Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

A series of bioactivity-variant neurotoxins from scorpion *Buthus martensii* Karsch: purification, crystallization and crystallographic analysis

Hong-Min Li, Tong Zhao, Lei Jin, Miao Wang, Ying Zhang and Da-Cheng Wang*

Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, People's Republic of China

Correspondence e-mail: wdc@pewdc.ibp.ac.cn

Received 9 January 1998
Accepted 6 May 1998

Three bioactivity-variant neurotoxins, BmK M1, M4 and M8, have been purified from Chinese scorpion BmK venom. They possess distinct toxic activities against mice *in vivo*. These proteins also have different electrostatic properties. The relative toxicities for BmK M1, M4 and M8 are 13.3:2.5:1 which, surprisingly, correspond to their respective p*I* values ranging from basic to acidic 9.01, 7.53 and 5.30, respectively. They have been crystallized in different crystal forms as orthorhombic, hexagonal and monoclinic, respectively. These crystals can diffract to 1.2 (BmK M1), 1.3 (BmK M4) and 1.8 Å (BmK M8) resolution and have been used in data collection. These toxins produced by natural mutagenesis or gene divergence should represent functionally distinct states, thereby forming a valuable system for studying structure–function relationships. The unusual relatively acidic component that first appeared in this series also provides a new concept for a more comprehensive understanding of scorpion neurotoxins.

1. Introduction

Scorpion neurotoxins are known to interact specifically with the voltage-dependent sodium channel (Catterall, 1979; Rochat *et al.*, 1979). They form a family of homologous proteins consisting of single polypeptide chains of about 60 amino-acid residues that are cross-linked by four disulfide bridges (Babin *et al.*, 1975; Possani *et al.*, 1975). Since Miranda and co-workers purified the first scorpion toxin in 1970 (Miranda *et al.*, 1970), over 100 scorpion toxins from different species have been isolated and nearly 60 sequences have been determined as shown in the PIR database. All of them, except in one case (Manueulle *et al.*, 1992), are reported to possess alkaline properties. Therefore, the scorpion neurotoxins are usually described as basic proteins. We recently found the three bioactivity-variant neurotoxins, BmK[1] M1, M4 and M8, from the venom of scorpion BmK distributed in China and East Asia. Within this series of toxins, a neutral (BmK M4) and an acidic toxin (BmK M8) were found, in addition to the normally basic toxin (BmK M1). It is interesting that the changes in p*I* values correlate with the variances in toxicity against mice *in vivo* as shown in the bioassays. Therefore, they should represent functionally distinct states. They all have been crystallized in our laboratory so as to form a valuable system for studying three-dimensional structure–function relationships. Furthermore, BmK M8 is the first acidic scorpion toxin reported so far, which therefore provides new information for understanding scorpion neurotoxins. Here we report the purification, characterization, crystallization and crystallographic data for this series of BmK toxins.

2. Materials and methods

2.1. Protein purification and characterization

The toxins were purified from the venom of scorpion BmK grown in the Henan Provinces of China. The purification included successive chromatography of Sephadex G-50 gel filtration (column 2.5 × 150 cm; buffer 0.05 *M* NH_4HCO_3, pH 8.0; flow rate 3 ml min^{-1}), Sp Sephadex C-25 ion exchange [column 1 × 80 cm; 0.1 *M* phosphate buffer, pH 6.0; salt gradient 0–0.3 1.0 *M* NaCl; flow rate 5 ml $(10\ min)^{-1}$], chromatography and preparative IEF electrophoresis (Ultrodex granule gel; p*I* range 7.0–10.0; power 10 W).

Purity was checked by HPLC (high-pressure/high-performance liquid chromatography) analysis running on a Bio-Rad series 400 system and a Waters system. The molecular weight and the isoelectric point of purified samples were determined by SDS–PAGE and IEF electrophoresis on a Pharmacia-LKB Multiphor II electrophoresis system. All media for chromatography and electrophoresis were from Pharmacia-LKB Biotechnology.

