Expression, Renaturation and Functional Analysis of an Excitatory Insect-Specific Toxin from Scorpion *Buthus martensii* Karsch

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Abstract: The cDNA of BmK IT-AP, an excitatory insect toxin from the scorpion *Buthus martensi* Karsch that has an analgesic effect on mammalian cells, was expressed in *E. coli* in the form of an inclusion body. Following denaturation and reduction, the recombinant protein was renatured and purified by liquid chromatography. The authenticity of the recombinant product was confirmed by bioassay and its electrophysiological effect on insect sodium channel.

Keywords: BmK IT-AP, *E. coli*, recombinant expression, denaturation and renaturation.

1. INTRODUCTION

Scorpion venom contains a large number of neurotoxins that affect the function of ion channels [1]. These toxins specifically interact with the Na^+, K^+, Ca^{2+} or Cl^- channels through affecting the ion permeability of excitable cells [2]. Scorpion toxins acting on voltage-depended sodium channels in excitable cells are mainly related to human envenomation; they are classified into -toxins that inhibit sodium current inactivation and -toxins that modify the activation process [3]. Scorpion toxins exhibit high selectivity towards animals belonging to different phyla. It has been shown that they can act on mammals, insects and crustaceans with high specificity [1]. The most intriguing property of scorpion toxins is that they can discriminate between insect and mammalian sodium channels. The highly insect-specific toxins are considered to be unique among the -toxins because they compete with -toxins for the same receptor-binding site [4]. These insect toxins include two distinct subgroups, the excitatory and the depressant [5]. Excitatory toxins induce a fast reversible paralysis in insect larva and repetitive firing in insect nerves, whereas depressant toxins cause a slow flaccid paralysis in insect larva [6,7]. These toxins are useful probes in the study of sodium channel gating mechanism in insects [1]. The anti-insect specificity has also been used in developing new insecticides [8,9]. However, it is very difficult to obtain a large quantity of highly purified and stable sample of the insect toxin because of the very low amounts of material. This has hampered relative studies and its potential applications. Use of the recombinant DNA approach for obtaining the insect toxin has also had its difficulties. Although more than ten scorpion toxins have now been expressed in different systems [11-19], most insect toxins are expressed either in very small amounts or in inclusion bodies. To date only one system has successfully been used for the expression of an excitatory scorpion insect toxin, Bj-xtrIT [20].

BmK IT-AP (insect toxin-analgesic peptide) is an excitatory insect-specific toxin from the scorpion *Buthus martensii* Karsch (BmK), which is widely distributed in China and East Asia and has been used as traditional medicine in China for more than 1000 years. It has 72 amino acid residues and is interesting because of exhibiting an obviously analgesic effect on mice [10]. A suitable recombinant expression system would allow performing in-depth structure-function studies of BmK IT-AP. Here we report the successful expression-reconstitution system of an excitatory insect-specific toxin, BmK IT-AP. Characterization of the purified toxin showed comparable bioactivity with the native toxin.

2. MATERIALS AND METHODS

2.1. Materials, Strains and Insects

The cDNA of BmK IT-AP was cloned in vector pGEM-T-ITAP by the procedure reported previously [10]. *E. coli* strains, DH5 and BL21(DE3), and expression vector pET3a were used from our lab. Restriction enzymes, RNase A, DNase I, *Taq* DNA polymerase and T4 DNA ligase were purchased from Gibco BRL (USA). Culture medium was obtained from Merck (Germany) and all other chemicals and reagents were of analytical grade made in China. Chromatography system ÄKTA purifier was from Pharmacia Biotech (Sweden). The insects used for the toxicity assay were kindly provided by Dr. Yinghong Pan from the Institute of Plant Protection, CAAS.

2.2. Construction of Expression Vector pET3a-ITAP

Based on the cDNA sequence of BmK IT-AP, a pair of synthetic oligonucleotides was used as primers in PCR. The forward primer (5'-GGG<u>CATATG</u>AAGAAGAATGGG-3') includes a *Nde*I restriction site and the reverse primer (5'-GCC<u>GGATCC</u>TTAACTTAAATCA-3') includes a *Bam*HI restriction site. PCR conditions are: 5 min at 94ºC followed by 30 cycles at 94ºC for 45 sec, 55ºC for 45 sec and 72ºC for 45 sec, using pGEM-T-ITAP as template. The PCR product was cut with *Nde*I and *Bam*HI, and then ligated into the

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*Nde*I-*Bam*HI site of pET3a. The ligated product was transformed into *E. coli* DH5 competent cells and identified by PCR. The recombinant pET3a-ITAP plasmid with correct reading frame was confirmed by DNA sequence analysis. PCR primers synthesis and DNA sequence analysis were accomplished by Sangon Biological Engineering Technology and Services Co. Ltd. (Shanghai, China).

