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Isolation, purification, crystallization and preliminary crystallographic studies of sagitoxin, an oligomeric cardiotoxin from the venom of *Naja naja saggitifera*

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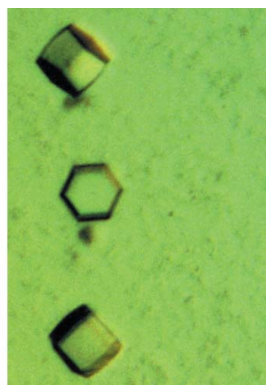
Sagitoxin, a novel cardiotoxin from the venom of *Naja naja saggitifera*, has been successfully isolated, purified to homogeneity and crystallized. The toxin was purified using successive separation steps on a CM-Sephadex C-50 column and a reverse-phase column. The 6.75 kDa toxin was sequenced by the Edman method using a PPSQ-21 protein sequencer. It was crystallized using the hanging-drop vapour-diffusion method. The hexagonal-shaped crystals diffracted to 3.0 Å resolution and belonged to space group $P6_4$, with unit-cell parameters $a = b = 111.1$, $c = 137.3$ Å, $\gamma = 120°$. There are 36 molecules in the unit cell, which has an approximate solvent content of 80%. Structure determination revealed that the molecules of sagitoxin associate in a hexameric form and create a pore in the centre which has functional significance.

1. Introduction

Cobra-venom cardiotoxins, which are the most abundant component of cobra venoms, belong to a family of all-β-sheet proteins (Housset & Fontecilla-Camps, 1996) which contain 60–70 residues. Cobra cardiotoxins are highly basic polypeptides that are effectively able to bind to both lipids and heparin and cause systolic cardiac arrest in victims of cobra snakebite (Dufton & Hider, 1988; Bougis *et al.*, 1981). They primarily act by binding to cell membranes, causing depolarization of the cardiomyocytes (Harvey & Hayashi, 1987; Hodges *et al.*, 1987), or by binding to phospholipids, causing fusion of sphingomyelin vesicles (Chien *et al.*, 1991). Cardiotoxins have also been shown to demonstrate anticancer activity (Chien *et al.*, 2008; Tsai *et al.*, 2006; Yang *et al.*, 2005) owing to their ability to induce apoptosis in cancer cells. Cardiotoxins can be classified into two forms, P- and S-types, depending on the presence of either proline at position 30 or serine at position 28, respectively, which are responsible for differential phosphotidylcholine membrane-binding activities (Chien *et al.*, 1994).

Although the crystal structures of several common cardiotoxins are known (Chen *et al.*, 2005; Lee *et al.*, 2005; Forouhar *et al.*, 2003; Sun *et al.*, 1997; Bilwes *et al.*, 1994; Rees *et al.*, 1990), the precise structure–function relationship of this molecule has not yet been defined. Here, we report for the first time the isolation, purification and preliminary crystallographic studies of sagitoxin, a novel oligomeric cardiotoxin from the Indian cobra subspecies *Naja naja saggitifera*.



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2. Materials and methods

2.1. Sagitoxin purification

The venom of *N. naja saggitifera* (South Indian Cobra) was obtained from Irula Cooperative Snake Farm, Tamil Nadu, India. 250 mg venom was dissolved in 20 ml 25 m*M* Tris–HCl buffer pH 8.0 and centrifuged at 8000*g* for 15 min to remove insoluble material. The supernatant was loaded onto a CM-Sephadex C-50 column (25 × 2 cm) which was equilibrated with 25 m*M* Tris–HCl pH 8.0. The bound protein was eluted with a continuous gradient of NaCl (0.0–1.0 *M* NaCl) in 25 m*M* Tris–HCl buffer pH 8.0. The peak eluted at 0.6 *M* NaCl was pooled and gel filtered using a Sephadex G-50 column (100 × 1.5 cm). The column was equilibrated with 25 m*M* Tris–HCl pH 8.0. The flow rate was adjusted to 5 ml h^{-1}. The samples of toxin were dialyzed against distilled water, applied onto a Vydac

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Space group	$P6_4$
Unit-cell parameters (Å, °)	$a = b = 111.1$, $c = 137.3$, $\gamma = 120$
No. of molecules in the unit cell	36
V_M (Å^3 Da^{-1})	6.0
Solvent content (%)	80
Resolution range (Å)	20.00–3.0 (3.90–3.0)
Total No. of measured reflections	191664
No. of unique reflections	16910
Completeness (%)	98.5 (90.3)
R_{merge} (%)	12 (42)
$I/\sigma(I)$	4.0 (1.8)