[1] Abbreviations used: BmK, *Buthus martensii* Karsch; LD_{50} the dose capable of statistically killing 50% of mice; IEF, isoelectric focusing; AaH, *Androctonus australis* Hector.

2.2. Toxicity bioassay

The toxicity test was carried out using Kunming mice with body weights of 20 ± 2 g. The lyophilized samples were dissolved in a modified Herreveld or Krebs solution and injected into each mouse by tail-vein injection. Quantitative estimation of the toxicity was based on the determination of LD_{50}, using the method described by Meier & Theakston (1986).

Table 1
Toxicity and pI values of BmK M1, M4 and M8.

Toxins	BmK M1	BmK M4	BmK M8
p*I* value	9.01	7.53	5.30
LD_{50} (mg kg^{-1} body weight)	0.75 ±0.034	4.00 ±0.025	10.00 ±0.034
Relative activity	13.3	2.5	1.0

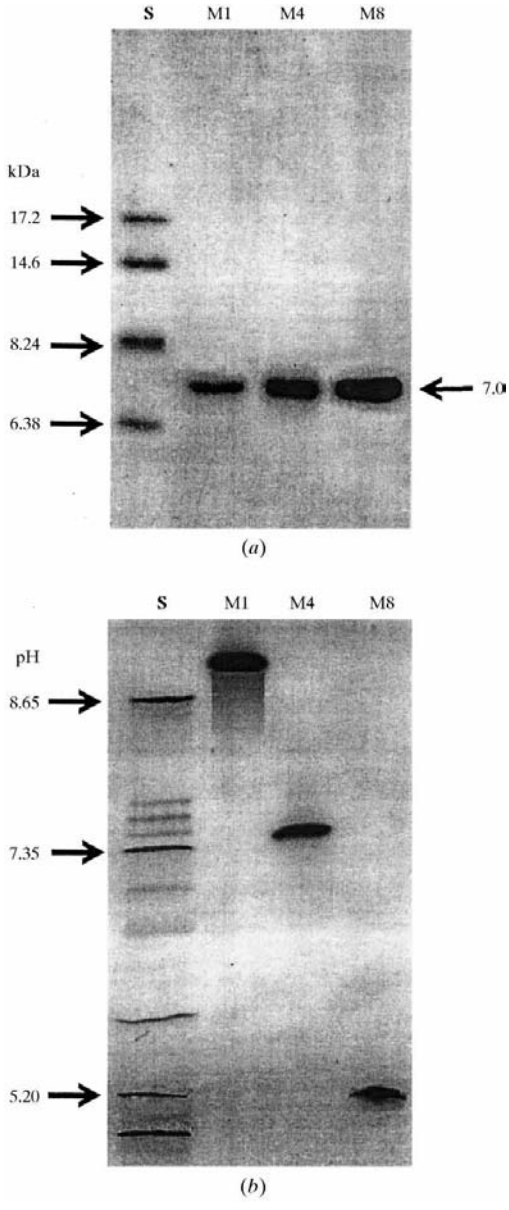

Figure 1
SDS–PAGE (*a*) and IEF (*b*) electrophoresis of BmK M1, M4 and M8. The p*I* values were determined on an enhanced laser densitometer (Ultroscan XL, LKB BROMMA).

2.3. Crystallization

The microbatch method and vapour-diffusion method were used in the crystallization. A sparse-matrix screening protocol (Jancarik & Kim, 1991) was used in the systematic search for the preliminary conditions which were then optimized in subsequent trials.

2.4. Crystal data determination and diffraction data collection

The crystallographic data were determined on a CAD-4 four-circle diffractometer (Enraf–Nonius). Values of V_m and the number of molecules per asymmetric unit were estimated by use of the Matthews statistics method (Matthews, 1968). The diffraction data of BmK M1 (*B*) and M8 were collected using a SIEMENS X-300B area detector. The high-resolution data of BmK M1 (*A*) and BmK M4 were collected at beamline BL6A of the Photon Factory in KEK, Tsukuba, Japan, by use of a screenless Weissenberg camera (Sakabe, 1983).

