). The purified protease was designated PL646.

The *L. psalliotae* protease Ver112 was purified from culture filtrates of the fungus as described previously (Yang, Huang, Tian, Wang *et al.*, 2005). The purified protease Ver112 was concentrated to 5 mg ml^{-1} in 10 m*M* PBS pH 6.0 and 50 m*M* NaCl using an Amicon (Millipore, USA) centrifugal filter device.

The tetrapeptide inhibitor MSU-AAPV was purchased from Sigma, USA. Its inhibition activity for Ver112 and PL646 was measured using the chromogenic substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Sigma, USA) as described previously (Wolf *et al.*, 1991). MSU-AAPV inhibited the hydrolytic activity of both Ver112 and PL646 (data not shown).

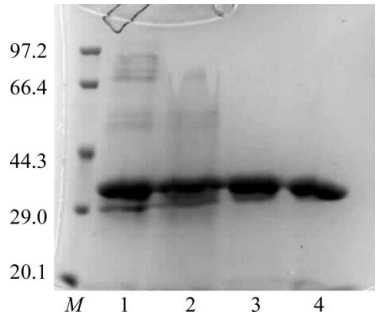
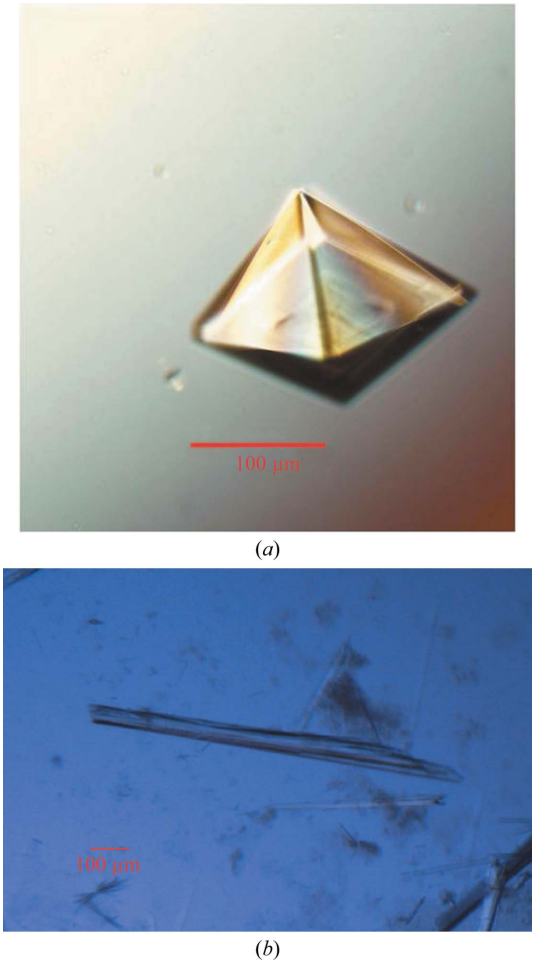


Figure 1
SDS–PAGE of PL646 during the purification process. SDS–PAGE was performed on a 12% gel and was stained with Coomassie Brilliant Blue R250. Lane *M*, marker proteins with molecular mass (kDa) indicated on the left; lane 1, culture filtrate; lane 2, crude enzyme; lane 3, protease purified using cation-exchange chromatography; lane 4, protease purified using gel filtration.



(*a*)

(*b*)

Figure 2
Crystals of cuticle-degrading proteases. (*a*) Ver112, (*b*) the PL646–inhibitor complex. The proteases Ver112 and PL646 were isolated from the nematophagous fungi *L. psalliotae* and *P. lilacinus*, respectively.

2.2. Crystallization

Crystallization of Ver112 was performed by the hanging-drop vapour-diffusion method at 289 K. The optimal crystallization condition was determined using PEG/Ion Screen, Crystal Screen Cryo, Grid Screen Ammonium Sulfate and Crystal Screen 2 (Hampton Research, USA). 1 µl protein solution was mixed with 1 µl reservoir solution. The mixture was equilibrated against 200 µl reservoir solution at 289 K. An octahedron-shaped crystal appeared within 3 d from well solution containing 2 *M* ammonium sulfate and 5% isopropyl alcohol (Fig. 2*a*).

