

Production and characterization of scorpine by MBP fusion technology in *Escherichia coli*

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Scorpine, a small cationic peptide isolated from venom glands of *Pandinus imperator*, has the anti-bacterial and anti-plasmodial activities, and shows potential important applications in the pharmaceutical industries. Its N-terminal amino acid sequence is similar to cecropins, whereas its C-terminal region has several disulfide bridges, similar to the structure of defensins. Here, we report the expression and purification of recombinant scorpine in *Escherichia coli*, using maltose-binding protein (MBP) fusion partner. The fusion protein was expressed in soluble form in *E. coli*, and verified by SDS-PAGE and Western blotting analysis. The fusion protein was purified to 90% purity by amylose resin, which binds to MBP. After the MBP-scorpine fusion protein was cleaved by the Factor Xa protease to remove the MBP tag, the cleaved sample was loaded to a Ni²⁺-NTA affinity chromatography column. Tricine/SDS-PAGE gel results indicated that scorpine had been purified successfully to more than 95% purity. The recombinantly expressed scorpine showed anti-bacterial activity against two bacteria, *Staphylococcus aureus* ATCC 29213 and *Acinetobacter baumannii* ATCC 19606. It also produced 100% reduction in *Plasmodium falciparum* parasitemia *in vitro*. Thus, the expression strategy presented in this study allows efficient and easy purification of recombinant scorpine for pharmaceutical applications in the future.

Keywords: Antimicrobial peptide, MBP fusion partner, recombinant scorpine, *Plasmodium*

Introduction

Scorpion, one of the oldest creatures, has existed on earth for more than 400 million years and is known to be widely distributed all over the world. So far, there are over 1500 species of scorpion reported¹. The scorpion venom, which is found in the telson, contains salts, nucleotides, biogenic amines, enzymes, such as, phospholipase, hyaluronidase, L-amino acid oxidase, metalloproteinase, serine protease, mucoproteins as well as small peptides, which are known to interact with various ion channels in excitable cell membranes, making them good candidates for drug design in the pharmaceutical industry^{2,3}.

The scorpine, the subject of the present study, isolated from the venom of *Pandinus imperator*, was shown to have anti-bacterial and anti-plasmodial activities⁴, and also a strong inhibition of dengue 2

virus (DENV-2) infection⁵. Compared to other known antimicrobial peptide (AMPs), the native scorpine purified from venom glands has a peculiar structure. Its N-terminal amino acid sequence is similar to cecropins, whereas its C-terminal region has several disulfide bridges, similar to the structure of defensins⁴.

Isolation of antibacterial peptides from natural sources is inefficient and time-consuming. For pharmaceutical applications, the *Escherichia coli* expression system remains one of the most attractive because of its ability to grow rapidly and at high density on inexpensive substrates, its well-characterized genetics and the availability of an increasingly large number of cloning vectors and mutant host strains^{6,7}. Although *E. coli* is usually the first choice as a recombinant expression organism, many eukaryotic proteins, especially proteins with disulfide bridges, cannot be expressed as soluble, active proteins in *E. coli*⁸. In the pMAL-p5X protein fusion and purification system, the signal peptide on

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pre-maltose binding protein (MBP) directs fusion proteins to the periplasm. And for fusion proteins that can be successfully exported, this allows folding and disulfide bond formation to take place in the periplasm of *E. coli*, as well as allowing purification of the protein from the periplasm⁹. This advantage might contribute to the recombinant expression of scorpine, because its C-terminal region has several disulfide bridges.

In the present communication, we tried to establish an efficient method for the production of recombinant form of scorpine in *E. coli*. A fusion protein consisting of a N-terminal MBP, hexa-histidine tag and scorpine was overexpressed in *E. coli*. After amylose resin purification, the fusion protein was cleaved by Factor Xa protease to remove the MBP tag. The recombinant scorpine was further purified using Ni²⁺-NTA affinity chromatography. And the anti-bacterial and anti-plasmodial activities of recombinant scorpine were investigated.

