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Crystallization and preliminary X-ray studies of a non-haemorrhagic fibrin(ogen)olytic metalloproteinase from the venom of *Agkistrodon acutus*

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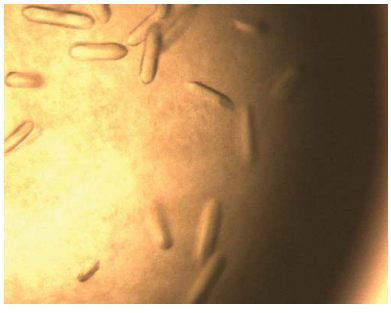

A non-haemorrhagic fibrin(ogen)olytic metalloproteinase from the venom of *Agkistrodon acutus* has been crystallized by the hanging-drop method. The crystals belong to space group $P3_121$, with unit-cell parameters $a = b = 80.57$, $c = 66.77$ Å and one molecule in the asymmetric unit. X-ray diffraction data were collected to 1.86 Å resolution.

1. Introduction

Snake-venom metalloproteinases (SVMPs) of the metzincin family are a group of zinc-dependent proteolytic enzymes contained in the venoms of poisonous snakes. More than 100 SVMPs have been reported to date and they are usually categorized into four classes based on their size and domain structure (Bjarnasson & Fox, 1994). Class P-I (the small enzymes) contain only the protease domain, whilst class P-II (the medium-size enzymes) also have a disintegrin-like domain at the carboxy-terminal region, class P-III, the most potent haemorrhagic toxins, have a cysteine-rich domain in addition to the same domains of P-II and class P-IV have a fourth lectin-like domain that follows the cysteine-rich domain. Most of the venom metalloproteinases are fibrin(ogen)olytic enzymes, preferentially cleaving the Aα-chain and more slowly cleaving the Bβ-chain of fibrinogen (Gutierrez *et al.*, 1995; Tu *et al.*, 1996; Markland, 1998; Siigur *et al.*, 1998). Some SVMPs can also degrade the vascular basement membrane or extracellular matrix components (Gutierrez & Rucavado, 2000) and other blood-coagulation factors (Kamigut *et al.*, 1996). The disintegrin/cysteine-rich domains of the larger SVMPs can inhibit platelet aggregation by competing specifically with endogeneous integrins, particularly with platelet receptors (Andrews & Berndt, 2000). These local and/or systemic actions on the blood system generally result in severe bleeding disorders.

Interestingly, some SVMPs, primarily those belonging to the P-I class, are devoid of haemorrhagic activity (Randolph *et al.*, 1992; Rodrigues *et al.*, 2000). The structural basis of this observation is not clear, although some comparative studies of haemorrhagic and non-haemorrhagic metalloproteinases have identified residues that may be required to exert this effect (Bolger *et al.*, 2001; Gasmi *et al.*, 2000). These types of non-haemorrhagic metalloproteinases have caught the attention of many investigators as potential thrombotic agents. Their characteristic direct action on fibrin(ogen) results in fewer complications than the currently available thrombolytic agents in clinical use, such as recombinant tissue plasminogen activator (t-PA), urokinase and streptokinase, which act on the thrombus indirectly *via* plasminogen activation (Ouriel, 2002).

A new kind of SVMP was isolated from the Chinese Anhui five-pace snake *Agkistrodon acutus* in our laboratory and was named FII (Chen *et al.*, 1993; Liang *et al.*, 2001). It has a molecular weight of 26 kDa, an isoelectric point of 4.6 and possesses the ability to directly degrade the α- and β-chain of fibrin and fibrinogen *in vitro* (Chen *et al.*, 1993; Liang *et al.*, 2001). It also dissolves thrombi effectively *in vivo* (Chen *et al.*, 1998), but does not have an influence on tissue-type plasminogen activator and plasminogen activator inhibitor-1 activities in the plasma of rats (Liang *et al.*, 2001). Histological examination of heart, liver and lung tissue showed no haemorrhage when FII

was applied at a dosage of 5 mg kg^{-1} (Liang *et al.*, 2001). All these results imply that the snake-venom metalloproteinase FII has potential therapeutic use in dissolving thrombi.

