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Crystallization and preliminary X-ray diffraction analysis of a protease inhibitor from the haemolymph of the Indian tasar silkworm *Antheraea mylitta*

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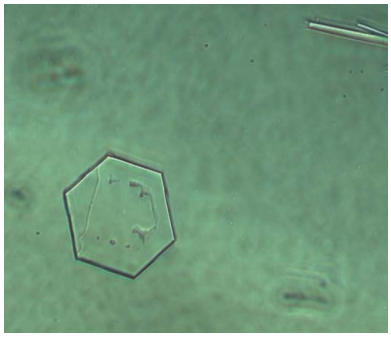

A protein with inhibitory activity against fungal proteases was purified from the haemolymph of the Indian tasar silkworm *Antheraea mylitta* and was crystallized using the hanging-drop vapour-diffusion method. Polyethylene glycol 3350 was used as a precipitant. Crystals belonged to space group $P6_322$, with unit-cell parameters $a = b = 60.6$, $c = 85.1$ Å. X-ray diffraction data were collected and processed to a maximum resolution of 2.1 Å.

1. Introduction

Insects lack the immune system of vertebrates involving antigen–antibody reactions, although they can protect themselves efficiently from entomopathogenic infections. The humoral part of the insect immune system is characterized by the rapid and transient synthesis of proteins with potent antibacterial and antifungal activity (Gillespie *et al.*, 1997). Entomopathogenic fungi infect susceptible hosts directly through the integument and utilize different proteases to carry out the digestion of cuticle proteins for colonization and to inactivate the host immune systems (Clarkson & Charnley, 1996; Frobius *et al.*, 2003). Insects protect themselves from this type of fungal infection by expressing a wide spectrum of protease inhibitors to inhibit the proteases. *Bombyx mori* produces fungal protease inhibitor F, which inhibits proteases from *Aspergillus melleus* and *Beauveria bassiana* as well as subtilisin, but not bovine trypsin or chymotrypsin (Eguchi, 1982). Larvae of the greater wax moth, *Galleria mellonella*, produce peptides such as the inducible serine protease inhibitors defencin and gallerimycin which are active against entomopathogenic fungi (Frobius *et al.*, 2000; Lee *et al.*, 2004; Schuhmann *et al.*, 2003). About 48 families of protease inhibitors have been identified (Rawlings *et al.*, 2004). Some of these inhibitors were found to inhibit serine proteases of mammalian origin such as trypsin, chymotrypsin and elastase. Several protease inhibitors from various sources have been studied by X-ray crystallography and NMR analysis. These include the structures of *Locusta migratoria* inhibitor D2 (Mer *et al.*, 1994), PMP-C (Mer *et al.*, 1996), active serpin 1K from *Manduca sexta* (Li *et al.*, 1999), chymotrypsin/cathepsin G inhibitor (AMCI-1) from *Apis mellifera* (Cierpicki *et al.*, 2000), the Michaelis complex of rat trypsin and *M. sexta* serpin 1B A353K (Ye *et al.*, 2001), PMP-C and PMP-D2v from *Schistocerca gregaria* in complex with bovine α-chymotrypsin (Roussel *et al.*, 2001) and natural crayfish trypsin in complex with *S. gregaria* trypsin inhibitor (Fodor *et al.*, 2005). Protease inhibitors of structurally different families can inhibit serine proteases, cysteine proteases, metalloproteases and aspartyl proteases through structural elements including N- or C-terminal exposed loops, either separately or in association with other similar structural elements (Otlewski *et al.*, 2005). Serine protease inhibitors are of three basic types: canonical, non-canonical and serpins. Canonical inhibitors are the largest family and are a widely distributed group, ranging in size from 14 to 200 amino-acid residues, which act through a standard mechanism of inhibition (Laskowski & Kato, 1980; Krowarsch *et al.*, 2003). These inhibitors are rigid, stable and mostly have purely β-sheet or mixed α/β topologies. Some can be only α-helical or are irregular proteins that are rich in disulfide bridges (Otlewski *et al.*, 2005). Non-canonical protease inhibitors such as hirudin and

Table 1
Essential crystallographic data.