Crystallization of the complex between PL646 and MSU-AAPV was performed by the hanging-drop vapour-diffusion method at 289 K. Specifically, 30 mg ml^{-1} enzyme and 10 m*M* inhibitor in 100 m*M* HEPES, 10 m*M* $CaCl_2$ pH 7.5 were mixed in a 1:1 ratio and the solution was incubated for 2 h at room temperature. 1 µl drops of this solution were mixed with 1 µl crystallization solution containing 0.2 *M* potassium dihydrogen phosphate, 20% PEG 3350 and equilibrated against 200 µl crystallization solution. Rod-shaped crystals with a length of 0.9 mm were obtained within 3 d (Fig. 2*b*).

2.3. X-ray diffraction data collection and processing

Crystals of Ver112 and PL646–MSU-AAPV were transferred to a cryoprotectant solution consisting of 25% glycerol in the crystal-

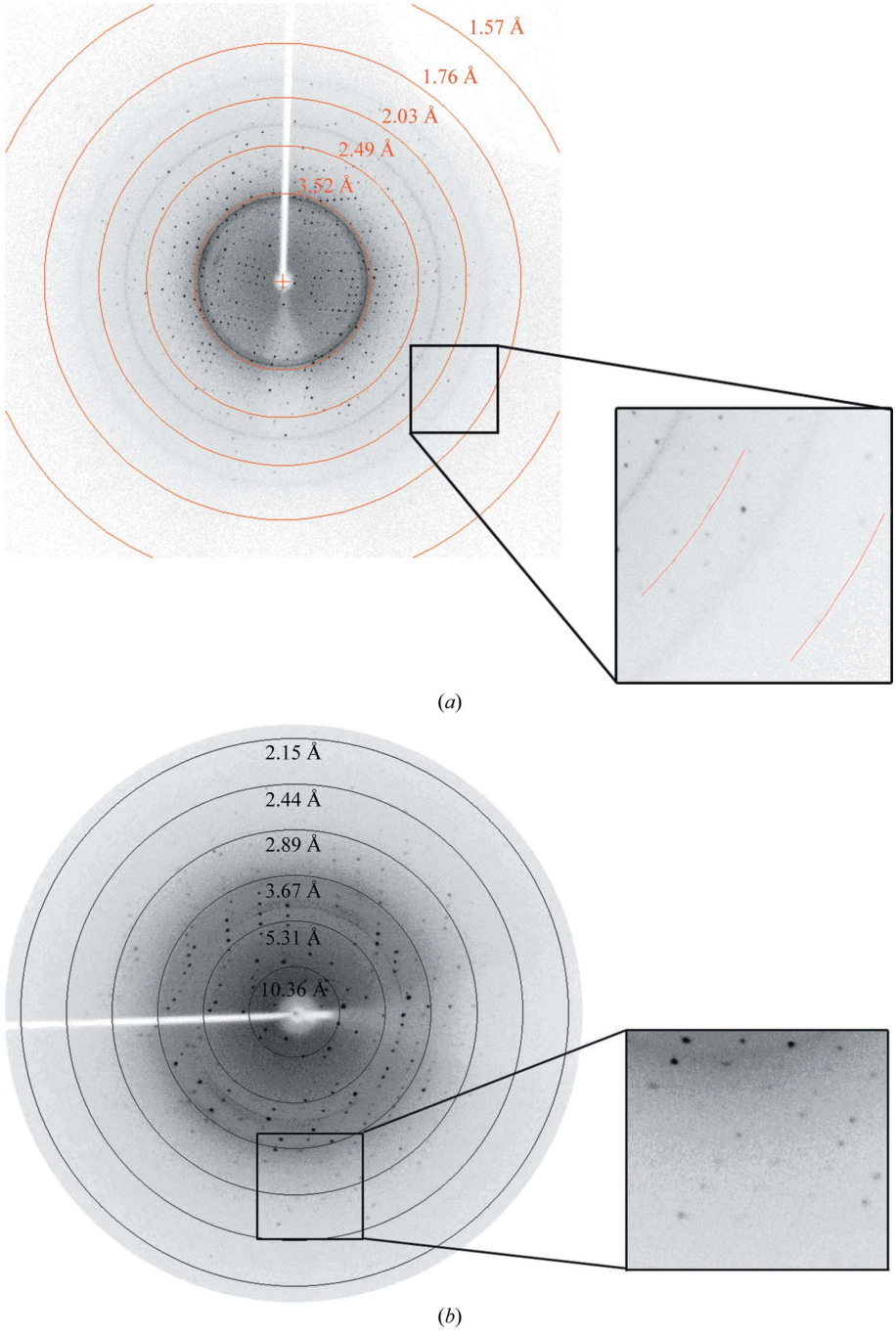


Figure 3
Typical diffraction patterns of crystals. (*a*) Ver112, (*b*) the PL646–inhibitor complex. The diffraction images were collected on a MAR345dtb image-plate detector.

Table 1
Summary of data-collection statistics for Ver112 and the PL646–inhibitor complex.

Values in parentheses are for the highest resolution shell.

	Ver112	PL646–MSU-AAPV
Space group	$P2_12_12_1$	$P2_1$
Unit-cell parameters (Å, °)	a = 43.7, b = 67.8, c = 76.3, $\alpha = \beta = \gamma = 90$	a = 65.1, b = 62.5, c = 67.6, β = 92.8
Resolution range (Å)	50.00–1.65 (1.71–1.65)	50.0–2.2 (2.3–2.2)
Total reflections	215774 (8017)	110259 (8026)
Unique reflections	26819 (2077)	27780 (2685)
Redundancy	8.0 (3.8)	4.0 (3.4)
Average $I/\sigma(I)$	46.9 (14.0)	12.1 (3.2)
$R_{\rm merge}$† (%)	3.4 (13.8)	13.1 (46.0)
Data completeness (%)	95.8 (75.4)	98.9 (96.4)
Matthews coefficient (Å^3 Da^{-1})	1.7	2.1
Assumed molecules in ASU	1	2

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $\langle I(hkl)\rangle$ is the mean intensity of the observations $I_i(hkl)$ of reflection hkl.

