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Crystallization and preliminary X-ray diffraction analysis of hemextin A: a unique anticoagulant protein from *Hemachatus haemachatus* venom

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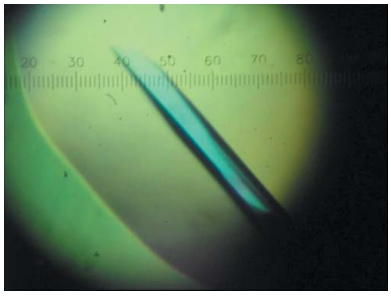
Hemextin A was isolated and purified from African Ringhals cobra (*Hemachatus haemachatus*). It is a three-finger toxin that specifically inhibits blood coagulation factor VIIa and clot formation and that also interacts with hemextin B to form a unique anticoagulant complex. Hemextin A was crystallized by the hanging-drop vapour-diffusion method by equilibration against 0.2 *M* ammonium acetate, 0.1 *M* sodium acetate trihydrate pH 4.6 and 30% PEG 4000 as the precipitating agent. The crystals belong to space group $P2_12_12_1$, with unit-cell parameters a = 49.27, b = 49.51, c = 57.87 Å and two molecules in the asymmetric unit. They diffracted to 1.5 Å resolution at beamline X25 at BNL.

1. Introduction

Snake venoms are veritable gold mines of pharmacologically active polypeptides and proteins (Kini, 2003; Koh *et al.*, 2006; Marsh & Williams, 2005). The venom from a single species of snake may contain several hundred different proteins, which may however be classified into a small number of superfamilies. The three-finger family, a toxin superfamily, contains proteins with molecular weights in the approximate range 6000–8000 Da and encompasses non-enzymatic polypeptides containing 60–74 amino-acid residues that confer lethality to *Elapidae* and *Hydrophidae* venoms (Kini, 2002). Proteins belonging to this family contain four or five disulfide bridges, of which four are conserved in all members. All proteins belonging to this family show a similar pattern of protein folding; they adopt a leaf-like shape comprising of three adjacent loops ('fingers') forming a large and flat β-pleated sheet that emerges from a small globular core confined by four conserved disulfide bridges, hence their name. Despite their similarity in structure, these proteins differ from each other in their biological activities. Members of this family include nicotinic and muscarinic toxins with selectivity towards distinct types of nicotinic and muscarinic acetylcholine receptors, fasciculins that inhibit acetylcholinesterase (Marchot, 1999; Taylor *et al.*, 1995), calciseptins that block the L-type calcium channels (Joseph *et al.*, 2004), cardiotoxins (cytotoxins) that exert their toxicity by forming pores in cell membranes (Kumar *et al.*, 1996; Menez *et al.*, 1990) and dendroaspins, which are antagonists of various cell-adhesion processes (Williams *et al.*, 1993).

Recently, we isolated and characterized a novel anticoagulant protein complex (hemextin A and B) from the venom of the elapid snake *Hemachatus haemachatus* (African Ringhals cobra; Banerjee *et al.*, 2005*a*,*b*). Hemextins A and B belong to the three-finger toxin family of snake-venom proteins. Individually, hemextin A exhibits mild anticoagulant activity, whereas hemextin B is inactive. However, hemextin B synergistically enhances the anticoagulant activity of hemextin A and their complex has potent anticoagulant activity. Thus, the formation of this unique synergistic complex of three-finger toxins is important for its ability to inhibit clot initiation. There are only a few noncovalent protein complexes in snake venoms that do not contain phospholipase A_2 as an integral part, such as rhodocetin (Wang *et al.*, 1999) and pseutarin C (Rao *et al.*, 2003). The hemextin AB complex is the only known snake-venom protein complex formed



by the interaction between two three-finger toxins and is the only known heterotetrameric complex of three-finger toxins. Here, we report the crystallization and preliminary diffraction studies of hemextin A, a unique anticoagulant protein, with the aim of understanding its structure and the function of this three-finger toxin in the anticoagulant process.

2. Methods and results

2.1. Purification of hemextin A

Hemextin A was purified using previously described methods (Banerjee *et al.*, 2005*b*). Briefly, crude *H. haemachatus* venom (100 mg in 1 ml distilled water) was applied onto a Superdex 30 gel-filtration column (1.6 × 60 cm) equilibrated with 50 m*M* Tris–HCl buffer pH 7.4 and eluted with the same buffer using an ÄKTA Purifier system (Amersham Biosciences, Uppsala, Sweden). Fractions containing potent anticoagulant activity were pooled and sub-fractionated on a Uno S-6 (Bio-Rad, Hercules, CA, USA; 6 ml column volume) cation-exchange column. The peaks containing hemextin A were further purified using RP-HPLC on a Jupiter C18 (1 × 25 cm) column (Fig. 1*a*). The protein was found to be homogeneous, with a molecular weight of 6835.00 ± 0.52 Da as determined by electrospray ionization mass spectrometry (ESI–MS; Fig. 1*c*).

