PEPTIDES 28 (2007) 2299–2305

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/peptides

Inducible antibacterial response of scorpion venom gland

Bin Gao, Caihuan Tian, Shunyi Zhu *

Group of Animal Innate Immunity, State Key Laboratory of Integrated Management of Pest Insects & Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, PR China

ARTICLE INFO

Article history:
Received 19 September 2007
Received in revised form
3 October 2007
Accepted 3 October 2007
Published on line 13 October 2007

Keywords:
Innate immunity
Buthus martensii
Antibacterial peptide
Inducible expression
Venom

ABSTRACT

Innate immunity is the first line defense of multicellular organisms that rapidly operates to limit aggression upon exposure to pathogen microorganisms. Although the existence of some antibacterial peptides in scorpion venoms suggests that venom gland could be protected by these effector molecules, antibacterial activity of venom itself has not been assessed. In this study, we reported the antibacterial activity of the venom of Chinese scorpion *Buthus martensii*. Protease K digestion test indicated that it is venom peptide/protein components, as key players, which are involved in such antibacterial response. As the first step toward studying molecular mechanism of scorpion venom gland immunity, we established an infection model which supports inducible antibacterial response of scorpion venom gland. A known *B. martensii* antibacterial peptide gene *BmKb1* was up-regulated at the transcriptional level after venom gland was challenged, suggesting its key defense role. This is further strengthened by the presence of several immune response elements in the *BmKb1* promoter region. Our work thus provides the first evidence supporting the role of venom antibacterial peptides (ABPs) in controlling scorpion venom gland infection and lays a basis for characterizing related components involved in regulation of scorpion venom gland ABP gene expression.

1. Introduction

Scorpion is one of the most ancient arthropods with about 430 millions of evolutionary history [18]. They developed the venom gland associated with specialized envenomation apparatus for prey and defense which compensates their physical limitation. Scorpion venoms contain rich biologically active constituents and majority of them are some polypeptides with neurotoxicity [19,21,24,27]. These neurotoxins fulfill their functions by targeting a variety of ion-channels present in excitable cells of their preys. Only recently some scorpion venom peptides with other functions have been reported, of which those with antibacterial activities have attracted more attention due to their therapeutic potential. Since the first two antibacterial peptides Hadrurin and Scorpine were respectively isolated from the scorpion *Hadrurus aztecus* and *Pandinus imperator*, several additional venom peptides had been characterized as antibacterial peptides with a broad-spectrum of bacterial targets [5–7,17,20,23,28]. Very recently, Zeng et al. identified and functionally characterized two antibacterial peptides (BmKb1 and BmKn2) from the scorpion *Buthus martensii* by the combined application of molecular cloning and polypeptide chemical synthesis techniques [26].

Despite the body of evidence supporting the role of antibacterial peptides (ABPs) in local immune defense of animal venom glands has been growing, it still remains unclear whether these venom-derived ABPs are essential to the immune protection of scorpion venom glands. In this work, we studied antibacterial properties of the venom of Chinese scorpion *B. martensii* and provided evidences for a major role of polypeptides in antibacterial response of *B. martensii* venom. We also established an infection model and

* *Corresponding author*. Tel.: +86 10 64807112.
E-mail address: Zhusy@ioz.ac.cn (S. Zhu).
0196-9781/$ – see front matter
doi:10.1016/j.peptides.2007.10.004

detected increased antibacterial activity after bacterial challenge, which indicated that an inducible expression mechanism may have been developed for the defense of scorpion venom gland.

2. Materials and methods

2.1. Venom collection

Scorpions were kindly provided by Dr Qilian Qin (Beijing, China). Venoms were obtained by electric stimulation of telson [18] with 128 Hz of frequency and 20 V of voltage and were collected into a 1.5 ml sterile Eppendorf tube. After centrifuged at 14,000 rpm for 15 min at 4 °C, the supernatant was transferred to a fresh tube and frozen at −20 °C for use.

2.2. Protein concentration determination

Bradford method was used to determine protein concentration [2]. Protein quantification kit was purchased from Beyotime Institute of Biotechnology (Shanghai, China). Absorbance was measured at 595 nm with DNM-9602 Microplate Reader and BSA was used as standard.