2.3. Expression, Refolding and Purification of Recombinant BmK IT-AP

pET3a-ITAP was transformed into *E. coli* BL21(DE3) and the recombinant cells were grown in LB medium at 37°C with the presence of 50 µg/ml ampicillin. The inducer IPTG was added to a final concentration of 0.4 mM at the cell density of OD_{600} = 0.6-0.8 and growth continued for an additional 3 hours. Cells from 1 liter were harvested by centrifugation. The pellets were suspended in 30 ml resuspending buffer (30 mM Tris-HCl (pH 8.0), 5 mM EDTA) and lysozyme was added to a final concentration 0.1 mg/ml, -mercaptoethanol to 15 mM, NaCl to 0.1 M. After incubating on ice for 30 min, RNase A and DNase I were added into the mixture to a concentration of 10 µg/ml and then an another incubation at room temperature was carried out for 30 min. The lysate was sonicated on ice and the insoluble pellet fraction was collected by centrifugation at 14,000 g for 20 min. The pellet, containing insoluble recombinant BmK IT-AP protein (inclusion bodies), was washed with a washing buffer (30 mM Tris-HCl (pH 8.0), 5 mM EDTA, 15 mM -mercaptoethanol, 2% (v/v) Triton X-100 and 1 M guanidinium hydrochloride). The pellet was then suspended in 10 ml denaturing solution (6 M guanidinium hydrochloride, 30 mM Tris-HCl (pH 8.0), 5 mM EDTA and 15 mM -mercaptoethanol) for overnight at 4°C.

The renaturation of denatured protein was initiated by dialyzing against 1 liter dialysis buffer A (1 M guanidinium hydrochloride, 30 mM Tris-HCl (pH 8.0), 1 mM EDTA and 1‰ Triton X-100) at room temperature. After 24 h, changed to 1 liter dialysis buffer B (0.5 M guanidinium hydrochloride, 30 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5‰ Triton X-100 and 5 mM cysteine) for 12 h. Then changed to 1 liter dialysis buffer C (30 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.1‰ Triton X-100 and 5 mM cysteine) for 12 h and with three buffer changes. After the dialysis renaturation was completed, the precipitation was removed by centrifugation and the soluble protein was concentrated by lyophilization. The freeze-dried protein was then solved in 1 ml 30 mM Tris-HCl (pH 8.0) and applied to size-exclusion column Superdex 30 on ÄKTA purifier system. The size-excluded peaks were pooled and analyzed by SDS-PAGE to confirm the expression and the purity of recombinant BmK IT-AP. The procedure of electrophoresis referred to a tricine-SDS-PAGE system [21].

2.4. Bioassay

The anti-insect toxicity of recombinant BmK IT-AP was tested with the larva of Asian corn borer (*Ostrinia furnacalis*) (average body weight 167.1 mg). The sample was dissolved in 0.9% sodium chloride and injected into an abdominal inter-segmental region of the larva. The

contraction paralytic unit CPU was defined as the acute dose that caused half of the recipient larva to immediately contract in 5 sec [22]. Five groups of larva were tested with different dose and each group contained 5 to 8 larva. The CPU value was calculated according to the sampling and estimation method [7].

2.5. Electrophysiological Test

Effects of recombinant BmK IT-AP on voltage-gated Na^+ channels ware tested on $Na_v1.2$ (rat), $Na_v1.5$ (human) and para (fruit fly) Na^+ channels, respectively, expressed in *Xenopus laevis* oocytes, using a two-electrode voltage-clamp technique [23-25]. Concentration of recombinant IT-AP used was 5 µM. The currents were evoked by depolarisations ranging from -20 to 10 mV depending on the channels, from a holding potential of -90 mV. The current traces were recorded before and after the addition of recombinant IT-AP.