In order to shed more light on the proteolytic mechanism of this enzyme, we embarked on the determination of its crystal structure by X-ray crystallography. Here, we report the crystallization and preliminary X-ray study of FII.

2. Materials and methods

2.1. Purification of FII

Lyophilized powdered *A. acutus* venom was purchased from Qimen snake farm (Anhui, China). The isolation procedures were performed at 277 K as described elsewhere (Chen *et al.*, 1993; Liang *et al.*, 2001) with some modifications. In short, crude venom dissolved in 0.05 *M* ammonium acetate pH 8.0 was applied onto a DEAE-Sephadex A-50 column (3.0 × 80 cm, Pharmacia) pre-equilibrated with 0.05 *M* ammonium acetate pH 8.0. Fractions were eluted with a linear gradient from 0.05 *M* ammonium acetate pH 8.0 to 1 *M* ammonium acetate pH 5.0. The second fraction eluted by ion-exchange chromatography was collected, lyophilized and dissolved in 0.01 *M* ammonium acetate pH 8.0 and then applied onto a Sephadex G-50 column (1.1 × 100 cm, Pharmacia) pre-equilibrated with the same buffer. The first fraction eluted by gel filtration was collected, dialyzed against distilled water and lyophilized and subsequently dissolved and applied again onto a Sephadex G-50 column. The first fraction eluted by the final gel filtration was collected, dialyzed against distilled water and lyophilized. After each isolation step, fractions were tested for fibrinolytic activity by using a modified fibrin-plate technique (Astrup & Mullertz, 1952).

The lyophilized FII powder was dissolved in 10 m*M* Tris–HCl pH 8.0, 0.15 *M* NaCl, applied onto a Superdex G75 gel-filtration column (Pharmacia) and eluted with the same buffer at a flow rate of 0.5 ml min^{-1}. The first fraction was collected and concentrated in the elution buffer to about 10 mg ml^{-1} using a 10 kDa molecular-weight cutoff ultrafiltration membrane (Filtron).

2.2. Crystallization and preliminary X-ray diffraction analysis of FII

The freshly prepared protein was crystallized using the hanging-drop vapour-diffusion method at 291 K. The conditions for crystallization were screened using Crystal Screens 1 and 2 (Hampton Research). 1 µl protein solution was mixed with 1 µl reservoir solution and equilibrated against 200 µl reservoir solution.

Preliminary X-ray diffraction analysis of FII crystals was performed at 100 K on an in-house Rigaku R-AXIS IV^{++} image-plate detector with a Rigaku Cu $K\alpha$ rotating-anode X-ray generator operating at 40 kV and 20 mA (λ = 1.5418 Å). All diffraction data were processed using the programs *DENZO* and *HKL*2000 (Otwinowski & Minor, 1997).