2.3. Antibacterial assays

Four bacterial strains used in the tests were respectively purchased from the Center for Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences (Beijing, China) (*Escherichia coli* ATCC 25922 and *Micrococcus luteus*) and China Center for Type Culture Collection (Wuhan, China) (*Bacillus megaterium* and *Agrobacterium tumefaciens*). Classical inhibition zone assay and liquid-growth inhibitory test were used to detect the antibacterial activity of *B. martensii* venoms [12,22]. For inhibition zone assay, 50 μl of bacteria were inoculated into 5 ml Luria-Bertaini's medium and grew overnight at 37 °C with shaking at 200 rpm. A 10 μl aliquot of each of the cultures was diluted in 6 ml pre-heated (approximately 45 °C) Luria-Bertaini's medium (1% bactotryptone, 0.5% bactoyeast extract and 0.5% NaCl) containing 0.8% agar. The mixture was spread on a 9-cm Petri dish, giving a depth of 1 mm. After settling, 3-mm wells were punched in the plate and then 3 μl of venom diluted with sterile ddH_2O to different concentrations was added to each well. The agar plates were incubated overnight at 37 °C. For liquid-growth inhibition assay, 50 μl of overnight *M. luteus* culture was inoculated into 5 ml Luria-Bertaini's medium and incubated at 37 °C for 1–3 h with shaking at 200 rpm until they grew to about 0.2 OD_{630}. Twenty microliters of crude venom and ddH_2O (as a control) were separately added to 80 μl culture of *M. luteus* and *B. megaterium*. Absorbance at 630 nm was measured per half hour.

2.4. Protease K digestion

To determine the role of polypeptides/proteins in the antibacterial activity of scorpion venom, we carried out the protease K digestion assay by treating the venom with protease K at 37 °C overnight. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and inhibition zone assay on plate of *M. luteus* were used to examine the change of the venom peptides/proteins after digestion.

2.5. Establishment of infection model

A standard method was applied to establish an infection model [25]. Briefly, 3 μl of combined bacterial mixture of *E. coli* ATCC 25922 and *M. luteus* which had been separately incubated overnight to 1.8 OD_{600} was injected into venom glands with a micro-injector. Average 10 scorpions were challenged once. Ten hours later, venoms were collected according to the method described above. Venoms from 10 non-injected scorpions were used as a control. The inhibition zone assay and semi-quantitative RT-PCR were chosen to evaluate the challenge effectiveness.

2.6. Preparation of total RNA and genomic DNA

For isolating venom gland total RNA, eight scorpion tails challenged 10 h by bacteria were cut off and ground into fine powder in liquid nitrogen. The Trizol reagent (SBS Genetech, Beijing) was used to prepare total RNA according to the supplier's instructions. This method was also applied to prepare non-challenged venom gland total RNA. Genomic DNA was isolated from legs of two scorpions according to a previously described method [29].

2.7. Semi-quantitative RT-PCR

Reverse transcription of total RNA was performed using RT-PreMix kit (SBS Genetech, Beijing) and a universal oligo (dT)-containing adaptor primer dT3AP. The first-strand cDNA amplification was performed using the *BmKb1* gene-specific primer GBF and the universal primer 3AP according to the method described by McPherson and Moller [16]. RPL19e was chosen as an internal control which was amplified by using primers L19F/3AP and the same cDNA template.

2.8. 5′RACE

According to the nucleotide sequence of *BmKb1* [15], we designed one reverse gene-specific primer (GBR) for 5′RACE to define its transcriptional start site. Briefly, total RNA was reverse-transcribed into the first-strand cDNAs using RT-PreMix kit (SBS Genetech, Beijing) and a universal oligo(dT)-containing adaptor primer (dT3AP). The purified first-strand cDNA mixture was tailed with terminal transferase and dCTP (Takara, Dalian). A PCR amplification of the tailed first-strand cDNAs was carried out using primers dG and GBR.

2.9. Isolation of the promoter region

Thermal asymmetric interlaced PCR (TAIL-PCR) [14] was used to amplify the upstream regulation region of the *BmKb1* gene. This method consists of three consecutive PCR reactions with three reverse nested gene-specific primers (Bm0, Bm1 and Bm2) designed according to the nucleotide sequence of *BmKb1* [15] and the shorter arbitrary degenerate primer AD3.