Values in parentheses are for the highest resolution shell.

Space group	$P6_322$
Unit-cell parameters (Å)	$a = b = 60.6$, $c = 85.1$
Unit-cell volume (Å^3)	270361.4
Resolution (Å)	25–2.1 (2.18–2.10)
No. of observations	56542
No. of unique reflections	5816 (544)
Completeness (%)	99.6 (96.8)
Redundancy	9.7 (6.9)
R_{sym}† (%)	13.1 (52.3)
$I/\sigma(I)$	12.9 (2.3)
V_M (Å^3 Da^{-1})	2.4
Solvent content (%)	47
Monomers per ASU	1

† $R_{sym} = \sum |I(h) - \langle I(h)\rangle| / \sum I(h)$, where $I(h)$ is the observed intensity and $\langle I(h)\rangle$ is the mean intensity of reflection h over all measurements of $I(h)$.

haemedin interact with the active site of serine proteases such as thrombin through their N-terminal tails (Grutter *et al.*, 1990; Richardson *et al.*, 2000). Finally, serpins are 45–55 kDa proteins that are mostly found in blood plasma which account for phagocytosis, coagulation, complement activation and fibrinolysis. They are composed of three β-sheets and eight or nine α-helices forming a single domain and inhibit through the reactive-site loop present at their C-terminus (Potempa *et al.*, 1994, Gettins, 2002).

Antheraea mylitta is one of the non-mulberry silkworms found in India. It produces tasar silk and is widely distributed. Previously, we have reported the presence of a 10.4 kDa fungal protease inhibitor, named AmFPI-1, in the haemolymph of the silkworm, the sequence of which showed 46% homology to the inducible serine protease inhibitor ISPI-1 of *G. mellonella*. This fungal protease inhibitor was found to inhibit serine proteases (Shrivastava & Ghosh, 2003). However, it has negligible sequence identity with serine protease inhibitors of known structure. In order to understand its structural properties, the mechanism of inhibition and to identify the reactive amino acids responsible for inhibition, we have purified native AmFPI-1 from the silkworm haemolymph, crystallized the protein and performed a preliminary diffraction analysis of these crystals.

2. Materials and methods

2.1. Insect and haemolymph

Fifth-instar larvae of *A. mylitta* were obtained from the local silk farm and haemolymph was collected in a cold tube by cutting larval abdominal appendages. After removing the haemocytes by centrifugation at 10 000*g* for 10 min, the supernatant was stored at 253 K.

2.2. Purification

The protease inhibitor was purified following the procedure reported by Shrivastava & Ghosh (2003) with some modifications. In brief, proteins were precipitated from 30 ml haemolymph supernatant overnight employing a 60% ammonium sulfate solution at 277 K, pelleted by centrifugation for 10 min at 277 K and resuspended in 10 ml citrate buffer (10 m*M* sodium citrate, 50 m*M* NaCl pH 6.0). The protein solution was dialyzed overnight against 10 m*M* citrate buffer, heat-treated at 353 K for 2 min and centrifuged at 10 000*g* for 10 min at 277 K to remove denatured proteins. The supernatant containing the heat-stable proteins was loaded onto a Q-Sepharose (Amersham Biosciences, Piscataway, New Jersey, USA) column equilibrated with 10 m*M* citrate buffer. Flowthrough fractions were collected, dialyzed against buffer containing 10 m*M* Tris pH 7.5 and 50 m*M* NaCl and applied onto a SP-Sepharose (Amersham Biosciences) column equilibrated with the same buffer. Proteins were eluted from the column with a linear gradient of 0.05–1 *M* NaCl. Fractions containing the protease inhibitor were pooled and loaded onto a Sephadex G-75 (Amersham Biosciences) column previously equilibrated with 10 m*M* Tris pH 7.5, 10 m*M* NaCl. Fractions containing the inhibitor protein were concentrated to 10 mg ml^{-1} using Centricon centrifugal devices and the homogeneity of the purified protein was verified by 15% SDS–PAGE. The extent of purification was checked by Western blot analysis at each step using anti-AmFPI-1 polyclonal antibody raised against recombinant protein expressed in bacteria.

