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Crystallization of a nonclassical Kazal-type *Carcinoscorpius rotundicauda* serine protease inhibitor, CrSPI-1, complexed with subtilisin

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Serine proteases play a major role in host–pathogen interactions. The innate immune system is known to respond to invading pathogens in a nonspecific manner. The serine protease cascade is an essential component of the innate immune system of the horseshoe crab. The serine protease inhibitor CrSPI isoform 1 (CrSPI-1), a unique nonclassical Kazal-type inhibitor of molecular weight 9.3 kDa, was identified from the hepatopancreas of the horseshoe crab *Carcinoscorpius rotundicauda*. It potently inhibits subtilisin and constitutes a powerful innate immune defence against invading microbes. Here, the cloning, expression, purification and cocrystallization of CrSPI-1 with subtilisin are reported. The crystals diffracted to 2.6 Å resolution and belonged to space group $P2_1$, with unit-cell parameters $a = 73.8$, $b = 65.0$, $c = 111.9$ Å, $\beta = 95.4°$. The Matthews coefficient (V_M = 2.64 $Å^3\,Da^{-1}$, corresponding to 53% solvent content) and analysis of the preliminary structure solution indicated the presence of one heterotrimer (1:2 ratio of CrSPI-1:subtilisin) and one free subtilisin molecule in the asymmetric unit.

1. Introduction

In invertebrates, the circulating haemocytes play an important role in protecting the host against invading microorganisms. The haemocytes participate in recognition, phagocytosis, melanization and cytotoxicity (Cerenius & Soderhall, 2004). Upon mechanical injury or wounding, several protease cascades and clotting enzymes are activated and released into the extracellular milieu (Armstrong, 2001). Serine protease inhibitors play a crucial role in the regulation of the host serine protease cascades for maintaining homeostasis and also target microbial serine proteases which the pathogen uses as a virulence factor to invade the host. Thus, the host serine protease inhibitor can exhibit antimicrobial activity (Jiang *et al.*, 2009). Like most invertebrates, the horseshoe crab innate immune system consists of a large number of multidomain serine protease inhibitors of the Kazal, Kunitz and α_2 macroglobulin families (Kanost, 1999). CrSPI-1, a Kazal-type serine protease inhibitor, was identified from the EST library of the hepatopancreas of a horseshoe crab infected with *Pseudomonas aeruginosa* (Ding *et al.*, 2005). CrSPI-1, a 9.3 kDa two-domain Kazal-type serine protease inhibitor, inhibits subtilisin with a K_i of 1.43 nM. CrSPI is classified into the nonclassical group of Kazal inhibitors. Here, we report the cloning, overexpression, purification and cocrystallization of CrSPI-1 (isoform 1) with subtilisin, with the aim of the determination of its structure in order to understand the mechanism of inhibition in regulating host–pathogen interactions.

2. Materials and methods

2.1. Cloning, expression and purification of recombinant CrSPI-1

CrSPI-1 (GenBank DQ090491.1) was initially cloned into pGEX-4T1 vector for overexpression. However, the protein yield was not sufficient for crystallization trials. A higher level of expression was achieved when the CrSPI-1 gene was cloned into pET-32 Ek/LIC vector (Novagen). The following primers were used for cloning:

