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PURIFICATION, CRYSTALLIZATION AND INITIAL STRUCTURAL SOLUTION OF A NEW α-LIKE TOXIN WITH CARDIAC TOXICITY FROM SCORPION *BUTHUS MARTENSII* KARSCH

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Abstract: An α-like toxin named BmK M7 active on both mammals and insects has been purified from the venom of scorpion *Buthus martensii* Karsch (BmK) recently. The electrophysiological experiments showed that M7 can bind to human cardiac Na^+-channel and modify its normal properties, hence can be considered as a cardiotoxin. Single crystals of M7 have been obtained by hanging-drop vapor diffusion method using ammonium sulfate as precipitant in Tris-HCl buffer at pH 8.5. A data set to 1.40 Å resolution was collected using synchrotron radiation and CCD detector in Photon Factory in Japan. Data analysis showed that the crystals belonged to space group $P3_121/P3_221$, with cell dimensions a=b=32.76 Å, c=176.82 Å. Assuming two molecules per asymmetric unit, the V_m value is 1.92 $Å^3$/Da. The initial structural analysis was carried out by molecular replacement, which showed the correct space group ($P3_121$), and the orientations and positions of the two molecules in the asymmetric unit.

Key words: Purification, Crystallization, Scorpion toxin, α-like toxin, Crystal data, *Buthus martensii* Karsch, Cardiotoxin

INTRODUCTION

Scorpion toxins are well-known probes for structure-function studies on the ion channels because they bind specifically to variant ion channels and exhibit wide variability in binding site and manner [1]. The Na^+-channel

specific toxins, which are composed of 60-72 amino acids in length cross-linked by 4 disulfide bridges, have been divided into two main groups, α toxins and β toxins [2], according to their pharmacological effects on Na^+ currents in electrophysiological preparations and their binding properties. The major effect of α toxins is to slow or inhibit the Na^+ currents inactivation and thus induce prolongation of action potentials. The high resolution crystal structures of two α toxins, AaH II [3] and BmK M8 [4], and a β toxin Csv3 [5] have revealed a general scaffold for the Na^+-channel toxins comprising an anti-parallel three strand-ed β-sheet and usually one stretch of α-helix tightly bound by 4 disulfide bridges. Based on these structures along with some pharmacological and biochemical studies, certain knowledge on the binding of the scorpion neurotoxin to sodium channel have been gained [4, 6, 7].

In the α toxins group a subgroup toxins, α-like toxins, which are toxic to both mammals and insects, have been recognized [8]. All α-like toxins reveal no competition for α- or β- toxin binding, which showed that their receptor binding site on sodium channel should be different from that of the classical α-toxins [8]. Several α-like toxins have been purified from the venom of scorpion *Buthus martensii* Karsch (BmK), which is widely distributed in Eastern Asia. The recent work on their high resolution structures [9, 10] showed a distinct feature has been found in all their structures: there is a non-proline *cis* peptide bond between the residues 9 and 10, which rarely occurs in proteins. However, the general structural feature and the functional significance of the *cis* peptide bond for the α-like toxins are still open. Furthermore, scorpion toxins as natural scaffolds have been used for protein engineering [11, 12], for which we need to know functional architectures. Accordingly, more detailed structural analyses of those toxins that possess certain structure-function distinctions are required. Here we are reporting the crystallization and initial structural solution of a new α-like toxin, BmK M7.

BmK M7 was purified from the BmK venom. The bioassay showed that it is toxic to both mammals and insects, so belongs to the α-like toxins. The most recent electrophysiological experiments showed that BmK M7 can bind to human cardiac Na^+ channel (hH1) and modify its normal properties, hence it can be considered as a cardiotoxin. The purification, crystallization, X-ray data collection and the initial structural solution of this toxin reported in this paper will set up a sound basis for structural analysis.