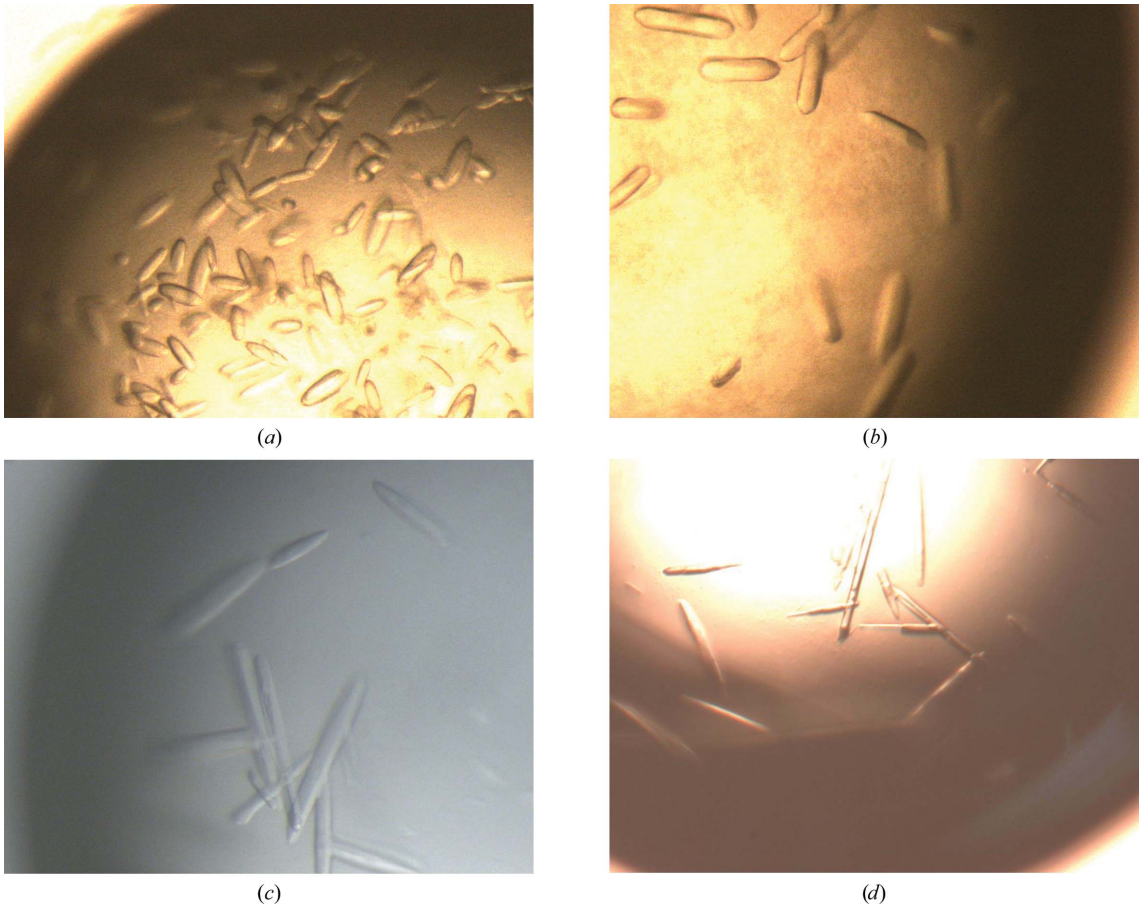

Figure 1
Crystals of FII. (*a*) Crystals grown in 1.4 *M* trisodium citrate, 0.1 *M* Na HEPES pH 7.5 (resolution 1.86 Å); (*b*) crystals grown in 2.0 *M* ammonium sulfate, 0.1 *M* sodium citrate pH 5.6, 0.2 *M* potassium/sodium tartrate (resolution 4.0 Å); (*c*) crystals grown in 30%(*w*/*v*) PEG 4000, 0.1 *M* Tris–HCl pH 8.5, 0.2 *M* lithium sulfate (resolution 3.5 Å); (*d*) crystals grown in 30%(*w*/*v*) PEG 8000, 0.1 *M* sodium cacodylate pH 6.5, 0.2 *M* ammonium sulfate (resolution 2.8 Å).

