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Crystallization and preliminary X-ray analyses of insect neurotoxins with analgesic effect from the scorpion *Buthus martensii* Karsch

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Three insect neurotoxins from the scorpion *Buthus martensii* Karsch, named BmK I1, BmK I4 and BmK I6, have been purified and crystallized. BmK I1 and BmK I4 show strong toxicity to insects, while BmK I6 is relatively weaker. They all exhibit an evident analgesic effect on mice; this is a novel biological function for scorpion insect toxins. Their crystals diffract to at least 3.5 (BmK I1), 2.8 (BmK I4), 2.8 (BmK I6 crystal form I) and 2.2 Å (of BmK I6 crystal form II) resolution on an ordinary X-ray source. Crystals of BmK I1 belong to space group *P*6, with unit-cell parameters $a = b = 66.2$, $c = 176.7$ Å. BmK I4 crystallized in the tetragonal space group *I*4, with unit-cell parameters $a = b = 134.5$, $c = 60.6$ Å. BmK I6 has been crystallized in two forms: form I belongs to space group *C*2, with unit-cell parameters $a = 46.5$, $b = 85.2$, $c = 32.6$ Å, $\beta = 110.5^\circ$; form II belongs to space group *R*3, with the hexagonal unit-cell parameters $a = b = 44.5$, $c = 164.7$ Å.

1. Introduction

Scorpion venoms are rich sources of various neurotoxins that bind to ionic channels at the surface of excitable cells and diversely modify their normal properties. These toxins are specific to mammals, insects and crustaceans (Possani, 1984; Gordon *et al.*, 1998). The toxins directed at insects are referred to as 'insect toxins' in this paper. Long-chain insect toxins, containing 60–80 residues, are able to bind to insect sodium channels (Gordon *et al.*, 1992; Zlotkin *et al.*, 1995). The investigation of these insect toxins is of great value from both academic and applicational viewpoints, in particular the investigation of the mechanism of their binding to sodium channels (Gordon, 1997). These potent toxins are an invaluable tool in developing pest-controlling transgenic plants and designing insecticides with higher performance and fewer side effects (Stewart *et al.*, 1991; Gurevitz *et al.*, 1996; Gershburg *et al.*, 1997). Therefore, the elucidation of the insect toxins' three-dimensional structures and structure–function relationship are of importance.

Recently, we obtained three insect toxins, BmK I1, I4 and I6, from the venom of the scorpion *B. martensii* Karsch (BmK), widely distributed in China, which displayed a 50-fold range in anti-insect toxicity *in vivo*. Very interestingly, these toxins display evident analgesic effects but are devoid of any toxicity to mice as shown by bioassay; this has never previously been reported for scorpion insect toxins. The analgesic activities of the three toxins are also different. Therefore, this series of BmK scorpion toxins forms a valuable bioactivity-distinctive system. Though all analgesic chemicals in common use, such as morphine, heroin and barbitone, are effective, they also have side effects, especially the addictive narcotic drugs. If the mechanism of the analgesic effect of this series of BmK toxins is elucidated, a small peptide designed to mimic such toxins should aid in the search for a potential medicine for analgesia without the danger of addiction. Because of this, the three-dimensional structures of the BmK toxins are of great significance.

Since the first insect toxin, AaH IT, was purified (Zlotkin *et al.*, 1971), many such toxins have been purified and characterized (Possani *et al.*, 1999). However, until now only one crystal structure of an insect toxin, Bj-xtrIT (Oren *et al.*, 1998), has been reported. Fortunately, the three BmK toxins described above have been crystallized in our laboratory. Furthermore, the gene encoding BmK I4 has been cloned (Xiong *et al.*, 1999) and expressed (unpublished results). Obviously, the next step is the three-dimensional structure determination. Here, we report the crystallization and preliminary X-ray analyses of these three toxins.

2. Experiments and results

2.1. Purification and characterization

BmK I1, BmK I4 and BmK I6 were purified from the venom of BmK scorpions from the Henan Province of China. The typical purification procedure is similar to that described

Table 1
Crystallization conditions for BmK I1, BmK I4 and BmK I6.