C18 analytical reverse-phase (RP) HPLC column (218TP54, 4.6 × 250 mm) and eluted at a flow rate of 1 ml min^{-1} with a linear gradient of 0–40% buffer *B* [acetonitrile containing 0.1%(*v*/*v*) trifluoroacetic acid] over 50 min after an equilibrium period of 3 min with buffer *A* [distilled water containing 0.1%(*v*/*v*) trifluoroacetic acid]. The complete amino-acid sequence of sagitoxin has been determined by Edman degradation using a PPSQ-21 amino-acid sequencer and has been deposited in the Swiss-Prot database with entry code P83345.

2.2. Sagitoxin crystallization

Sagitoxin was crystallized using the hanging-drop vapour-diffusion method at 291 K using Hampton Research Crystal Screen HR2-108. The protein samples were dissolved to a concentration of 20 mg ml^{-1} in bis-tris propane pH 7.0 buffer. Small crystals were obtained within 7 d of initial screening using sodium chloride as precipitant in bis-tris propane pH 7.0 buffer. This condition was further refined to produce improved crystals by adding 2.5% methylpentanediol to the protein drop and allowing it to equilibrate against reservoir solution containing 5% methylpentanediol. Hexagonal crystals formed within 14 d and grew to maximum dimensions of 0.25 × 0.1 × 0.1 mm (Fig. 1).

2.3. Data collection and processing

A complete data set for sagitoxin was collected using a MAR Research 345 imaging-plate scanner mounted on a rotating-anode X-ray generator operating at 50 kV and 100 mA. Data were collected at 283 K using a crystal-to-detector distance of 250 mm with a rotation range of 1.0° for each image and an exposure time of 20 min per image. The crystals diffracted to 3.0 Å resolution and belonged to space group $P6_4$, with unit-cell parameters $a = b = 111.1$, $c = 137.3$ Å, $\gamma = 120°$. The crystals showed a mosaicity of 0.95°. Assuming the presence of 36 molecules of cardiotoxin in the unit cell, the Matthews coefficient (Matthews, 1968) was calculated to be 6.0 Å^3 Da^{-1}, which corresponds to a solvent content of approximately 80%. Data processing and reduction were performed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). Crystal parameters and diffraction data statistics are summarized in Table 1. The structure of cardiotoxin from *N. naja atra* (PDB code 2bhi; Wang *et al.*, 2006) was used as a model for molecular replacement. A number of attempts were made to solve the structure of sagitoxin using various molecular-replacement programs as implemented in the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994) with various monomeric, dimeric and trimeric models of known cardiotoxin structures (Chen *et al.*, 2005; Lee *et al.*, 2005; Sun *et al.*, 1997; Rees *et al.*, 1990; Wang *et al.*, 2006; Bilwes *et al.*, 1994; Forouhar *et al.*, 2003). However, these attempts were not successful. The structure solution was finally obtained with the program *Phaser* (McCoy *et al.*, 2005) using a single-molecule model (PDB code 2bhi). The successful solution has a *Z* score of 21.6, which is an indicator of a good solution. Further calculations using the self-rotation function (Rossmann & Blow, 1962) revealed the presence of sixfold noncrystallographic symmetry in the asymmetric unit, indicating the presence of a hexameric form of the cardiotoxin. This is the first time a hexameric form of cardiotoxin has been observed. It also revealed the formation of a pore filled with solvent molecules. Further efforts to refine the structure are in progress.

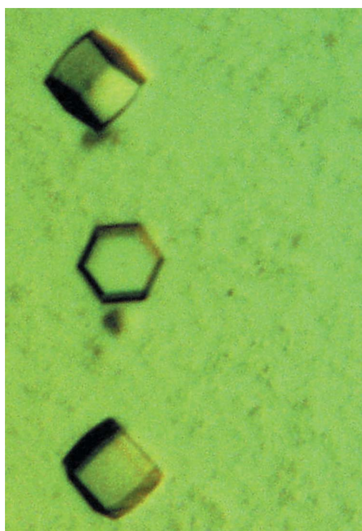

Figure 1
Crystals of sagitoxin.

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