2. EXPERIMENTAL

2.1. Purification

BmK M7 was purified from the venom of scorpion BmK. The crude venom 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 the same buffer. The second fraction from G-50 column was then subjected to a SP Sephadex C-25 ion-exchange column (1.6×80 cm) pre-equilibrated with 0.02 M sodium phosphate buffer at pH 6.5. Then the column was

eluted with a step gradient of NaCl (first from 0.1 M to 0.43 M, then from 0.43 M to 0.6 M, and then from 0.6 M to 0.8 M). The seventh peak (Figure 1) was collected and desalted on a Sephadex G25 column (1.6×120 cm). Further purification was carried out using MONO S HR 5/5 (Pharmacia) column, from which the second fraction (Figure 2) was pooled and desalted. The resulting sample of BmK M7 was lyophilized and stored at –20 °C before use. The molecular weight of M7 was determined to be 7137.4 by the MALDI-TOF mass spectrum on BIFLEX III MS (Bruker, USA), and the pI value is 8.2 shown by the experiment of the isoelectrofocusing (IEF) electrophoresis with Phast Gel IEF 3-9 on PhastSystem (Pharmacia).

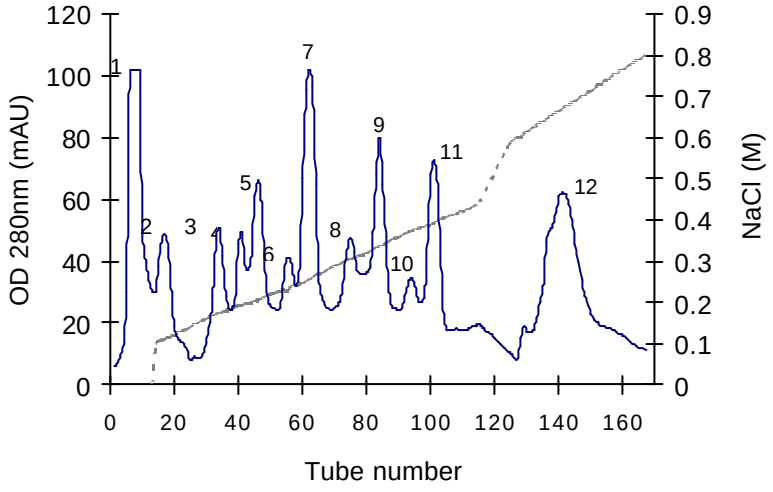

Figure 1. Separation of the second peak from Sephadex G-50 on the SP Sephadex C-25 column eluted with 0.02 M sodium phosphate at pH 6.5. The dash line indicates the gradient of sodium chloride dissolved in the above buffer. The seventh elution peak was collected for further purification.

2.2. Bioassay

The anti-insect toxicity of BmK M7 was tested with the larvae of the house fly as described earlier [13]. The fly larvae weighing about 30 mg were selected for the experiments. The sample was dissolved in 0.9% sodium chloride and injected into the lower part of the larva's abdomen at different doses. Ten fly larvae as a group were tested with the same dose. After the injection the larva contracted rapidly without further extension. The CPU (contraction paralysis unit, defined as the acute dose that caused half of the recipient larvae immediately contracted in 5 s) value for M7 is 13.3 μg/body (~ 30 mg), which indicates that M7 has relatively weak toxicity on insects.