The presence of hemextin A was confirmed using size-exclusion chromatography (SEC). All SEC experiments were carried out at room temperature on a pre-packed Superdex 75 gel-filtration column (1.6 × 60 cm) using the same ÄKTA Purifier system. The column was eluted with 50 m*M* Tris–HCl buffer pH 7.4 at a flow rate of 1 ml min^{-1}. The sample volume applied onto the column was 4 ml.

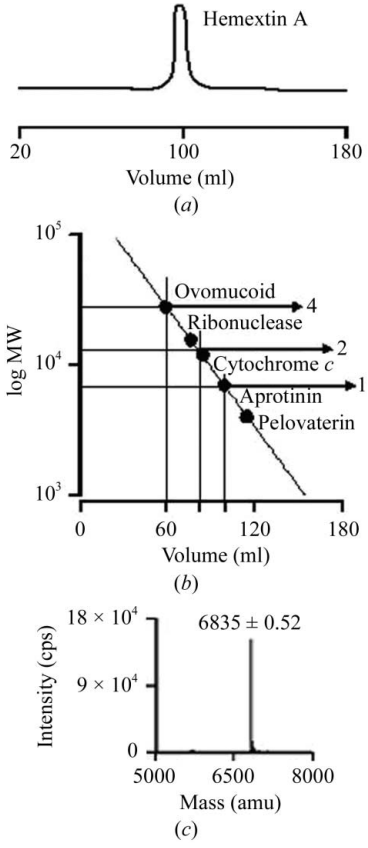


Figure 1
(*a*) Size-exclusion chromatography elution profile of hemextin A. (*b*) Molecular-weight standards. (*c*) Mass spectrum of hemextin A.

The column was calibrated using ovomucoid (28 kDa), ribonuclease (15.6 kDa), cytochrome *c* (12 kDa), apoprotinin (7 kDa) and pelovaterin (4 kDa) as molecular-weight markers (Fig. 1*b*). The void volume was determined by running Blue Dextran. The column was equilibrated with at least two bed volumes of elution buffer prior to each run.

2.2. Crystallization

Crystallization trials for hemaxtin A were conducted using the hanging-drop vapour-diffusion method and a wide range of conditions were tested using Hampton Research Crystal Screens I and II. The protein concentration was kept at approximately 30 mg ml^{-1} and the drops were prepared by mixing equal volumes (1 µl) of protein solution in 50 m*M* Tris buffer pH 7.4 and crystallization solution. The screens were set up at 295 K using VDX plates from Hampton Research. 500 µl reservoir solution was placed in each well. The initial screen identified a PEG-based crystallization condition. By systematic optimization around the preliminary condition, we obtained diffraction-quality crystals of hemextin A. The best crystals were from a condition consisting of 0.2 *M* ammonium acetate, 0.1 *M* sodium acetate trihydrate, 30%(*w*/*v*) PEG 4000 pH 4.6. The rod-shaped crystals appeared after 12–15 d (Fig. 2).

2.3. Data collection

We have collected a complete native data set for hemextin A. Prior to data collection, crystals of hemaxtin A were briefly soaked in a cryoprotectant solution consisting of reservoir solution supplemented with 25%(*w*/*v*) glycerol, picked up with a nylon cryo-loop and frozen at 100 K in a nitrogen-gas cold stream (Cryostream cooler; Oxford Cryosystems, Oxford, England). Synchrotron data were collected at beamline X25, NSLS, Brookhaven National Laboratory. Data sets were collected using a Quantum 4 CCD detector and were processed using *HKL*-2000 (Otwinowski & Minor, 1997; Table 1). The crystals diffract to 1.5 Å resolution. The space group was $P2_12_12_1$, with unit-cell parameters a = 49.27, b = 49.51, c = 57.87 Å. The Matthews coefficient V_M of 2.92 Å^3 Da^{-1} (Matthews, 1968) is within the expected range for two monomers in the asymmetric unit and corresponds to a solvent content of 57.89%.