lization reservoir solution. The crystal was mounted on a nylon loop and flash-cooled in a cold nitrogen-gas stream at 100 K using an Oxford Cryosystems cryostream. Diffraction data were collected on a MAR345dtb (MAR Research, Hamburg) image-plate detector at 100 K using a Rigaku MM-007 rotating-anode home X-ray generator operating at 40 kV and 20 mA (λ = 1.5418 Å). A total of 360 frames of data were collected (Fig. 3). All intensity data were indexed, integrated and scaled with the *HKL*-2000 package (Otwinowski & Minor, 1997). Diffraction data statistics are given in Table 1.

3. Results and discussion

The crystal of Ver112 was found to belong to space group $P2_12_12_1$, with unit-cell parameters a = 43.7, b = 67.8, c = 76.3 Å, $\alpha = \beta = \gamma = 90°$. Initial molecular replacement of Ver112 was performed using the crystal structure of proteinase K complexed with methoxysuccinyl-Ala-Ala-Pro-Ala-chloromethyl ketone (Wolf *et al.*, 1991; PDB code 3prk) as the initial search model. Proteinase K shares 64% identity with Ver112 (Yang, Huang, Tian, Sun *et al.*, 2005). This procedure was performed using *CNS* v.1.2 (Brünger *et al.*, 1998) and *Phaser* (McCoy, 2007). Both programs gave obvious and correct solutions according to clear rotation and translation functions. We initially tried to cocrystallize Ver112 and the tetrapetide inhibitor MSU-AAPV under various crystallization conditions, but did not succeed. Fortunately, another cuticle-degrading protease PL646 isolated from the nematophagous fungus *P. lilacinus* formed a complex with the inhibitor and rod-shaped crystals grew within 3 d in the crystallization condition 0.2 *M* potassium dihydrogen phosphate, 20% PEG 3350 and diffracted to a resolution of 2.2 Å. The PL646–MSU-AAPV crystal belonged to space group $P2_1$, with unit-cell parameters a = 65.1, b = 62.5, c = 67.6 Å, β = 92.8°. Molecular replacement of PL646 complexed with MSU-AAPV was performed with *Phaser* using the crystal structure of the proteinase K complex mentioned above as the search model. A clear solution was found according to the rotation and translation functions. Electron density for the bound peptide was also found clearly.

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