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Crystallization and preliminary X-ray analysis of a depressant insect toxin from the scorpion *Buthus martensii* Karsch

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Depressant insect toxins are a distinct group of scorpion neurotoxins for which no three-dimensional structures are yet available. A depressant insect toxin named BmK dITAP3 from the scorpion *Buthus martensii* Karsch (BmK) has been purified and crystallized. Single crystals of dITAP3 grew in the presence of the detergent CYMAL-6 using the hanging-drop vapour-diffusion method with ammonium sulfate as precipitant. A set of diffraction data to 2.6 Å resolution has been collected. Preliminary analysis of the diffraction data indicated that the crystal belonged to space group *R*3, with unit-cell parameters $a = b = 73.29$, $c = 68.90$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. Assuming two molecules in the asymmetric unit, the estimated solvent content is 53.4%.

1. Introduction

The Na^+-channel-specific toxins from scorpion venoms are peptides composed of 60–76 amino acids cross-linked by four disulfide bridges (Possani *et al.*, 1999). Of these, the toxins highly specific to insects are classified into two groups, the excitatory and the depressant toxins (Zlotkin, 1991). The excitatory toxins cause a fast contraction paralysis in injected animals and induce repetitive firing in insect nerves, while the depressant toxins cause a slow depressory flaccid paralysis owing to depolarization of the nerve membrane and blockage of the sodium conduction in axons (Zlotkin, 1991; Pelhate & Zlotkin, 1982; Zlotkin *et al.*, 1985). Because of their great value as a tool for studying the pharmacological properties of insect sodium channels and as a promising candidate in developing new bio-pesticides (Stewart *et al.*, 1991; Tomalski & Miller, 1991), these insect toxins have been widely studied by many research groups. However, the depressant toxins have been as yet less investigated.

In fact, of the Na^+-channel-specific toxins, the depressant toxins are currently the least-known. Although a series of three-dimensional structures of Na^+-channel toxins have been solved (Possani *et al.*, 1999) either by X-ray crystallography or NMR methods, including some mammalian toxins (*e.g.* Fontecilla-Camps *et al.*, 1988; Li *et al.*, 1996; He *et al.*, 1999, 2000) and excitatory insect toxins (Oren *et al.*, 1998; Darbon *et al.*, 1991), no structures of depressant toxins have yet been reported. According to the functional classification of Na^+-channel-specific toxins, the depressant insect toxins belong to a distinct group (Possani *et al.*, 1999). Recent investigation of various scorpion toxins showed that each toxin group binds to a distinct receptor site on the Na^+ channel, although they possess a general molecular scaffold (Gordon *et al.*, 1998). Therefore, it is significant to elucidate the fine structure of each toxin in the group so as to uncover the structural basis of the respective toxin–receptor binding specificity and functional determinant. Therefore, it is important to determine the three-dimensional structure of the depressant insect toxin by X-ray crystallography.

One of the most important problems that has hampered advances in the structural determination of the depressant insect toxin is the difficulty in preparing suitable single crystals, as samples with high purity and the correct fold are not easy to produce from native isolation or cDNA recombination. Recently, we have set up a suitable procedure to purify the depressant insect toxin BmK dITAP3 in sufficient quantity and purity from the venom of the scorpion *B. martensii* Karsch (BmK), which is widely distributed in East Asia. On the basis of this, the toxin dITAP3 has been crystallized. In this paper, we report the crystallization and preliminary X-ray analysis of this toxin, which has established a sound basis for the structural determination.

2. Experimental and results

2.1. Purification

The crude venom of the scorpion BmK was dissolved in 0.05 *M* NH_4HCO_3 and applied to a Sephadex G-50 column (2.6 × 150 cm) which was previously equilibrated with 0.05 *M* NH_4HCO_3. The second fraction from the G-50

Table 1
Crystal data and data-collection statistics.

Values in parentheses refer to the highest resolution shell (2.74–2.60 Å).

X-ray source	BL18B, PF
Wavelength (Å)	1.0
Resolution (Å)	2.6
Space group	*R*3
Unit-cell parameters (Å, °)	$a = b = 73.29$, $c = 68.90$, $\alpha = \beta = 90$, $\gamma = 120$
No. of observations	24087
No. of unique reflections	4246
$I/\sigma(I)$	7.0 (2.0)
Completeness (%)	99.7 (97.5)
$R_{\rm merge}$ (%)	9.7 (36.2)

column was then subjected to an SP Sephadex C-25 ion-exchange column (1.6 × 80 cm) pre-equilibrated with 0.02 *M* sodium phosphate buffer pH 6.5. The flowthrough peak from the SP C-25 column was pooled and desalted. Further separation was carried out by preparative isoelectrofocusing electrophoresis on an LKB 2117 Multiphor II system with Ultrodex granule gel in ampholytes pH range 3.5–9.5. The sample focused at pH 6.5 in the gel was recovered by eluting the gel with 5% acetic acid. The final step of the purification was completed on a Resource RPC 3 ml column (Pharmacia Biotech), which was eluted with a linear gradient of acetonitrile.