Materials and Methods

Bacterial Strains, Vectors and Enzymes

E. coli DH5a (maintained in our laboratory) was used for subcloning and plasmid amplification. *E. coli* BL21 (DE3) (Novagen, USA) was used as the expression host. Strains of *Staphylococcus aureus* ATCC 29213 and *Acinetobacter baumannii* ATCC 19606 were purchased from China General Microbiological Culture Collection Center (CGMCC), China and cultured according to the method provided by CGMCC. pMAL-p5x vectors, amylose resin, Factor Xa protease and anti MBP monoclonal antibodies were purchased from New England Biolabs, Inc. (Beijing, China). All the restriction enzymes and T4 DNA ligase were purchased from Takara Biotech Co. Ltd. (Dalian, China).

Construction of Expression Vectors

The scorpine gene of interest was a gift from Fang's laboratory¹⁰. The PCR fragments were separated using 1.0% gel electrophoresis and purified with a DNA gel extraction kit (Takara, China). The resulting PCR product was digested with *NdeI* and *BamHI*, and ligated into the pMAL-p5x plasmid at the corresponding restriction sites¹¹. The ligation mixture was transformed into *E. coli* DH5a cells for verification by sequencing (Nanjing Genscript Bio. Co. Ltd.).

Expression and Characterization of MBP Fusion Protein

The pMAL-p5x/scorpine plasmid that had been constructed was transformed into competent *E. coli*

BL21 (DE3). Three colonies were picked and cultured in 4 mL sterilized Luria-Bertani (LB) medium with vigorous shaking (220 rpm) at 37°C to an optical density (A600) of 0.6. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) (0.5 mM) was then added to induce the expression of the recombinant protein at 28°C for 8 h.

SDS-PAGE and Western Blotting Analyses

The SDS-PAGE analysis was performed according to Laemmli¹² using 12% polyacrylamide gel. The samples from cell lysates after induction were mixed with equivalent sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.005% bromophenol blue & 10% 2-mercaptoethanol). Gels were stained with Coomassie brilliant blue R-250.

The Tricine/SDS-PAGE analysis for the resolution of proteins smaller than 30 kDa was performed according to the reference¹³ using 16.5% polyacrylamide gel. Gels were stained with Coomassie brilliant blue R-250.

For Western blotting, the same protein sample was separated on a 12% polyacrylamide gel under reducing conditions and then transferred to a polyvinylidene difluoride (PVDF) membrane (Roche Applied Science). The Western blotting was performed as described¹⁴. The rabbit IgG secondary antibody was used against MBP-tag primary antibody. The blots were developed using TMB immunoblotting system.

Purification of MBP Fusion Protein

BL21(DE3)-pMAL-p5x/scorpine strains were cultured in 200 mL sterilized LB medium with vigorous shaking (220 rpm) at 37°C to an optical density (A600) of 0.6. IPTG (0.5 mM) was then added to induce the expression of the recombinant protein at 28°C for 8 h. Cultures were collected by centrifugation at 12,000× g, at 4°C for 10 min. The pellet from 200 mL culture was then resuspended in 20 mL lysis buffer and lysed on ice by sonication at 400 W for 100 cycles (4 sec working, 8 sec free). The supernatant of the cell lysate resulting from centrifugation at 12,000× g at 4°C for 20 min was purified through an amylose resin (New England Biolabs) equilibrated in lysis buffer, eluted with amylose elution buffer (lysis buffer supplemented with 10mM maltose) according to the manufacturer's instruction. The fusion protein were pooled and dialyzed overnight at 4°C against phosphate buffered saline (PBS, pH 8.0).

Purification of His-scorpine

The dialyzed fusion protein was reacted with the Factor Xa protease to remove the MBP tag, according to the manufacturer's instruction. Briefly, after the MBP fusions were cleaved, the sample was loaded onto a nickel-nitrilotriacetic acid (Ni^{2+} -NTA, Novagen) affinity chromatography column according to the manufacturer's instruction. After extensive washing with binding buffer (10 column volumes) (20 mM Tris, 500 mM NaCl, 20 mM imidazole, and 10 mM phenylmethylsulfonyl fluoride, pH 8.0), the proteins was eluted with five column volumes of elution buffer (20 mM Tris, 500 mM NaCl, and 250 mM imidazole, pH 8.0). The peak fractions with high-UV values at A280, which were detected by LPDataView (BIORAD), and the eluates containing the His-scorpine proteins were pooled and dialyzed overnight at 4°C against phosphate buffered saline (PBS, pH 8.0). The purified proteins were checked on SDS-PAGE and stored at -80°C for activity assay.