3. RESULTS AND DISCUSSION

### 3.1. Expression and Purification of Recombinant BmK IT-AP

The cDNA of BmK IT-AP was amplified by PCR and recombined into expression vector pET3a correctly, which encoded the full-length of 72 amino acid of IT-AP with no additional residues at N- or C- terminus except an initial Met. Recombinant IT-AP was obviously expressed as inclusion body with an apparent molecular weight corresponding to the expected value (8 kDa) (Fig. **1**). The expressed IT-AP was then subjected to refolding by the procedure described above. After refolding, the soluble protein was purified by chromatography on Superdex 30 size-exclusion column (Fig. **2**) on ÄKTA purifier system. Two separate peaks were pooled. The recombinant BmK IT-AP was mainly in the second peak which was confirmed by tricine-SDS-PAGE (Fig. **3**). The expression level was about 0.5 mg per liter of culture medium.
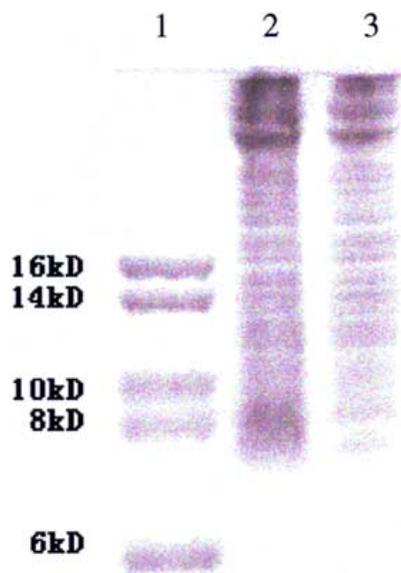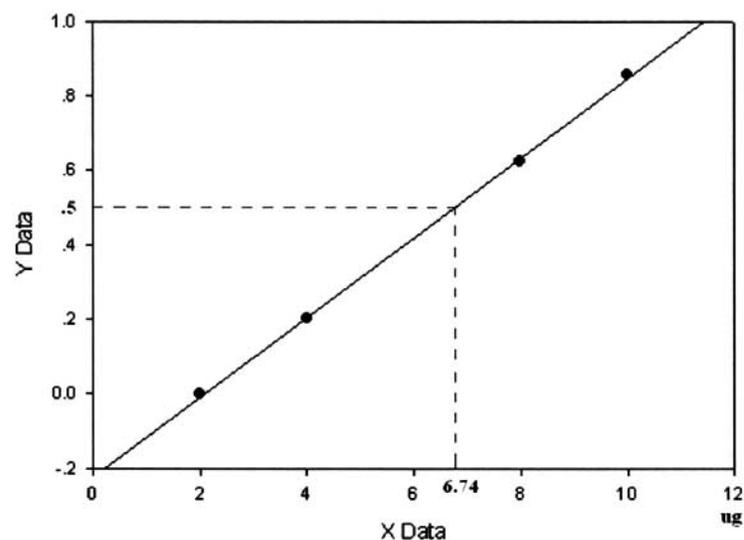

Figure 1. The tricine-SDS-PAGE analysis of expressed BmK IT-AP. Lane 1, molecular weight markers; 2, sample induced with IT-AP; 3, sample without IT-AP.
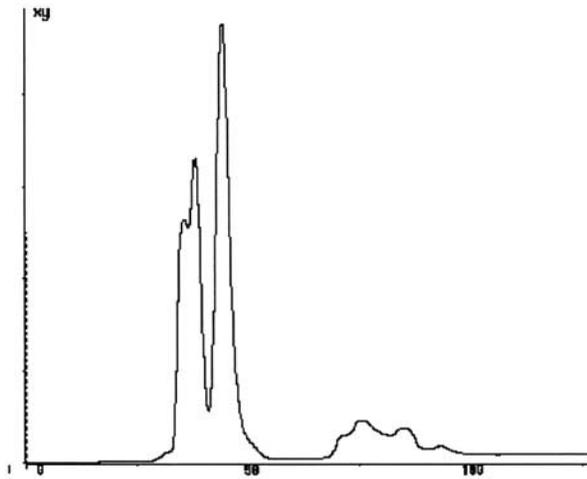
Figure 2. Purification of the expressed BmK IT-AP by chromatography on a size-exclusion column Superdex30. The Y axis denotes the absorbance at 280 nm; the X axis is the elution volume (ml). The recombinant IT-AP is mainly in the second peak.
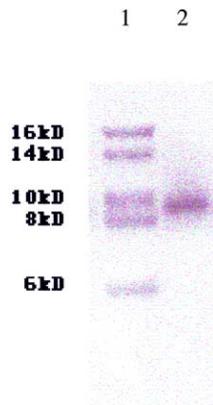
Figure 3. The tricine-SDS-PAGE of purified BmK IT-AP. Lane 1, molecular weight markers; 2, purified IT-AP by Superdex 30, which shows a single band for the recombinant IT-AP.
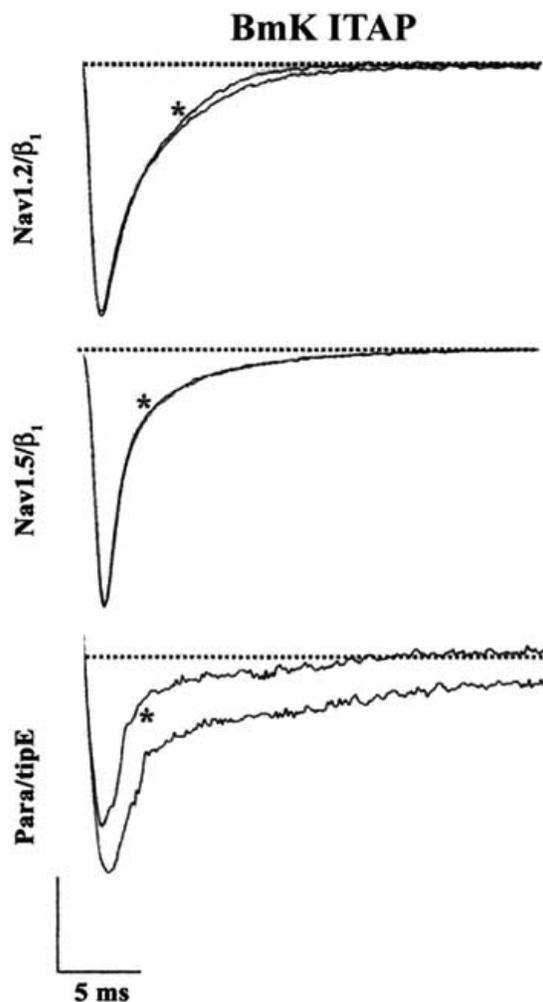