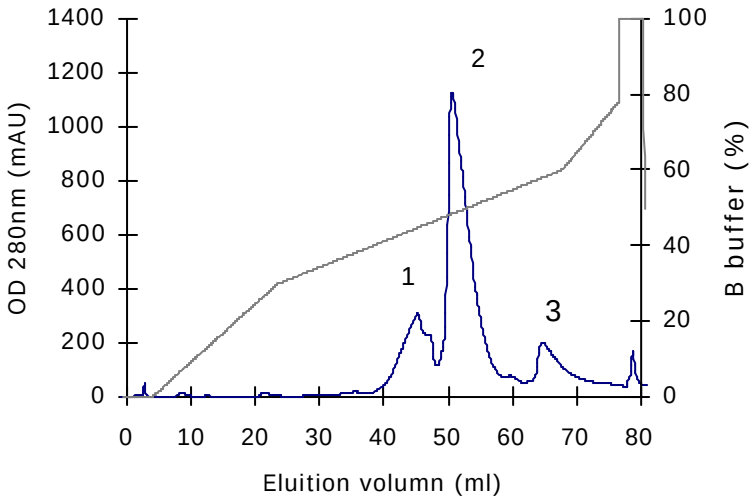

Figure 2. Further isolation of the desalted 7th peak from SP Sephadex C-25 column on a MONO S column. The dash line indicates the gradient of the B buffer (sodium chloride dissolved in 0.05 M acetic acid at pH 4.5). The second fraction was pooled and lyophilized.

The method suggested by Meier and Theakston [14] was used in the LD_{50} determination of M7. According to Meier and Theakston, the plot of D as a function of D/T should be a straight line, and the interception of this line at the D axis should represent the LD_{50} value (D is the dose in mg/kg, and T is the time from the injection to the death of the mouse in second). The toxicity of M7 on mammals was tested with ICR mice (male, SPF level, 18-20 g in body weight) taken from Beijing Experimental Animal Center. 100 μl of the sample dissolved in 0.9% sodium chloride at various concentrations was injected into the mouse through the tail vein, and the time from injection to the death of mouse was recorded. Ten mice as a group were tested with the same dose. The urination of the mouse was taken as the standard of death. After the experiments, the data were processed according to the method. The plot of D~D/T was shown in figure 3, from which the LD_{50} of M7 could be obtained as 0.93 mg/kg.

Most recently, the electrophysiological characterization of M7 was carried out on an expressed human cardiac Na^+ channel (hH1), with the method similar to that described by Goudet et al. [15]. The result shows that M7 can bind to the cardiac Na^+ channel and modify its normal electrophysiological properties, and hence affect the heart rhythm. So, this indicates that BmK M7 has cardiac toxicity and can be considered as a cardiotoxin. The detailed experimental data and results will be published in elsewhere.

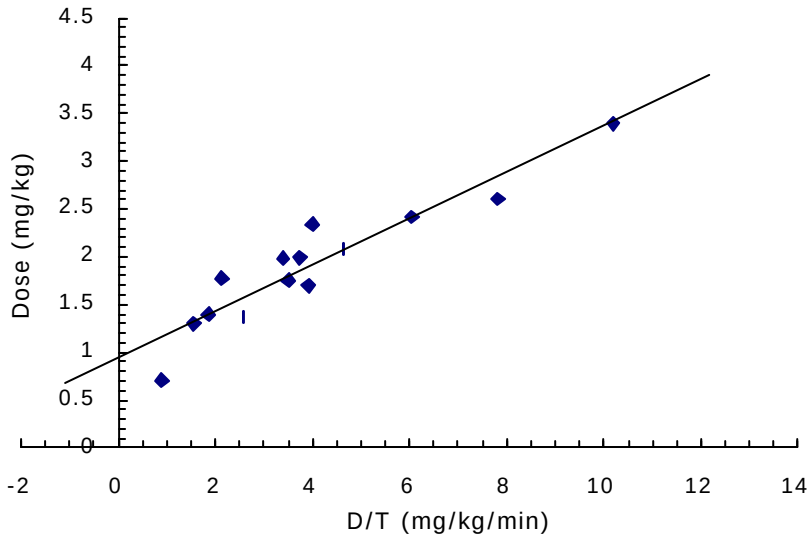

Figure 3. The activity curve of BmK M7 in the determination of its toxicity to mouse. Plotted is the D as a function of D/T, where the D is the injection dose (mg/kg) and T is the time from injection to the death of the mouse (s). The interception of the straight line with the D axis represent the LD_{50} of BmK M7, which is 0.93 mg/kg seen from the figure.