forward, 5′-ACG **GAC GAC GAC AAG** ATG TGT CCT CAT ACT TAC AAA-3′; reverse, 5′-ACG **GAG GAG AAG CCC GG**T TTA CAA GCA AGC TTC TAG TGG-3′. The 5′-end of the primers incorporated the ligation-independent cloning (LIC) sequences (shown in bold). The amplified product was ligated into the expression vector pET-32 Ek/LIC (Novagen, USA) after being treated with LIC-qualified T4 DNA polymerase. This created a fusion with an N-terminal thioredoxin tag, His tag, thrombin cleavage site, S-tag and an enterokinase cleavage site followed by the CrSPI-1 protein. The plasmid was then transformed into *Escherichia coli* strain DH5α and the positive clones were confirmed by colony PCR with gene-specific/vector-specific primers and DNA sequencing. In order to express the CrSPI-1 protein, *E. coli* expression strain BL21 (DE3) was transformed with the recombinant plasmid. The successfully transformed *E. coli* were picked from a single colony and grown overnight at 310 K in Luria–Bertani (LB) medium (0.5% yeast extract, 1% Bacto tryptone, 1% NaCl) supplemented with 100 µg ml^{-1} ampicillin. The culture was induced with 300 µ*M* isopropyl β-D-1-thiogalactopyranoside for 4 h at an $OD_{600\,nm}$ of 0.6. After induction, the bacterial cells were harvested by centrifugation at 6000*g* for 10 min. This pellet was resuspended in phosphate-buffered saline pH 7.4 (PBS buffer; 137 m*M* NaCl, 2.7 m*M* KCl and 10 m*M* phosphate) and sonicated. The sonicated cells were centrifuged at 18 000*g* for 45 min to remove insoluble material. The supernatant was bound to Ni–NTA (Novagen, USA) matrix pre-equilibrated with PBS pH 7.4, 10 m*M* β-mercaptoethanol (BME). The protein bound to nickel–NTA affinity beads (Qiagen) was washed with equilibration buffer containing 0.1% Triton X-100 to remove nonspecifically bound impurities. Recombinant CrSPI-1 fusion protein was eluted with four column volumes of 1×PBS pH 7.4 containing 300 m*M* imidazole. The protein was eluted in 300 m*M* imidazole. The fusion tags were cleaved by a 2 h incubation with 0.1 U enterokinase (Sigma) at room temperature.

2.2. Characterization of CrSPI-1

The purified CrSPI-1 was 84 residues in length. Native and SDS polyacrylamide gel electrophoresis (SDS–PAGE) were performed to estimate the purity of CrSPI-1 prior to cocrystallization with subtilisin. Subtilisin was obtained from Sigma in the lyophilized form. 18 mg subtilisin was dissolved in 1 ml 9 mg ml^{-1} CrSPI-1 kept in 1×PBS (137 m*M* NaCl, 2.7 m*M* KCl and 10 m*M* phosphate) pH 7.4 in an approximate molar ratio of 1.2:1 (inhibitor:protease). The mixture was then incubated for 1 h at 310 K. The complex was then passed through a Superdex-75 gel-filtration column (Amersham Pharmacia Biotech, USA) and the fractions were pooled and concentrated to 20 mg ml^{-1}. The complex fractions eluted at a volume corresponding to a heterotrimer (Fig. 1). 1 µl CrSPI–subtilisin complex (20 mg ml^{-1}) was resolved on 12% nonreducing SDS–PAGE and stained with Coomassie Blue. CrSPI-1 and subtilisin were resolved on the same gel, which served as a control (Fig. 2). The purified complex was further characterized by a dynamic light-scattering (DLS) experiment using different concentrations. DLS experiments performed on a DynaPro MS/X (Protein Solutions) instrument indicated the presence of homogeneous species in solution. The apparent molecular weight of 70 kDa observed in DLS experiments corresponded to the molecular weight of a heterotrimer consisting of two molecules of subtilisin and one molecule of CrSPI-1.

2.3. Crystallization

Crystallization screening was carried out manually using the hanging-drop vapour-diffusion method with Crystal Screen and Crystal Screen 2 (Hampton Research) and JBScreen Classic 1–10 (Jena BioScience, Germany) at room temperature (298 K). A total of 336 conditions were tried. The drops were set up in 24-well plates with 500 µl reservoir solution. Each 2 µl drop consisted of a mixture of 1 µl reservoir solution and 1 µl protein-complex solution. The initial crystallization condition was derived from Hampton Research Crystal Screen 2 condition No. 22 (0.1 *M* MES pH 6.5 and 12% polyethylene glycol 20 000). The condition was further optimized by varying the precipitant concentration from 8 to 15%. Diffraction-quality crystals were obtained after 5 d from an optimized crystallization condition consisting of 11%(*w*/*v*) polyethylene glycol 20 000 in 0.1 *M* MES pH 6.5 at 298 K. Crystals grew to approximate dimensions of 0.2 × 0.1 × 0.05 mm within 5 d.