Anti-bacterial Assays

To measure effects of recombinant scorpine on the growth of bacteria, the bacteria were transferred into 96-well flat-bottomed polystyrene microtiter plates (BD Falcon, SanJose, CA, USA) at approx 10^5 CFU/mL in TSB in the presence of recombinant His-scorpine (5 & 10 μM) and cultured at 37°C for 24 h under static conditions, aerobically. After cultivation, the supernatant was serially diluted 10-fold with 0.1% sterile BPW, and the dilutions were pour-plated with TSA. The numbers of bacteria were determined and calculated by the counts on agar plates.

Anti-plasmodial Assay

P. falciparum FCC1/HN strain was used for the assay. The FCC1/HN line was isolated from Hainan Island, China¹⁵. The strain was cultured in human erythrocytes type A+ at 5% hematocrit and RPMI (Gibco) medium supplemented with HEPES 25 mM, glutamine 2 mM, glucose 2 g/L, NaHCO_3 2 g/L, hypoxanthine 29.25 mg/L, gentamicin 60 mg/L and albumax 1.6% at pH 7.4, kept in 96% nitrogen, 3% CO_2 and 1% oxygen atmosphere at 37°C, and exchange medium was done every 24 h¹⁶.

P. falciparum at 2.5% of parasitemia was further cultured in the presence of recombinant His-scorpine (5 & 10 μM) for 24, 48 and 72 h. Every 24 h, the medium was discharged and fresh medium with the appropriate recombinant scorpine concentration was added. The percentage of infected red blood cells was determined by microscopic examination of thin blood

films stained with Giemsa. Each recombinant scorpine concentration was tested by duplicate. Controls with recombinant scorpine solvent (Schneider culture medium) and nontreated infected erythrocytes were included⁵.

Statistical Analysis

Results were expressed as mean \pm SD from at least three independent experiments. Statistical analysis was performed according to Student's t-test by one-way analysis of variance. Significant difference was taken as $P < 0.05$.

Results

Plasmid Construction and Expression of MBP Fusion Protein

The construct for scorpine expression, containing the MBP and His-tag for affinity purification, was depicted in Fig. 1. The recombinant plasmid pMAL-p5x/scorpine sequence was verified by DNA sequencing (Nanjing Genscript Bio. Co. Ltd.). The correct construct was transformed into the expression host *E. coli* BL21 (DE3). As shown in Figs 2A and C, there was an obvious protein band after IPTG induction, which could be detected using the Western blotting method with anti-MBP tag antibody. The apparent mol wt of the MBP fusion protein was about 54 kDa (the scorpine gene encodes a protein of 75 amino acids with 8 kDa plus MBP of about mol wt of 44.2 kDa).

Purification of MBP Fusion Protein

As described above, amylose resin was used for the fusion protein purification. The MBP-His-scorpine fusion protein was eluted with more than 90% purity using amylose elution buffer (lysis buffer supplemented with 10 mM maltose) (Fig. 2B). The purity was estimated through SDS-PAGE gels stained with Coomassie blue.

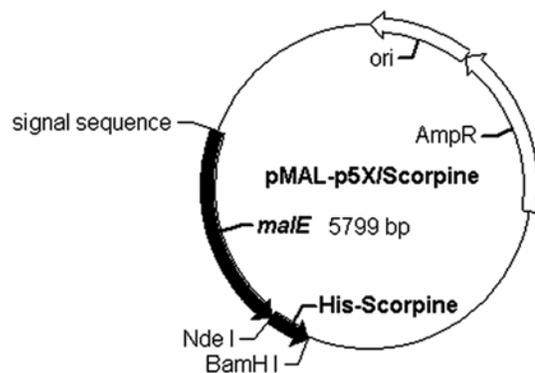


Fig. 1—Schematic representation of the expression vector pMAL-p5x/scorpine. Scorpine was expressed as a fusion protein with a N-terminal MBP and hexa-histidine tag.