3. Results

3.1. Purification and characterization of toxins BmK M1, M4 and M8

After two steps of gel filtration on Sephadex G-50 and Sp Sephadex C-25, eight fractions were isolated from the crude venom.

Among others, fraction 1 showed a sharp peak in the HPLC analysis, indicating a high purity. Fraction 8 was further precipitated out from the elution buffer as microcrystals by setting at 277 K which provided a purified sample. Since the preliminary IEF analysis showed a neutral component in fraction 4, a preparative IEF was carried out to obtain a purified sample as shown in Fig. 1. These three purified toxins are referred to as BmK M1, BmK M4 and BmK M8, respectively.

SDS–PAGE showed that BmK M1, M4 and M8 possess similar molecular weights, about 7000 Da (Fig. 1*a*). However, the IEF analysis demonstrated that they have obviously distinct p*I* values, 9.01, 7.53 and 5.30 for BmK M1, M4 and M8, respectively (Fig. 1*b*).

3.2. Variant toxicity

The toxicity bioassay showed that the LD_{50} values of BmK M1, M4 and M8 were 0.75 ±0.034, 4.00 ±0.025 and 10.00 ±0.034 mg kg^{-1} body weight, respectively, as shown in Fig. 2 and Table 1. The results showed that BmK M1, M4 and M8 were a toxicity-variant series of mammalian-directed neurotoxins. It is quite interesting that the potencies of these toxins are associated with the dramatic differences in the electrostatic properties of these molecules: from acidic to basic ones, they have relative toxicities of 1:2.5:13.3. In fact, the higher the p*I* value, the stronger is the toxicity (see Table 1).

3.3. Crystallization

The methods used in the successful crystallization of BmK M1, M4 and M8 are listed in Table 2. Under these conditions, single crystals (see Fig. 3) suitable for X-ray structural analysis could be obtained. Among others, BmK M1 provided two types of crystal which have the same space group, but obviously different unit-cell dimensions (see Table 3).

3.4. Crystal data and diffraction data

The preliminary X-ray analysis on a diffractometer demonstrated that the crystal forms of BmK M1, M4 and M8 are orthorhombic, hexagonal and monoclinic, respectively. It showed that these toxins also possess distinct crystallization behaviours. They all diffracted to well beyond 2.0 Å (1 Å = 10^{-8} cm) resolution. Details are given in Table 3.

Diffraction data for BmK M1 (*B*) and M8 were collected at 1.7 and 1.8 Å resolution with an $R_{\rm merge}$ of 0.0501 and 0.0523, respectively, on a SIEMENS X-2000B area detector. The diffraction of BmK M1 (*A*) and M4 each reached a resolution limit of 1.2 and 1.3 Å, respectively, for synchrotron radiation with 1 Å wavelength at KEK of Tsukuba, Japan. The data sets were collected with an $R_{\rm merge}$ of 0.0687 and 0.0723, respectively, by use of a screenless Weissenberg camera with a film cassette at 429.7 mm. The X-ray structural analyses of these toxins are in progress.

4. Discussion

Among the more than 100 scorpion toxins purified so far, few have shown properties similar to those of this series of BmK. However, the existence of these toxins is not surprising from an evolutionary point of