2.4. Data collection

For data collection, the crystal was transferred to a cryoprotectant solution, which was prepared by mixing equal amounts of reservoir solution and 50% glycerol. The crystals were flash-cooled directly in liquid nitrogen. A complete data set was collected using an oscillation range of 0.5° and a crystal-to-detector distance of 200 mm on the X-29A synchrotron beamline at Brookhaven National Laboratories, Upton, USA. These crystals diffracted to a maximum resolution of

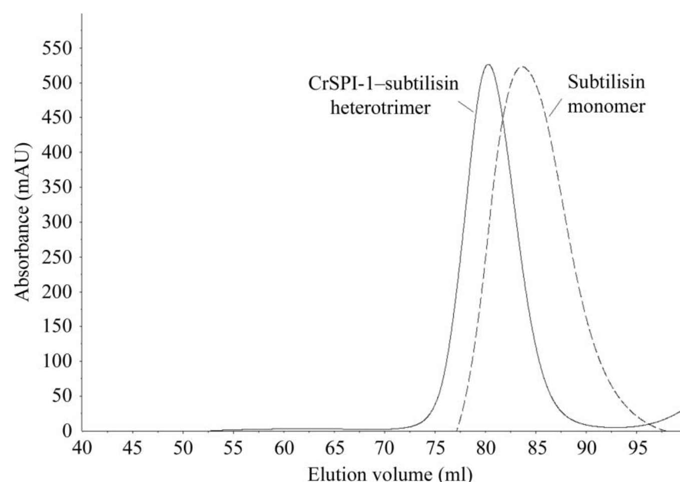

Figure 1
Gel-filtration profile of the CrSPI-1–subtilisin complex together with subtilisin as control run on a Superdex 75 column.

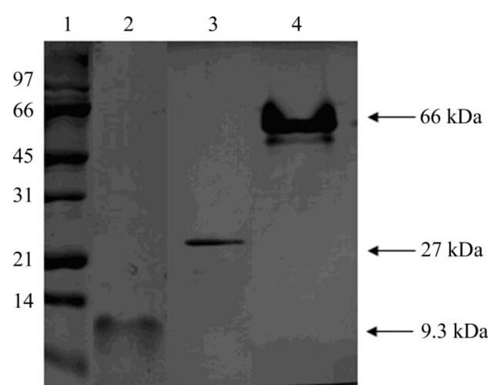

Figure 2
Nonreducing SDS gel of the CrSPI-1–subtilisin complex. Lane 1, protein markers (kDa); lane 2, CrSPI-1; lane 3, subtilisin; lane 4, CrSPI-1–subtilisin complex with a molecular weight corresponding to a 1:2 ratio of CrSPI-1:subtilisin. Notably, CrSPI-1 runs slow on this gel. The actual size of this recombinant protein is 9.3 kDa and has been verified by mass spectrometry.