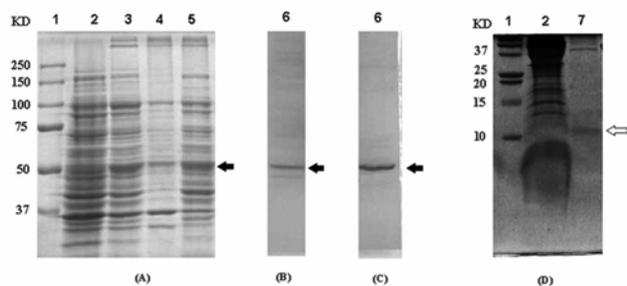


Fig. 2 (A-D)—The expression analysis of recombinant scorpine expressed in *E. coli* BL21 by 12% SDS-PAGE (A & B), Western blotting (C) and 16.5% Tricine-SDS-PAGE (D). [Lane 1: Protein mol wt marker; lane 2: Crude cells extracts of uninduced *E. coli* BL21 containing pMAL-p5x/scorpine; lane 3: Crude cells extracts of induced *E. coli* BL21 containing pMAL-p5x/scorpine; lane 4: Sonicated precipitation of induced *E. coli* BL21 containing pMAL-p5x/scorpine; lane 5: Sonicated supernatant of induced *E. coli* BL21 containing pMAL-p5x/scorpine; lane 6: Purified supernatant on amylose resin; lane 7: Recombinant His-scorpine with cleavage of MBP tag by Factor Xa protease; Black arrow showed the MBP-His-scorpine and white arrow showed the His-scorpine with cleavage of MBP tag.]

Purification of His-scorpine

The purified MBP-His-scorpine fusion protein was competently cleaved after incubation with Factor Xa protease, according to the method from New England Biolabs. After the cleaving sample was loaded to a Ni^{2+} -NTA column to remove MBP and protease, and finally purified His-scorpine was obtained with a yield of about 60 mg/L. The 16.5% Tricine/SDS-PAGE gel results indicated that His-scorpine had been purified successfully to more than 95 % purity (Fig. 2D). The purified His-scorpine was filtered through a 0.22 μm filter membrane and stored at -80°C for activity assays.

Anti-bacterial Activity of Recombinant Scorpine

It was very important to demonstrate that recombinant His-scorpine was capable of producing inhibition of bacteria growth similar to that originally shown for native scorpine isolated from *P. imperator* venom⁴. As shown in Fig. 3, the recombinant His-scorpine was able to inhibit the growth of two standard bacteria, *S. aureus* ATCC 29213 (Fig. 3A) and *A. baumannii* ATCC 19606 (Fig. 3B) at 24 h post-treatment. The maximum inhibitory effect was observed at 10 μM concentration of recombinant His-scorpine ($p < 0.05$).

Anti-plasmodial Activity of Recombinant Scorpine

The trophozoite stage cultures of *P. falciparum* were exposed to recombinant His-scorpine. As shown in Fig. 4, both the tested concentrations (5 & 10 μM)

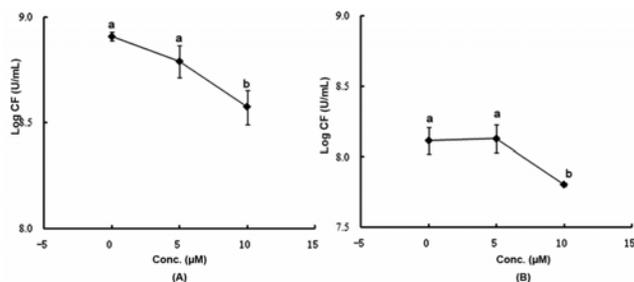


Fig. 3 (A & B)—Effect of recombinant scorpine on the growth of (A) *S. aureus* ATCC 29213 and (B) *A. baumannii* ATCC 19606, cultured in nutrient broth in the presence of recombinant His-scorpine for 24 h. [Different letters (a-b) are significantly different within treatments at $p < 0.05$.]