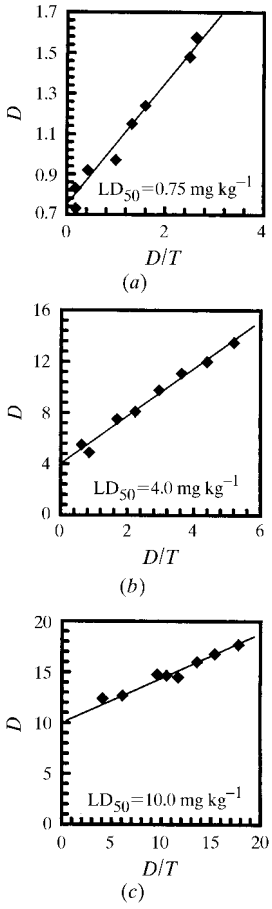

Figure 2
LD_{50} determination of BmK M1 (*a*), M4 (*b*) and M8 (*c*) using dose/survival-time observation. The point where the regression line intersects the ordinate is the LD_{50}. *D*, dose (mg kg^{-1}) of toxin used in experiments; *T*, survival time (min) of mice after injection of toxins.

Table 2
Methods for crystallization of BmK M1, M4 and M8.

	BmK M1			
Samples	*A* type	*B* type	BmK M4	BmK M8
Solution *A*	1 m*M* HAc	0.03 N HCl	1 m*M* HAc	0.03 N HCl
(protein)	20 mg ml^{-1}	20 mg ml^{-1}	20 mg ml^{-1}	10 mg ml^{-1}
Solution *B*	2.8 *M* NaH_2PO_4	0.2 *M* NaH_2PO_4	4.8 *M* NH_4Ac	0.2 *M* NaH_2PO_4
Solution *C*		MPD		
pH	4.53	7.50	6.80	4.55
Method	Vapour diffusion	Batch	Vapour diffusion	Batch
Droplet	5 µl *A* + 5 µl *B*	4 µl *A* + 8 µl *B*	5 µl *A* + 5 µl *B*	5 µl *A* + 5 µl *B*
Reservoir	500 µl *B*	12 µl *C*	500 µl *B*	

Table 3
Crystal data of BmK M1, M4 and M8.

	BmK M1			
Samples	*A* type	*B* type	BmK M4	BmK M8
Crystal system	Orthorhombic	Orthorhombic	Hexagonal	Monoclinic
Space group	$P2_12_12_1$	$P2_12_12$	$P6_1/P6_5$	$P2_1$
V_m (Å^3 Da^{-1})	1.92	2.88	2.07	1.84
Molecule/asymmetric unit	2	1	1	1
Resolution limit (Å)	1.2	1.7	1.3	1.8

	BmK M1			
Cell parameters	*A* type	*B* type	BmK M4	BmK M8
a (Å)	76.39	83.42	54.88	23.32
b (Å)	52.77	41.09	54.88	38.56
c (Å)	27.12	23.93	33.76	29.17
α (°)	90	90	90	90
β (°)	90	90	90	107.48
γ (°)	90	90	120	90

view. Scorpions first appeared on Earth about 400 million years ago. Since then, they have been subjected to many evolutionary pressures for venom proteins and carried multiple toxin genes (Bougis *et al.*, 1989). In fact, every scorpion venom contains a homologous set of toxins within which changes in toxicity and receptor-binding properties accompany differences in sequence. The existence of relatively acidic and inactive toxins may be a vestige of venom proteins in the early evolutionary stages. In the course of evolution, the enhancement of toxicity seems to be associated with an increment in the overall positive-charge potential on the molecule, since this is required by the high efficacy of the toxin–receptor binding (*e.g.* Kharrat *et al.*, 1990). Consequently, multiple forms of the molecule, from acidic to basic, could reasonably appear in this process. We have observed that a significant decrease in toxicity usually accompanies the qualitative lowering of the isoelectric point in the same class of toxins. Therefore, the finding of relatively acidic toxins throws a new light on our understanding of scorpion neurotoxins.