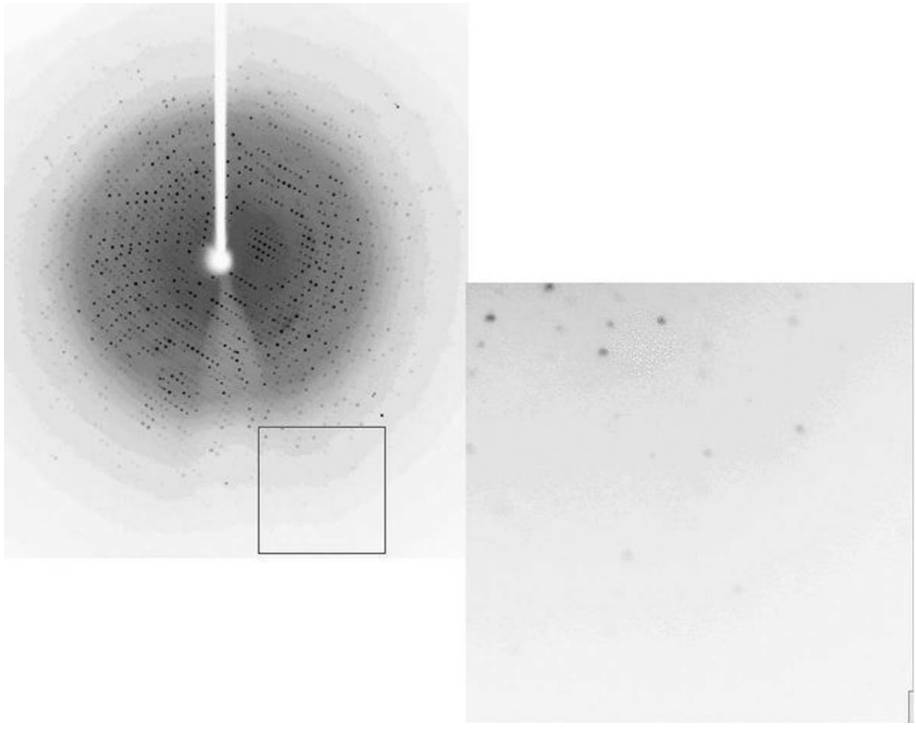

Figure 2
Typical diffraction pattern for a crystal grown in 1.4 *M* trisodium citrate, 0.1 *M* Na HEPES pH 7.5. The contents of the rectangle in the top left image are magnified in the bottom right part of the figure.

Table 1
X-ray diffraction data-collection statistics.

Values in parentheses are for the highest resolution shell.

Crystal-to-film distance (mm)	150
Oscillation range (°)	1
$R_{\rm merge}$† (%)	7.1 (45.5)
Resolution range (Å)	50.0–1.86 (1.95–1.86)
Completeness (%)	99.5 (100.0)
Redundancy	7.8 (7.4)
Reflections (all)	183192
Reflections (unique)	23489
Mean $I/\sigma(I)$	21.2 (6.9)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith intensity measurement of reflection hkl, including symmetry-related reflections, and $\langle I(hkl)\rangle$ is its average.

3. Results and discussion

Well shaped crystals appeared under several conditions (Fig. 1) and crystals grown in 1.4 *M* trisodium citrate, 0.1 *M* Na HEPES pH 7.5 showed good diffraction to 1.86 Å resolution. These crystals appeared on the second day and reached final dimensions of about 50 × 50 × 200 µm after four weeks. The parameters for X-ray diffraction data collection are shown in Table 1. The crystals belong to space group $P3_121$, with unit-cell parameters $a = b = 80.57$, $c = 66.77$ Å. A diffraction pattern is shown in Fig. 2.

Following the initial report of the crystal structure of adamalysin II, a P-I class SVMP from the eastern diamondback rattlesnake *Crotalus atrox* (Gomis-Ruth *et al.*, 1994), several three-dimensional structures of P-I class SVMPs obtained either by X-ray diffraction (Gong *et al.*, 1998; Kumasaka *et al.*, 1996; Watanabe *et al.*, 2003; Zhu *et al.*, 1999) or by computer modelling (Rodrigues *et al.*, 2000; Bolger *et al.*, 2001) have been reported. As these enzymes share the same topology and overall structure (especially in the zinc-binding region required for proteolytic reaction), they can be used as models for molecular-replacement trials. The elucidation of the FII structure and its comparison with other SVMPs will help to understand the structure–function relationships of this family of enzymes, facilitating further exploration of their pharmacological use. The determination of the structure is in progress.

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