2.3. Crystallization

BmK M7 was crystallized at room temperature by the hanging-drop vapor diffusion method using Linbro culture plates. Preliminary crystallization conditions were searched using a screen of several common precipitating reagents with pH ranging from 4.0 to 9.0. The hanging drops contained equal volumes of 2 μl of the protein solution (10 mg/ml) and 2 μl of the reservoir solution. In the beginning, small crystals with regular shape appeared after 3 days using a reservoir solution composed of 1.2 M ammonium sulfate and 100 mM Tris-HCl buffer at pH 8.5. Different additives were tried to control the nuclei number and the drop volume was enlarged to get larger crystals. After optimizing the crystallization condition, the best block shape single crystals of M7 were obtained in 0.65 M ammonium sulfate and 100 mM Tris-HCl at pH 8.5 in the presence of 1% (v/v) ethanol added as an additive. The largest crystal could grow up to approximately maximum dimensions of 0.5 × 0.2 × 0.2 mm^3 within one month (Figure 4).

2.4. Data collection and processing

The best crystals were mounted in thin wall sealed quartz capillaries in the presence of a small amount of mother liquor. Diffraction data were collected at room temperature using synchrotron radiation (λ=1.0 Å) on an ADSC Quantum 4 CCD detector at the beam line BL18B of the Photon Factory in Tsukuba, Japan. The crystal

could diffract to at least 1.4 Å. Due to one of the cell parameters is very long (~ 176 Å), the distance from crystal to detector had to be set 230.0 mm and the 2θ angle was set to 20° to collect high resolution data. The exposure time for each image was 50 s, and an oscillation angle of 1° for a total of 120° was collected.

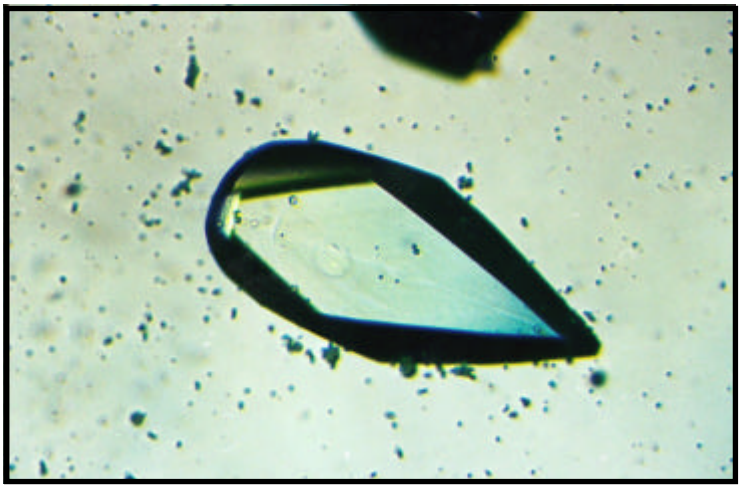

Figure 4. Crystal photo of BmK M7 grown with 0.65 M ammonium sulfate in 0.1M Tris-HCl at pH 8.5. 1% Ethanol was added as an additive. The size of the crystal shown is 0.5 × 0.2 × 0.2 mm^3 approximately.

Processing and analyses of the diffraction data using the programs *DPS/MOSFLM/CCP4* and *SCALA* [16-18] indicated that the crystal belonged to space group $P3_121$ or $P3_221$ with cell parameters a=b=32.76 Å, c=176.82 Å and α=β=90°, γ=120°. Assuming 2 molecules in the asymmetric unit, the calculated solvent content was 39.7% with a V_m value of 2.04 $Å^3$/Da [19]. The detailed crystal data and data collection statistics for M7 were listed in Table 1.