The amino-acid sequences of BmK M1, M4 and M8 have been determined by protein analysis and cDNA sequencing (Li *et al.*, 1996; Luo *et al.*, 1997; Xiong, Ling, Zhao *et al.*, 1997) and are shown in Fig. 4. The encoding genes have also been cloned recently in our laboratory (Xiong, Ling, Wang *et al.*, 1997; Xiong, Ling, Zhao *et al.*, 1997). The results showed that these three proteins are indeed different gene products

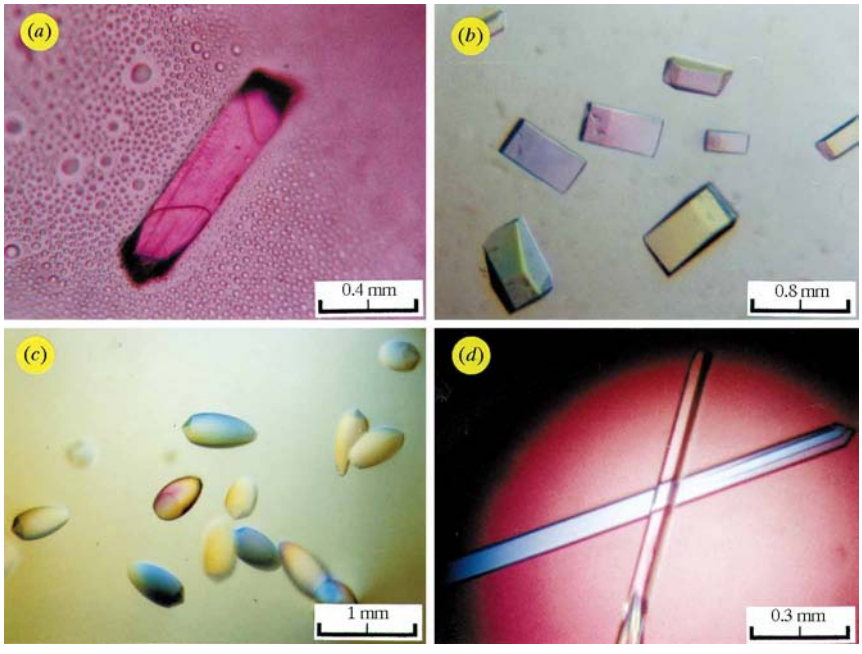

Figure 3
Crystal photographs of BmK M1-*A* (*a*), M1-*B* (*b*) orthorhombic, M4 (*c*) hexagonal and M8 (*d*) monoclinic.

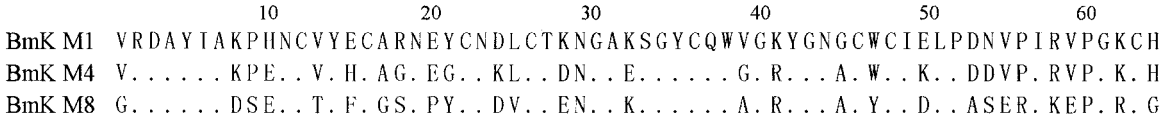

Figure 4
Amino-acid sequences of BmK M1 (from Xiong, Ling, Zhao *et al.*, 1997), M4 (from Luo *et al.*, 1997) and M8 (from Li *et al.*, 1996).

and not variants resulting from chemical modification.

BmK is a representative scorpion species in China and East Asia. In contrast to some other scorpions, *e.g.* AaH from North Africa which usually causes serious symptoms, even leading to death, scorpion BmK has been used as a traditional medicine in Chinese folk remedies fro about a thousand years. In the first authoritative Chinese pharmacopoeia *Ben Cao Gang Mu* (*Compendium of Material Medica*) edited by Li Shi-Zhen in 1578, the pharmaceutical properties and the curative effects of BmK were described in detail, including the treatment of some neural diseases such as apoplexy, hemiplegia, epilepsy, facial paralysis *etc.* Death from scorpion stings has never been reported in China. This special property of BmK, *i.e.* its mild toxicity, may also be relevant to the appearance of a certain amount of relatively inactive venom proteins such as BmK M8.

