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Crystallization and preliminary X-ray analysis of crinumin, a chymotrypsin-like glycosylated serine protease with thrombolytic and antiplatelet activity

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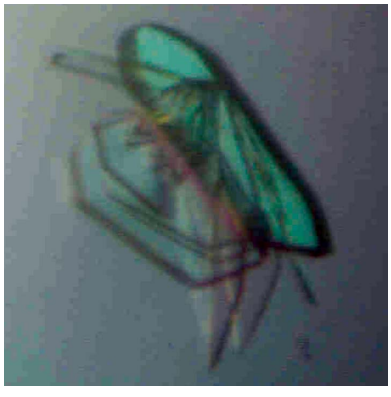

Crinumin, a novel glycosylated serine protease with chymotrypsin-like catalytic specificity, was purified from the medicinally important plant *Crinum asiaticum*. Crinumin is a 67.7 kDa protease with an extraordinary stability and activity over a wide range of pH and temperature and is functional in aqueous, organic and chaotropic solutions. The purified protease has thrombolytic and antiplatelet activity. The use of *C. asiaticum* extracts has also been reported for the treatment of a variety of disorders such as injury, joint inflammation and arthritis. In order to understand its structure–function relationship, the enzyme was purified from the plant latex and crystallized by the hanging-drop vapour-diffusion method. X-ray diffraction data were collected from a single crystal and processed to 2.8 Å resolution. The crystal belonged to the monoclinic space group $C2$, with unit-cell parameters $a = 121.61$, $b = 95.00$, $c = 72.10$ Å, $\alpha = \gamma = 90$, $\beta = 114.19^\circ$. The Matthews coefficient was 2.81 Å^3 Da^{-1}, corresponding to a solvent content of 56%, assuming one molecule in the asymmetric unit. Structure determination of the enzyme is in progress.

1. Introduction

The serine protease chymotrypsin is a digestive enzyme that preferentially hydrolyzes peptide amide bonds where the carboxyl side of the amide bond is a tyrosine, a tryptophan or a phenylalanine (Appel, 1986). Chymotrypsin is produced as an inactive precursor protein in the pancreas and becomes activated upon secretion into the small intestine. Together with other pancreatic enzymes, it contributes to the digestion of food. Chymotrypsin is used as a medication for a variety of diseases and disorders, including as an anti-inflammatory agent to treat inflammation and reduce swelling (Swamy & Patil, 2008; Goodman & Gilman, 1975), for the relief of symptoms related to episiotomy (Goodman & Gilman, 1975) and for the debridement of necrotic wounds, ulcers, abscesses and fistulas (Osol & Hoover, 1975). It has also been used for the liquefaction of blood (Osol & Hoover, 1975).

Serine proteases with chymotrypsin-like specificity can be isolated from many species. However, enzymes from plant sources have an advantage owing to their broad substrate specificity, good solubility, high stability and activity over a wide range of pH and temperature (Antão & Malcata, 2005; Tomar *et al.*, 2008). Therefore, it is useful to study the basic molecular mechanisms of these plant proteins, which are of interest to the biomedical and biotechnological community for therapeutic applications. One potentially interesting target is the protein crinumin, which is a 67.5 kDa glycosylated serine protease with chymotrypsin-like substrate specificity. Crinumin has been purified from the plant *Crinum asiaticum*, which belongs to the Amaryllidaceae family (Singh *et al.*, 2010). The plant is considered to be a medicinal source and is used to treat inflamed joints and sprains (Samud *et al.*, 1999). Its bulb shows cytotoxicity towards tumour cells (Min *et al.*, 2001) and the plant extract demonstrates inhibition of calprotectin-induced cytotoxicity (Yui *et al.*, 1998).

Crinumin can be purified with a high yield (33% recovery) and remains stable and active under adverse conditions (Singh *et al.*, 2010). Recently, it has been demonstrated that crinumin has thrombolytic and antiplatelet activity (Singh *et al.*, 2011). We aim to

elucidate its three-dimensional structure using X-ray crystallography in order to obtain insight into its exceptional stability, enzymatic mechanism and structure–function relationship. Here, we report the crystallization and preliminary X-ray diffraction data of crinumin.