2.5. Initial structural solution

The self-rotation functions were calculated with program *POLARRFN* in the CCP4 package [18], in order to check the non-crystallographic symmetry. All data in the range of 20~3.0 Å with a radius of integration in the Patterson function of 30 Å were used in the calculation. No significant peak was observed in the self-rotation function corresponding to κ=180° (Figure 5), which indicates an absence of non-crystallographic two-fold rotation symmetry or alternatively a coincident of the crystallographic and non-crystallographic axes. Evidence for the later came from the results of molecular replacement, which showed that the direction of the peudo-2-fold axis is very close to the crystallographic 2-fold axis.

Table 1 Crystal data and data collection statistics for BmK M7

X-ray source	BL18B, PF
Wavelength (Å)	1.0
Detector	ADSC Quantum 4 CCD
Resolution (Å)	1.4
Space group	$P3_121$
Unit-cell parameters (Å, º)	a=b=32.76, c=176.82, α = β=90, γ=120
Number of observations	57131
Number of unique reflections	19260
Completeness† (%)	85.8 (74.6)
R_{merge} † (%)	4.0 (20.1)
I/σ(I) †	10.0 (3.6)

† Values in parentheses refer to the outer resolution shell 1.48-1.40 .Å.

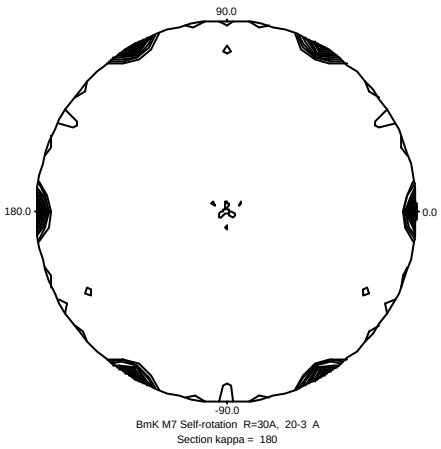

Figure 5. Representation of the κ=180° section of the self-rotation function from the crystals of BmK M7. The peaks around the edge indicate the crystallographic two-fold axes. No peak for the two-fold non-crystallographic axis was observed because they are coincident with crystallographic ones.

The molecular replacement calculation was carried out by the program *AMoRe* [20]. A homologous model of BmK M8 with PDB code 1snb [4] was used as probe. The rotation function was calculated using the data from 8-3 Å, 8-3.5 Å, 8-4 Å, in parallel, all displaying no obvious peak above the mean. Thus a total of 99 rotation function peaks from the 8-3 Å data was submitted to translation function calculation and different resolutions were tried in parallel. The two possible space groups $P3_121$ and $P3_221$ were also tried. It was lucky to find that in resolution range 8-3 Å with the space group $P3_121$, the highest function peak, corresponding to the 32th high

rotation function peak, had a 3.3 σ peak-height above the mean value. The best solution after rigid-body refinement corresponding to a 4.6 σ peak-height (correlation coefficient=34, R factor=0.50) indicated a potential location of one molecule, named molecule I. Then molecule I was fixed and the second molecule, molecule II, was soon found using the *AMoRe* multiple-molecule protocol to search for the second orientation and location. The peak height goes to 20 σ (correlation coefficient 57, R factor 0.42) when the locations of the two molecule together was submitted to rigid-body refinement. By applying the crystallographic symmetry on molecule II, it goes to a new position where a peudo-2-fold axis was found between the two molecules. The direction of the pseudo axis is (0.9984, -0.0229, 0.0613), close to the "a" axis. The two molecules were checked for packing with program *O* [21]. No overlapping of the main chain has been found and the distances between molecules were proper.

The model was then subjected to initial refinement using *X-PLOR* [22]. After rigid-body refinement, positional refinement and simulated annealing, as well as the model rebuilding by hand, the R factor and free R factor (allocating 10% of the data for cross validation) reached 0.274 and 0.316, respectively. Further refinement is now under way.

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