Sample	BmK I1	BmK I4	BmK I6 (I)	BmK I6 (II)
Solution *A* (mg ml^{-1} protein)	20	20	20	20
Solution *B*	1.4 *M* $(NH_4)_2SO_4$, 0.1 *M* CHES†	2.7 *M* $(NH_4)_2SO_4$, 0.1 *M* Tris	2.2 *M* $(NH_4)_2SO_4$, 0.2 *M* NaAc‡	0.8 *M* sodium citrate, 0.2 *M* NaAc
Solution *C*	4 m*M* Zwittergent 3-10	Dioxane	2-propanol	2-propanol
pH	9.5	8.5	5.0	5.0
Drop	2 µl *A* + 2 µl *B* + 0.6 µl *C*	2 µl *A* + 2 µl *B*	2 µl *A* + 2 µl *B*	2 µl *A* + 2 µl *B*
Reservoir	600 µl *B*	600 µl *B* + 30 µl *C*	600 µl *B* + 60 µl *C*	600 µl *B* + 60 µl *C*
Temperature (K)	287	295	295	295
Time	< One week	1–2 weeks	< One week	One month
Crystal size (mm)	0.3 × 0.15 × 0.15	0.3 × 0.1 × 0.1	0.4 × 0.1 × 0.1	0.5 × 0.3 × 0.2

† CHES, 2-(*N*-cyclohexylamino)-ethanesulfonic acid. ‡ NaAc, sodium acetate.

previously (Xiong *et al.*, 1999). The products were analyzed on a C8 reverse-phase column on the ÄKTA Purifier system (Pharmacia, Sweden), which showed that all the resulting toxins possessed purities of higher than 90%. Compared with other insect toxins, these three toxins are rather acidic; their isoelectric points (pI) are 4.0, 4.3 and 6.5 for BmK I1, I4 and I6, respectively, as determined by isoelectrofocusing PAGE. The molecular masses of BmK I1, I4 and I6 obtained from the MALDI–TOF mass spectrum on a BIFLEX III-MS System (Bruke, USA) are 8141.0, 8149.3 and 6722.2 Da, respectively.

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          1                                                  50
Bj-xtrIT  KKNGYPLDRN GKTTECSGVN AIAPHYCNSE CTKVYYAESG YCCWGACYCF
              .. ..   ..  ...  .....   .          ..      ...
 BmK I4   KKNGYAVDSS GKVAECLFNN ------YCNNE CTKVYYADKG YCCLLKCYCF

                                    77
Bj-xtrIT  GLEDDKPIGP MKDITKKYCD VQII-PS
            .    ... .. .  .         ..
 BmK I4   GLADDKPVLD IWDSTKNYCD VQIIDLS
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Figure 1
Amino-acid sequence of BmK I4 and comparison with Bj-xtrIT from the scorpion *Buthotus judaicus* (Oren *et al.*, 1998).

Analysis of amino-acid components showed BmK I1, I4 and I6 contained 72, 72 and 61 residues; they are all crosslinked by four disulfide bridges. The full sequence of BmK I4 has been determined by protein analysis and cDNA sequencing (Xiong *et al.*, 1999). The sequence shows about 61% homology with that of Bj-xtrIT from the scorpion *Buthotus judaicus* (Fig. 1). At the present time, only 15 residues at the N-terminals of BmK I1 and I6 have been sequenced; their cDNA clonings are in progress.

The insect-specific toxicities of the purified toxins were tested on the larvae of house flies weighing about 30 mg, as described previously (Zlotkin *et al.*, 1985; Xiong *et al.*, 1999). The CPU (contraction paralysis unit) was defined as the acute dose that caused half of the recipient larvae to contract in 5 s. The toxins strongly induced a sustained contraction paralysis when injected into the fly larvae and their CPU values were 0.02, 0.01 and 0.5 µg per body for BmK I1, I4 and I6, respectively. Their relative toxicities are 25:50:1. They all also displayed an obvious analgesic effect on mice. The analgesic activity was assessed by a mouse-twisting model (Fennessy & Lee, 1975) using a procedure reported previously (Xiong *et al.*, 1999). At a dose of 1 mg kg^{-1}, BmK I4 exhibited a 54.4% inhibition of the mouse-twisting reaction; however, BmK I1 and I6 exhibited 47.0 and 42.7% inhibition efficiency, respectively, at a dose of 5 mg kg^{-1}. When using a painkiller (Tranquilizer), a dose of 80 mg kg^{-1} only displayed 62.4% inhibition, which was much less potent than these toxins. Interestingly, when 10 mg kg^{-1} toxins were injected into mouse through its tail vein, no paralytic symptoms were observed. Thus, these toxins were devoid of mammalian neurotoxicity.