Figure 4. Larvae of *Ostrinia furnacalis* before (above) and after (below) injection of purified BmK IT-AP, body-length of larvae in the native state is about 2.2 cm; after injection recombinant IT-AP the larvae showed a rapid contraction paralysis and the body-length is shorted to about 1.4 cm.

3.2. Biological Activity of Recombinant BmK IT-AP

The purified recombinant BmK IT-AP strongly induced a sustained contraction paralysis when injected into the larva of corn borer (Fig. **4**). A plot of the percent of corresponding larva versus dose was shown in Fig. **5**, from which the CPU was determined as 6.74 μg/body.

Electrophysiological test of the recombinant BmK IT-AP on the cloned voltage-gated Na$^+$ channels was showed in Fig. **6**. No effects were found on two Na$^+$ channels from mammalian, Na$_v$1.2 (rat) and Na$_v$1.5 (human). On a Na$^+$ channel from insect, para/tipE (fruit fly), recombinant BmK IT-AP induced a small increase in the peak conductance and a slowing of its turning off, which was a typical effect of excitatory insect toxins on Na$^+$ channels [7].

In this study, we successfully constructed an expression system of excitatory insect-specific toxin BmK IT-AP in *E. coli* and obtained 0.5 mg active recombinant protein from 1 liter culture through *in vitro* refolding and purification strategy. Since scorpion toxins specific for Na$^+$ channels are

Figure 5. CPU determination of the recombinant BmK IT-AP. Y data presents the percent of corresponding larvae under different injection dose; X data is the dose (μg) of IT-AP used. The CPU is determined as 6.74 μg/body.

BmK ITAP

Figure 6. The effects of recombinant BmK IT-AP on the cloned voltage-gated Na$^+$ channels expressed in *Xenopus laevis* oocytes. * represents control conditions where no toxin was added. Current traces were evoked by depolarisations ranging from -20 to 10 mV depending on the channels, from a holding potential of -90 mV. Scale bar: Y-axis sacle for Na$_v$1.2/β_1 = 1 µA; Na$_v$1.5/β_1 = 1 µA; para/tipE = 0.3 µA.

61-76 amino acid peptide containing 4 pair of disulfide bridges, it is difficult to express active toxins in *E. coli*. The refolding of recombinant inclusion bodies has been an important method to get enough proteins and mutants for further research. Thus far, several toxins from BmK, including -toxin and depressant insect toxin, have been successfully expressed in different system [12;15;26]. BmK IT-AP is the first excitatory insect-specific toxin from BmK been expressed. This work will facilitate the structure-function studies of excitatory insect-specific toxins and an examination of its potential as a biological insecticide in pest control.

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