2. Materials and methods

2.1. Purification

The enzyme was purified from the latex of *C. asiaticum* using cation-exchange chromatography (Singh *et al.*, 2010). Fresh latex was obtained from leaves of the plant *via* incisions on the midrib of the leaves. The latex was collected into 10 m*M* acetate buffer pH 5.0 and stored at 253 K for 36 h. It was then thawed at room temperature and centrifuged at 15 000*g* (Sorvall RC-5C Plus) for 30 min to remove insoluble materials. After filtration, the clear supernatant was applied onto a CM-Sepharose column (GE Healthcare) pre-equilibrated with the same buffer. The bound protein was eluted in 4.0 ml fractions with a 0–0.8 *M* NaCl gradient in the same buffer. Crinumin typically eluted around 0.4 *M* NaCl. The protein was quantified by measuring the absorbance at 280 nm. Fractions were also assayed for protease activity using casein as a substrate as described previously (Sarath *et al.*, 1989; Singh *et al.*, 2010). An aliquot of enzyme was incubated in a final volume of 500 µl 50 m*M* Tris–HCl buffer pH 7.5 at 310 K. After 15 min, 0.5 ml 1%(*w*/*v*) casein solution was added to the reaction mixture and the reaction was allowed to proceed at 310 K. After 90 min, the reaction was terminated by the addition of 0.5 ml 10% TCA. The precipitate was removed by centrifugation and TCA-soluble peptides in the supernatant were estimated from the absorbance at 280 nm. One unit of enzyme activity was defined as the amount of enzyme that gives rise to an increase of one unit of absorbance at 280 nm per minute of digestion under the given assay conditions. The specific activity is the number of units of activity per milligram of protein.

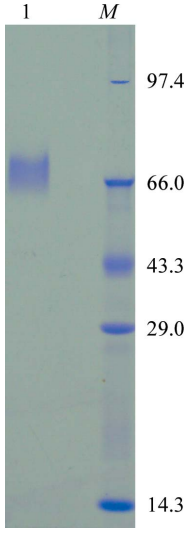

Figure 1
SDS–PAGE of crinumin under nonreducing conditions. Lane 1, crinumin after CM-Sepharose purification; lane *M*, molecular-weight markers (kDa).

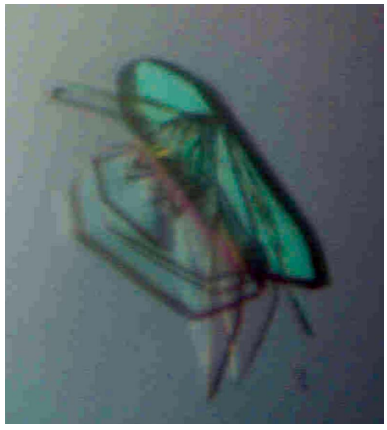

Figure 2
Crystals of crinumin grown in a hanging drop equilibrated against 6%(*w*/*v*) PEG 6000, 90 m*M* NaBr in 0.1 *M* Tris–HCl buffer pH 8.5. The largest crystals are approximately 0.45 × 0.30 × 0.07 mm in size.

The homogeneity of the protein in the fractions was judged by SDS–PAGE. Active fractions with high homogeneity were pooled, concentrated using an Amicon concentrator with a YM10 membrane (Millipore) and stored at 253 K. Prior to crystallization screening, the purity of the protein was determined by SDS–PAGE and it was concentrated to 6 mg ml^{-1} using an Amicon ultrafiltration centrifugal device followed by concomitant buffer exchange to 10 m*M* sodium acetate buffer pH 5.0 containing 50 m*M* NaCl.

At present, the protein sequence of crinumin remains unknown. Protein identification using mass spectrometry revealed the presence of a single peptide that could be matched to the subtilase family of proteins from various plants (Singh *et al.*, 2010), indicating that crinumin shares some homology with these serine proteases.

2.2. Crystallization

Nanolitre crystallization screening was performed using commercial 96-well crystallization screening solutions from Qiagen: JCSG+ and PACT (Newman *et al.*, 2005), Classics, Protein Complex, Compass and Opti-Salts. Crystallization solutions (75 µl each) were dispensed by a Hydra II robot (Matrix) into the reservoirs of 96-well CrystalQuick Low Profile crystallization plates (Greiner Bio-One) and 200 nl sitting drops consisting of 100 nl protein solution and 100 nl reservoir solution were prepared using a Mosquito nanolitre-dispensing robot (TTP LabTech). Plates were stored at 293 K in a Crystal Farm Imaging System (Bruker-AXS) and images were collected according to a predefined schedule (*i.e.* on day 1 to day 90).

2.3. Data collection and processing

Crystals were transferred to a cryoprotectant solution consisting of crystallization solution supplemented with 25% glycerol and with an increase in the PEG 6000 concentration to 20% before flash-cooling at 100 K by plunging them into liquid nitrogen. Crystals were tested for diffraction at our home source: a MICROSTAR Ultra II (Bruker-AXS) X-ray source equipped with a MAR345 image-plate detector (MAR Research). Diffracting crystals were used for further X-ray diffraction analysis on beamline ID23-1 at the ESRF synchrotron facility (Grenoble, France) using an ADSC Q315R CCD detector. The diffraction data set was indexed and integrated using *MOSFLM* (Leslie, 1992) and scaled using *SCALA* (Evans, 1997).

3. Results and discussion

The glycosylated serine protease crinumin could be purified to >90% purity in a single cation-exchange step as judged by Coomassie R-250-stained SDS–PAGE (Fig. 1). About 89 mg protein could be isolated from 267 mg crude plant latex.