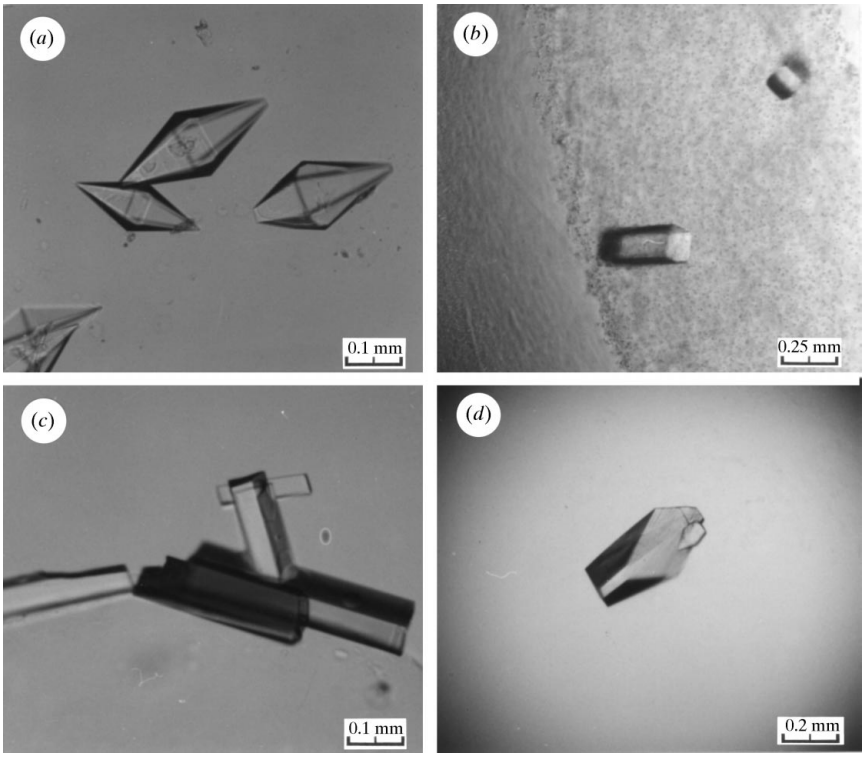

Figure 2
Crystal photographs of (*a*) hexagonal BmK I1, (*b*) tetragonal BmK I4, (*c*) monoclinic BmK I6 and (*d*) rhombohedral BmK I6.

2.2. Crystallization

Initially, the Crystal Screen and Crystal Screen II kits (Hampton Research, USA) were used to screen the initial crystallizing conditions for BmK I1, BmK I4 and BmK I6; however, no crystals were found. With the solubility information provided by the screening experiments, a wide range of precipitants and pH values were tried using the grid-screen method (Samudzi & Fivash, 1992). After optimization of the conditions under which crystals appeared, good single crystals suitable for diffraction of these toxins were finally obtained (Fig. 2). The detailed crystallization conditions are listed in Table 1. It should be mentioned that in the crystallization of BmK I4, lowering the dioxane concentration to slightly below 5% led to the appearance of another crystal

Table 2
Crystal data for BmK I1, BmK I4 and BmK I6.

Sample	BmK I1	BmK I4	BmK I6 (I)	BmK I6 (II)
Resolution (Å)	3.5	2.8	2.8	2.2
Space group	*P*6	*I*4	*C*2	*R*3
Unit-cell parameters (Å, °)	$a = b = 66.2$ (1), $c = 176.7$ (2)	$a = b = 134.5$ (2), $c = 60.6$ (1)	$a = 46.5$ (1), $b = 85.2$ (1), $c = 32.6$ (1), $\beta = 110.5$ (1)	$a = b = 44.5$ (1), $c = 164.7$ (2)
V_m (Å^3 Da^{-1})	2.29	2.10	2.25	2.33
Solvent content (%)	46.3	41.4	45.3	47.2
Molecules per asymmetric unit	6	8	2	2

form of hexagonal shape which showed very weak X-ray diffraction.

2.3. X-ray crystallographic analyses

The best single crystals of BmK I1, BmK I4 and BmK I6 were selected for use in data collection. The sizes of the crystals used are listed in Table 1. All diffraction data were collected at room temperature on a 345 mm MAR Research imaging-plate detector using Cu $K\alpha$ radiation ($\lambda = 1.5418$ Å) from a generator operating at 40 kV and 50 mA. The programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997) were used for data processing and analysis. The Matthews coefficients and solvent contents were calculated using the Matthews equation (Matthews, 1968). The results are listed in Table 2. The structure determination of BmK I1 and I4 are currently under way using the molecular-replacement method, with the structure of Bj-xtrIT (PDB code 1bcg) as the initial model.

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