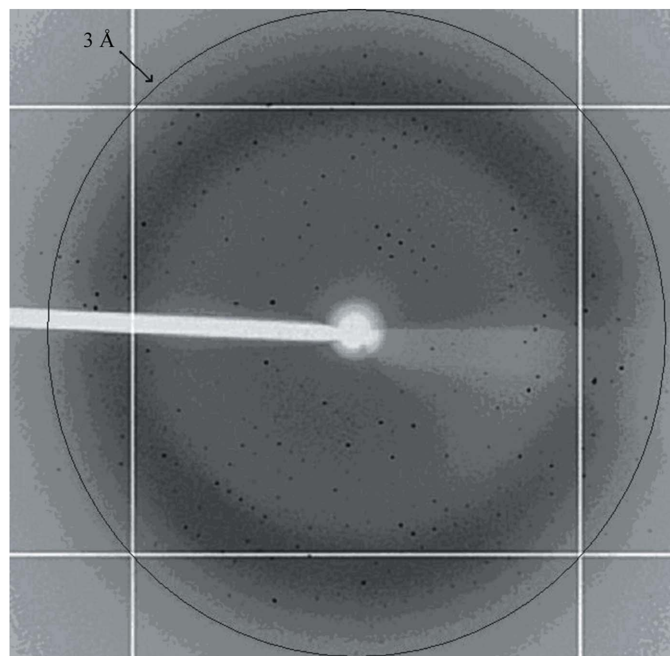

Figure 3
X-ray diffraction pattern of the CrSPI-1–subtilisin complex crystal. This crystal diffracted to 2.6 Å resolution. Data were collected on beamline X-29A at Brookhaven National Laboratories, USA.

2.6 Å. Fig. 3 shows a diffraction image of the crystal of the complex. Data-collection statistics are provided in Table 1. The data set was processed using the *HKL*-2000 program suite (Otwinowski & Minor, 1997). The Matthews constant (Kantardjieff & Rupp, 2003) was calculated using the *CCP*4 package. The structure solution was obtained using the molecular-replacement method with the program *MOLREP* (Vagin & Teplyakov, 1997). Initial refinement was carried out using *REFMAC* (Murshudov *et al.*, 1997) and *CNS* (Brünger *et al.*, 1998).

3. Results and discussion

CrSPI-1 is a double-domain Kazal-type inhibitor that is exclusively expressed in the hepatopancreas of *Carcinoscorpius rotundicauda*. The two-domain inhibitor is involved in the immunomodulation of host serine proteases such as CrFurin and defence against the bacterial protease subtilisin (Jiang *et al.*, 2009). Sequence analysis shows that both domains of CrSPI-1 belong to the nonclassical family of Kazal inhibitors owing to their unusual disulfide pattern (Hemmi *et al.*, 2005). The characterization and crystallization of the CrSPI-1–subtilisin complex will lead to structure determination and this will aid in understanding the inhibition mechanism of CrSPI-1.

CrSPI-1 was cloned and overexpressed in *E. coli*. The protein was obtained in the soluble fraction and purified to homogeneity by single-step affinity chromatography; it was subsequently complexed with subtilisin. The complex was crystallized by the hanging-drop vapour-diffusion method. Preliminary analysis of the X-ray diffraction data suggests that the crystals belong to space group $P2_1$, with unit-cell parameters a = 73.8, b = 65.0, c = 111.9 Å, β = 95.4°. The Matthews coefficient V_M of 2.64 Å^3 Da^{-1} best corresponds to the presence of a heterotrimer (1:2 inhibitor:protease ratio) in the asymmetric unit with 53% solvent content. A total of 208 523 observed reflections were merged to 31 832 unique reflections in the 50.0–2.6 Å resolution range. The overall completeness of the data was 97.8%, with an R_{merge} of 10.7%. Structure solution was obtained by the molecular-replacement method using the program *MOLREP* (Murshudov *et al.*, 1997). The atomic coordinates of subtilisin Carlsberg from *Bacillus licheniformis* (PDB code 1sca) were used as the starting search model. The structure solution clearly indicates that there are two subtilisin molecules bound to one inhibitor molecule, *i.e.* each subtilisin molecule binds each of the two domains of the CrSPI-1 inhibitor molecule (1:2 ratio of CrSPI-1:subtilisin) and one free subtilisin molecule in the asymmetric unit. We are currently in the process of refining the structure using *CNS* (Brünger *et al.*, 1998).

Table 1
Data-collection statistics.

Wavelength (Å)	0.9600
Resolution limits (Å)	50.0–2.6 (2.69–2.6)
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 73.8, b = 65.0, c = 111.9, $\alpha = \gamma$ = 90, β = 95.4
Total No. of observed reflections	208523
No. of unique reflections	31832 (2756)
R_{merge}†	0.107 (0.326)
Completeness (%)	97.8 (86.0)
Redundancy	6.6 (4.2)
Overall $I/\sigma(I)$	5.1 (2.3)

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

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