2.2. Bioassay

The anti-insect toxicity of BmK dITAP3 was tested with house-fly larvae as described previously (Zlotkin *et al.*, 1985). Fly larvae weighing 30 ± 2 mg were selected for the bioassay. The sample was dissolved in 0.9% sodium chloride solution and then injected into the lower part of the larva's abdomen at different doses. The larvae were the inspected for 5 min to see what effect would occur. The result showed that dITAP3 caused a fast contraction and then a delayed, slow and progressive depressant paralysis in the larvae, which are the typical symptoms of depressant toxicity. The FPU (flaccid paralysis unit, defined as the amount of toxin that causes half of the recipient larvae to become flaccid within 5 min of injection) value for dITAP3 was 0.5 µg per body (∼30 mg), which indicated that the depressant insect toxicity of dITAP3 was relatively weak.

Figure 1
Crystals of BmK dITAP3. The crystal shown in the photograph is of dimensions 0.1 × 0.1 × 0.1 mm.

2.3. Crystallization

It has taken a long time to obtain good crystals of BmK dITAP3. Firstly, the initial crystallizing conditions for dITAP3 were searched for using the Crystal Screen and Screen II kits (Hampton Research, USA), but no crystals were found. Therefore, a wide range of precipitants and pH values were searched for by the grid-screen method. Since the sample could be precipitated by ammonium sulfate easily, even at low concentration, combinations of ammonium sulfate with many kinds of organic solvents as well as the detergents in Detergent Screen Kit I (Hampton Research, USA) were tried. Crystals of dITAP3 were found under several conditions, but the quality was quite disappointing, with the crystals either being fragile or being strongly twinned. It took a long time to optimize the crystallization conditions. When detergents were introduced into the conditions, the situation was greatly improved. Finally, the best crystal (Fig. 1) was grown in a hanging drop consisting of 1 µl protein (5 mg ml^{-1}), 1 µl reservoir solution (1.0 *M* ammonium sulfate, 0.2 *M* sodium acetate pH 4.8) and 0.5 µl CYMAL-6 (0.56 m*M*), which was equilibrated with the same reservoir solution.

2.4. Crystallographic analysis and data collection

A single crystal of dITAP3 of dimensions 0.1 × 0.1 × 0.1 mm was used in the crystallographic analysis and data collection. The preliminary X-ray analysis showed that the number of molecules in the asymmetric unit was two and the solvent content estimated according to Matthews (1968) was 53.4%, corresponding to a $V_{\rm M}$ of 2.64 Å^3 Da^{-1}. The crystal of dITAP3 belongs to space group *R*3; the unit-cell parameters are listed in Table 1.

Diffraction data were collected at room temperature using synchrotron radiation (λ = 1.0 Å) on an ADSC Quantum 4 CCD detector at the BL18B experimental station in the Photon Factory (Tsukuba, Japan). The crystal-to-detector distance was set to 100.0 mm. The exposure time for each image was 70 s and an oscillation angle of 1.5° over a total range of 120° was used. Data processing and analysis were performed using the programs *DPS*/*MOSFLM*/*CCP*4 and *SCALA* (Nielsen *et al.*, 1998; Leslie, 1992; Collaborative Computational Project, Number 4, 1994). The data statistics are shown in Table 1. The structure determination is currently under way using this data set.

3. Discussion

Since there are four disulfide bridges in depressant toxins, it is very difficult to obtain the correctly refolded recombinant molecules at overexpression level for X-ray or NMR experiments. The purification from venom is an effective method at present. However, the isolation of dITAP3 from many components with pI values close to each other is crucial in the procedure. For this problem, ion-exchange chromatography even with a high-resolution column is not effective. Our experiment showed that the preparative isoelectrofocusing electrophoresis (pIEF) is a good method in this case. The dITAP3 purified from the pIEF provided suitable single crystals. The result indicates that the pIEF is very sensitive to the isolation of charge isomers.

To date, there is no report on the crystallization of depressant scorpion toxins, which involves some particular difficulties. In fact, in our laboratory we have crystallized a series of BmK scorpion toxins (Li *et al.*, 1996, 1999; He *et al.*, 1999, 2000), all of which could diffract to very high resolution. However, we encountered problems growing suitable crystals of dITAP3. Fragile or strongly twinned crystals often appeared in hanging drops when using conditions that succeeded in the crystallization of homologous molecules. The use of detergent played an important role in overcoming such difficulties. As shown in the results, when the detergent CYMAL-6 was introduced into conditions which only produced fragile crystals previously, perfect single crystals appeared. The X-ray diffraction analysis showed that the quality of these crystals was greatly improved. It seems that, in addition to their use with membrane proteins, detergents could also be useful in the optimization of soluble protein crystallization.

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