Crystallization hits were found by screening nearly 500 different sitting-drop vapour-diffusion conditions. Initial crystals were obtained

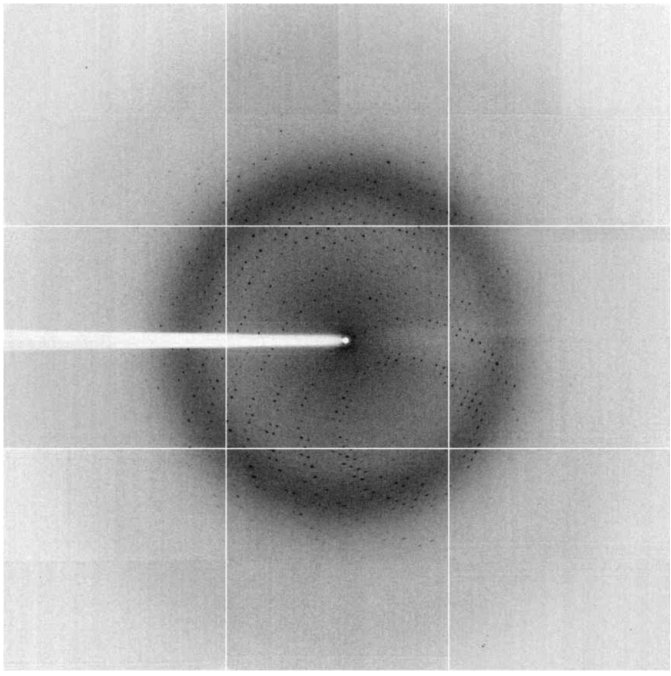

Figure 3
Diffraction image of a crinumin crystal collected on beamline ID23-1 at the ESRF. The resolution limit at the edge is 1.9 Å.

in a solution consisting of 0.1 *M* Tris–HCl pH 8.5, 10% PEG 6000 (Protein Complex Suite condition No. 48) and could be improved by using the same solution supplemented with 175 m*M* NaBr from the Opti-Salt Suite (condition Nos. 47 and 48).

During subsequent optimization, the concentration of NaBr and the ratio of protein to crystallization solution were varied. Solutions were prepared manually and sitting-drop crystallization trials were set up by mixing protein solution (6 mg ml^{-1}) and precipitant solution in different ratios. Drops (3–5 µl) were equilibrated against 750 µl reservoir solution. Suitable crystals for X-ray diffraction experiments were obtained using 0.1 *M* Tris–HCl pH 8.5, 90 m*M* NaBr, 10% PEG 6000. A protein:crystallization solution ratio of 3:1 was found to be optimal, but the thickness of the crystals was still not sufficient. Further optimization was performed using the hanging-drop vapour-diffusion technique by varying the PEG 6000 concentration as well as the ratio of protein to crystallization solution. Finally, well diffracting crystals with typical dimensions of 0.4 × 0.3 × 0.07 mm (Fig. 2) were grown at 293 K in hanging drops consisting of 0.1 *M* Tris–HCl pH 8.5, 90 m*M* NaBr, 6% PEG 6000 with a protein: crystallization solution ratio of 2:3. SDS–PAGE analysis of the crystals showed that the mobility of the crystallized protein was identical to that of the crinumin stock solution, indicating that the full-length protein had been crystallized (data not shown).

Diffraction data were collected at 100 K to a resolution of 2.8 Å (crystal-to-detector distance of 197.9 mm; Fig. 3). A total of 220 frames with a 1° oscillation range per frame and 2 s exposure per frame were collected from a single crystal at 100 K on the ID23-1 beamline at the ESRF. Diffraction analysis of the crystal showed that it belonged to the monoclinic space group *C*2, with unit-cell parameters $a = 121.61$, $b = 95.00$, $c = 72.10$ Å, $\alpha = \gamma = 90$, $\beta = 114.19°$ (see Table 1 for statistics). Volume calculations based on the unit-cell parameters and the molecular weight of the protein indicated that there was one molecule in the asymmetric unit, with a solvent content of 56% and a Matthews coefficient of 2.81 Å^3 Da^{-1}. Attempts to solve the structure by molecular replacement using models of existing serine protease structures are in progress.

Table 1
Experimental conditions and data-collection statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	1.29
Temperature (K)	100
Space group	*C*2
Unit-cell parameters (Å, °)	$a = 121.61$, $b = 95.00$, $c = 72.10$, $\alpha = \gamma = 90$, $\beta = 114.19$
Resolution limits (Å)	20–2.8 (2.95–2.80)
Total reflections	72099 (10599)
Unique reflections	18458 (2681)
Completeness (%)	99.7 (100)
$R_{\rm merge}$† (%)	14.3 (52.9)
Mean $I/\sigma(I)$	7.7 (2.6)
Multiplicity	3.9 (4.0)
Wilson *B* factor (Å^2)	47.8
$V_{\rm M}$ (Å^3 Da^{-1})	2.81
Z	4
Solvent content (%)	56

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl) \times 100\%$, where $I_i(hkl)$ is the measured intensity and $\langle I(hkl)\rangle$ is the average intensity over symmetry-equivalent reflections.

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