So far most of the information on molecular structure, function and binding properties of scorpion toxins comes from investigations on the common basic toxins (*e.g.* Aah I–IV) (*e.g.* Almassy *et al.*, 1983; Fontecilla-Camps *et al.*, 1988; Zhao *et al.*, 1992; Houset *et al.*, 1994; Kharrat *et al.*, 1990; Tejedor & Caterall, 1988; Thomsen & Caterall, 1989). The bioactivity-variant series of BmK toxins reported here could be considered as functionally distinct states of the scorpion toxin produced by natural mutagenesis or gene divergence. Thus, the finding and crystallization of these scorpion neurotoxins provide an opportunity for gaining insight into their structure–function relationships.

We thank Professor N. Sakabe and Dr N. Watanabe for the kind help in data collection at the Photon Factory of KEK in Tsukuba, Japan. This work was supported by grants from the National Natural Science Foundation of China, the 863 High-Tech Program and the Climb (A) Program (95-Yu-34).

References

Almassy, R. J., Fontecilla-Camps, J.-C., Suddath, F. L. & Bugg, C. E. (1983). *J. Mol. Biol.* **170**, 497–527.
Babin, D. R., Watt, D. D., Goos, S. M. & Mlejnek, R. V. (1975). *Arch. Biochem. Biophys.* **166**, 125–134.
Bougis, P. E., Rochat, H. & Smith, L. A. (1989). *J. Biol. Chem.* **264**, 19259–19265.
Catterall, W. A. (1979). *J. Gen. Physio*. **74**, 357–391.
Fontecilla-Camps, J.-C., Habersetzer-Rochat, C. & Rochat, H. (1988). *Proc. Natl Acad. Sci. USA*, **85**, 7443–7447.
Houset, D., Habersetzer-Rochat, C., Astier, J. & Fontecilla-Camps, J.-C. (1994). *J. Mol. Biol.* **238**, 88–103.
Jancarik, J. & Kim, S. H. (1991). *J. Appl. Cryst.* **24**, 409–441.
Kharrat, R., Dabon, H., Rochat, H. & Granier, C. (1990). *Toxicon*, **28**, 509–523.
Li, H. M., Wang, D. C., Zeng, Z. H., Jin, L. & Hua, R. Q. (1996). *J. Mol. Biol.* **261**, 415–431.
Luo, M. J., Xiong, Y. M., Wang, M., Wang, D. C. & Chi, C. W. (1997). *Toxicon*, **35**, 723–731.
Manueulle, P., Martin, M. F., Rochat, H. & Granier, C. (1992). *Natural Toxins*, **1**, 61–69.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 395–401.
Meier, J. & Theakston, R. D. G. (1986). *Toxicon*, **24**, 395–401.
Miranda, F., Kopeyan, C., Rochat, H., Rochat, C. & Lissitzky, S. (1970). *Eur. J. Biochem.* **17**, 477–484.
Possani, L. D., Martin, B. M., Svendsen, I., Rode, G. S. & Erickson, B. W. (1975). *Biochem. J.* **229**, 739–750.
Rochat, H, Bernard, P. & Couraud, F. (1979). *Advances in Cytopharmacology*, Vol. 3, edited by B. Cecarelli & F. Clementi, pp. 325–334. New York: Raven Press.
Sakabe, N. (1983). *J. Appl. Cryst.* **16**, 542–547.
Tejedor, F. J. & Catterall, W. A. (1988). *Proc. Natl Acad. Sci. USA*, **85**, 8742–8746.
Thomsen, W. J. & Catterall, W. A. (1989). *Proc. Natl Acad. Sci. USA*, **86**, 10161–10165.
Xiong, Y. M., Ling, M. H., Wang, D. C. & Chi, C. W. (1997). *Toxicon*, **35**, 1025–1031.
Xiong, Y. M., Ling, M. H., Zhao, T., Wang, D. C. & Chi, C. W. (1997). *Acta Biochem. Biophys. Sin.* **29**, 200–205.
Zhao, B., Carson, M., Ealick, S. E. & Bugg, C. E. (1992). *J. Mol. Biol.* **227**, 239–252.