3. Discussion

Hemextin A, a three-finger toxin, was successfully purified from crude *H. haemachatus* venom. As shown in Fig. 1(*a*), hemextin A exists as a monomer in solution. Hemextin A was crystallized using

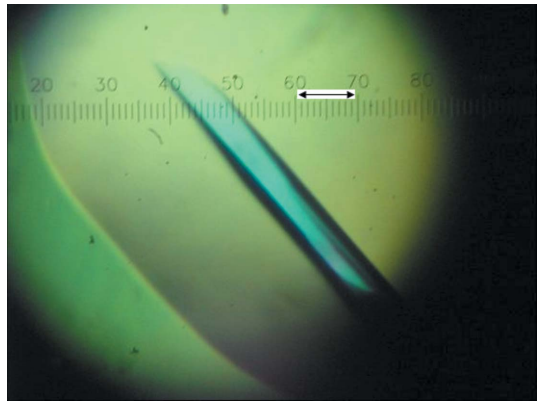


Figure 2
Crystal of hemextin A. Ten divisions of the scale is equivalent to 0.17 mm.

Table 1
Data-collection statistics for hemextin A.

Values in parentheses are for the highest resolution shell.

Beamline	X25, NSLS, BNL
Wavelength (Å)	0.97950
Oscillation range (°)	1
Space group	$P2_12_12_1$
Unit-cell parameters	
a (Å)	49.27
b (Å)	49.51
c (Å)	57.87
Resolution limits (Å)	50–1.5 (1.55–1.50)
No. of molecules in ASU	2
Observed *hkl*	287505
Unique *hkl*	23374
Redundancy	12.3
Completeness (%)	100 (100)
Overall $I/\sigma(I)$	10.5
R_{sym}† (%)	0.071 (0.26)

† $R_{sym} = \sum |I_i - \langle I \rangle| / \sum |I_i|$, where I_i is the intensity of the ith measurement and $\langle I \rangle$ is the mean intensity of the reflection.

the hanging-drop vapour-diffusion method. The X-ray diffraction data showed that the crystal belongs to the orthorhombic system, space group $P2_12_12_1$, with two molecules in the asymmetric unit. Using a three-finger toxin homology structure (PDB code 1ug4) as a search model, the rotation function gave two clear solutions corresponding to two molecules in the asymmetric unit. The translation function calculated in the resolution range 8–4 Å using the program *AMoRe* (Navaza, 1994) had two peaks that were significantly above the background and were of nearly equal height. The translation function confirmed that each of these peaks corresponded to an orientation of one of the two molecules in the asymmetric unit. Rigid-body refinement performed with *AMoRe* after combining the two solutions gave a correlation coefficient of 0.62 and an *R* factor of 0.40. We are now in the process of building the model and refining the structure. The structure of hemextin A will help in detailed understanding of its anticoagulant activity.

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References

Banerjee, Y., Mizuguchi, J., Iwanaga, S. & Kini, R. M. (2005*a*). *Pathophysiol. Haemost. Thromb.* **34**, 184–187.
Banerjee, Y., Mizuguchi, J., Iwanaga, S. & Kini, R. M. (2005*b*). *J. Biol. Chem.* **280**, 42601–42611.
Joseph, R., Pahari, S., Hodgson, W. C. & Kini, R. M. (2004). *Curr. Drug Targets Cardiovasc. Haematol. Disord.* **4**, 437–459.
Kini, R. M. (2002). *Clin. Exp. Pharmacol. Physiol.* **29**, 815–822.
Kini, R. M. (2003). *Toxicon*, **42**, 827–840.
Koh, D. C., Armugam, A. & Jeyaseelan, K. (2006). *Cell. Mol. Life Sci.* **63**, 3030–3041.
Kumar, T. K., Lee, C. S. & Yu, C. (1996). *Adv. Exp. Med. Biol.* **391**, 115–129.
Marchot, P. (1999). *J. Soc. Biol.* **193**, 505–508.
Marsh, N. & Williams, V. (2005). *Toxicon*, **45**, 1171–1181.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Menez, A., Gatineau, E., Roumestand, C., Harvey, A. L., Mouawad, L., Gilquin, B. & Toma, F. (1990). *Biochimie*, **72**, 575–588.
Navaza, J. (1994). *Acta Cryst.* A**50**, 157–163.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Rao, V. S., Swarup, S. & Kini, R. M. (2003). *Blood*, **102**, 1347–1354.
Taylor, P., Radic, Z., Hosea, N. A., Camp, S., Marchot, P. & Berman, H. A. (1995). *Toxicol. Lett.* **82–83**, 453–458.
Wang, R., Kini, R. M. & Chung, M. C. (1999). *Biochemistry*, **38**, 7584–7593.
Williams, J. A., Lu, X., Rahman, S., Keating, C. & Kakkar, V. (1993). *Biochem. Soc. Trans.* **21**, 73S.