2.3. Crystallization

Crystallization trials were performed using the sitting-drop vapour-diffusion method using drops consisting of 2 µl protein solution and 2 µl mother liquor equilibrated against 100 µl reservoir solution using Index HT from Hampton Research at room temperature in 96-well Corning plates (Hampton Research, Aliso Viejo, CA, USA). Preliminary crystallization conditions were observed with 0.2 *M* ammonium sulfate, 0.1 *M* Bis-Tris pH 6.5 and 25%(*w*/*v*) polyethylene glycol 3350. A fine screen around this preliminary condition assayed variations in pH, ionic strength and precipitant concentration. Hexagonal plates with dimensions of 0.6 × 0.3 × 0.06 mm were obtained after 5 d at 293 K (Fig. 1) from 0.2 *M* ammonium sulfate, 0.1 *M* Bis-Tris pH 6.5 and 30%(*w*/*v*) polyethylene glycol 3350 using hanging-drop vapour diffusion in standard 24-well Linbro plates (Hampton Research, Aliso Viejo, CA, USA), in which 2 µl protein solution was mixed with 2 µl reservoir solution and equilibrated against 1000 µl reservoir solution.

2.4. Diffraction data collection and processing

Diffraction data were collected on a MAR Research MAR-345dtb image-plate detector attached to a Rigaku RU-H3R rotating-anode generator producing Cu $K\alpha$ radiation equipped with an Osmic mirror system and operated at 50 kV and 100 mA. A total of 94° of data were collected with 1° oscillation increments per frame with a crystal-to-detector distance of 175 mm. X-ray data were processed using *DENZO* (Otwinowski & Minor, 1997) and subsequent scaling and merging of intensities was carried out using *SCALEPACK* (Otwinowski & Minor, 1997). Crystals were flash-cooled in a liquid-

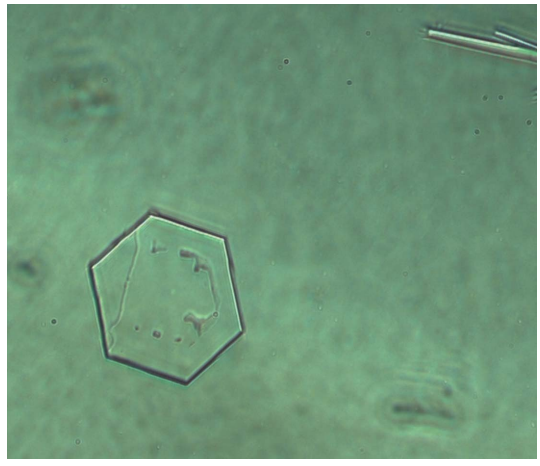

Figure 1
A hexagonal-shaped crystal of *A. mylitta* fungal protease 1 with maximum dimensions of 0.6 × 0.3 × 0.06 mm.

nitrogen stream at 100 K using an Oxford cryostream controller. The mother liquor was suitable as a cryoprotectant owing to its high polyethylene glycol concentration.

3. Results

The protease inhibitor was purified to homogeneity from silkworm haemolymph. Protein concentrated to 10 mg ml^{-1} was used for crystallization experiments. Hexagonal crystals were obtained that diffracted to a maximum resolution of 2.10 Å and analysis of the diffraction data indicated that they belonged to space group $P6_322$. The unit-cell parameters were found to be $a = b = 60.6$, $c = 85.1$ Å. Determination of the Matthews coefficient indicated the presence of 47% solvent content in the unit cell (V_M = 2.4 Å^3 Da^{-1}) with one molecule in the asymmetric unit. The mosaicity of the crystal was found to be 0.63°. The overall completeness of the data set was 99.6%, with an R_{sym} of 13.1% (Table 1). We attempted to solve the structure by Patterson-search techniques using homology models, but none yielded a clear solution because of the very low sequence identity. Currently, heavy-atom derivatives are being prepared in order to solve the structure by the multiple isomorphous replacement (MIR) method.

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