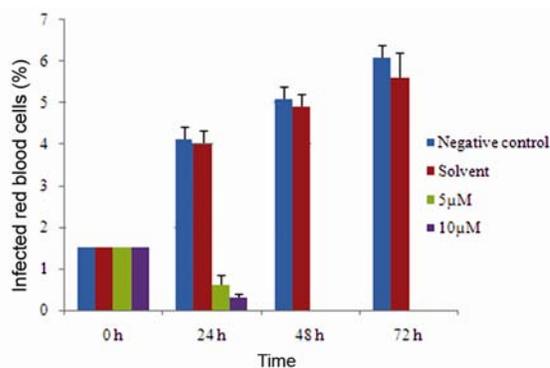


Fig. 4—Effect of recombinant scorpine on *P. falciparum* FCC1/HN trophozoite development. [Recombinant His-scorpine with cleavage of MBP tag by the Factor Xa protease was added at different concentrations (5 & 10 μM) to *P. falciparum* FCC1/HN cultures. The number of infected erythrocytes was counted as described in the 'Materials and Methods'.]

of recombinant His-scorpine reduced the parasite density over the time course of the experiment in relation to controls. At 24 h post-treatment, the maximum inhibitory effect was observed at 10 μM concentration of recombinant His-scorpine. However, a critical dependence on exposure time was also observed in recombinant His-scorpine treated cultures. At 48 h of exposure, no parasites were observed at 5 μM or above concentration of recombinant His-scorpine. In addition, no infected erythrocytes were detected after 72 h at all the concentrations tested.

Discussion

Scorpions have developed a negative reputation due to their stings and envenomation, usually resulting in pain, swelling, hypertension, cardiac arrhythmia and other systemic manifestations¹⁷. However, human beings have also derived benefits

from the scorpions. In China, fried scorpions are popularly consumed as food, while scorpion or snake wines are used to strengthen the immune system¹⁸. Scorpion venoms are rich source of peptides with a variety of pharmacological functions, especially those that interact with membrane permeability of excitable and non-excitable cells for Na⁺, K⁺, Ca²⁺ and Cl⁻¹⁹⁻²¹. Several antimicrobial peptides have been described from scorpions. Among the peptides isolated from the venom of the African scorpion, *P. imperator*, a small cationic peptide named scorpine was identified⁴, showing the anti-bacterial and anti-plasmodial activities^{4,5}, with the possibility of being used as an anti-microbial and anti-plasmodial agent in the future. Native scorpine purified from venom glands has a molecular mass of 8350 Da. It has a peculiar structure compared to other known AMPs. Its N-terminal amino acid sequence is similar to cecropins, whereas its C-terminal region has several disulfide bridges, similar to the structure of defensins⁵. Isolation and purification of antibacterial peptides from natural sources is inefficient and time-consuming, while the *Escherichia coli* expression system remains one of the most attractive because of its ability to grow rapidly and at high density on inexpensive substrates^{6,7}. In the present study, we have established an efficient method for the production and purification of recombinant scorpine in *E. coli* using the MBP fusion partner.

Similar to that original native scorpine isolated from *P. imperator* venom⁴, the recombinant His-scorpine was able to inhibit the growth of the bacteria, *S. aureus* ATCC 29213 and *A. baumannii* ATCC 19606 (Fig. 3). Our results clearly showed that the recombinant scorpine has the potential of clinical use in future.

Malaria, caused by *Plasmodium* infection, is one of the most debilitating parasitic diseases of humans, with an estimated 225 million clinical cases and 781,000 deaths per year²². The native scorpine was shown to have the anti-plasmodial activities^{4,5}. So, we investigated effects of recombinant scorpine on the growth of *P. falciparum*. As shown in Fig. 4, in the present study, both the concentrations of recombinant scorpine tested on *P. falciparum* trophozoite stage cultures reduced the parasite density over the time course of the experiment. These results suggested that recombinant scorpine is similar to that of original native scorpine and could inhibit the growth of *Plasmodium* spp.

In summary, the MBP fusion partner and customized expression and purification protocol described here have further improved the efficiency and lowered the costs of scorpine production and laid the foundation for pharmaceutical applications in the future.

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