2.10. DNA sequencing

PCR products were ligated into the pGEM-T Easy Vector following purification using PCR purification kit (Tiangen Biotech, Beijing). *E. coli* DH5α was used for plasmid propagation. Recombinant clones were analyzed by PCR using two vector primers (SP6 and T7) and gel electrophoresis. Positive clones were sequenced through the chain termination method using the primers SP6 and T7. The nucleotide sequence of the *BmKb1* promoter has been deposited in the GenBank database (http://www.ncbi.nlm.nih.gov) under the accession number of EU158251.

2.11. Promoter analysis and DNA secondary structure prediction

Potential transcription factor (TF)-binding sites in the upstream regulation region of *BmKb1* promoter was searched using the P-Match program against the TRANSFAC database under default parameters [3]. Promoter secondary structure prediction was performed at the DNA mfold server (http://www.bioinfo.rpi.edu/).

2.12. Primer sequences

All primers used in this study are synthesized by SBS Genetech (Beijing, China) and listed in Table 1.

3. Results

By using classical inhibition zone assay, we for the first time show that *B. martensii* venom is active against gram-positive bacteria *M. luteus* and *B. megaterium* (Fig. 1A. only *M. luteus* treated using 2 mg/ml shown) in which a clear inhibition zone can be formed. However, the venom appears to lack activity against the gram-negative bacteria *E. coli* ATCC 25922 and *A. tumefaciens* even in 10 mg/ml of venom concentration (data not shown). Liquid-growth inhibition assay showed that the growing bacterial culture of *M. luteus* and *B. megaterium* displayed a fast response when treated with *B. martensii* venoms. A decrease in OD_{630} revealed the bactericidal effect of the *B. martensii* venom (Fig. 1B, only *M. luteus* shown).

Because previous studies have identified several antibacterial peptides from venoms derived from different scorpion

Table 1 – PCR primers

Name	Sequence	Usage
dT3AP	5′-CTGATCTAGAGGTACCG-GATCCTTTTTTTTTTTTTTTT-3′	Reverse transcription
3AP	5′-CTGATCTAGAGGTACCG-GATCC-3′	Semi-quantitative RT-PCR
GBF	5′-CAGAATATTCGAAACTCGGC-3′	Semi-quantitative RT-PCR
L19F	5′-AAGAAGGCCGAAAATT-CACGTC-3′	Semi-quantitative RT-PCR
Bm0	5′-TTAGAAATC TGGGA-GAGTTGGGTCCT-3′	TAIL-PCR
Bm1	5′-TCCTATGTAAACTTAGGAA-TACTCACC-3′	TAIL-PCR
Bm2	5′-GCAGGACTAAGAAGACGG-TAAGAAG-3′	TAIL-PCR
AD3	5′-NTCGASTWTSGWGTT-3′ (N = A/T/G/C; S = G/C; W = A/T)	TAIL-PCR
dG	5′-AT-GAATTCGGGGGGGGGGGGG-3′	5′RACE
GBR	5′-GAGAGACTAACGACTGTAG-TA-3′	5′RACE

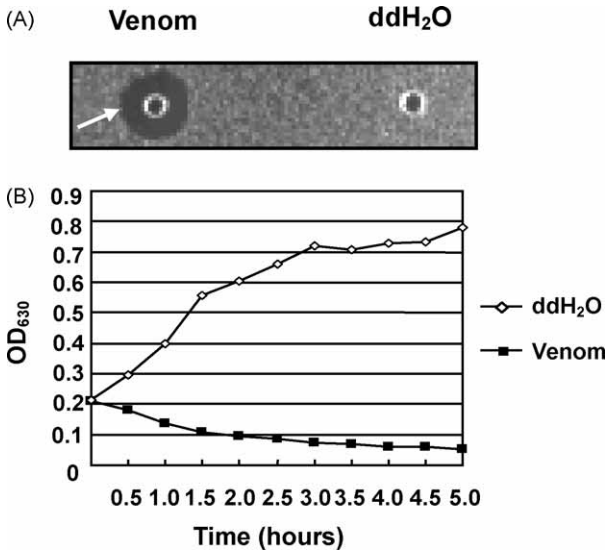

Fig. 1 – Antibacterial activity of *Buthus martensii* venom against *Micrococcus luteus* identified by inhibition zone assay (2 mg/ml used) (A) and liquid-growth inhibition (crude venom used) (B). Arrow labels the edge of inhibition zone.

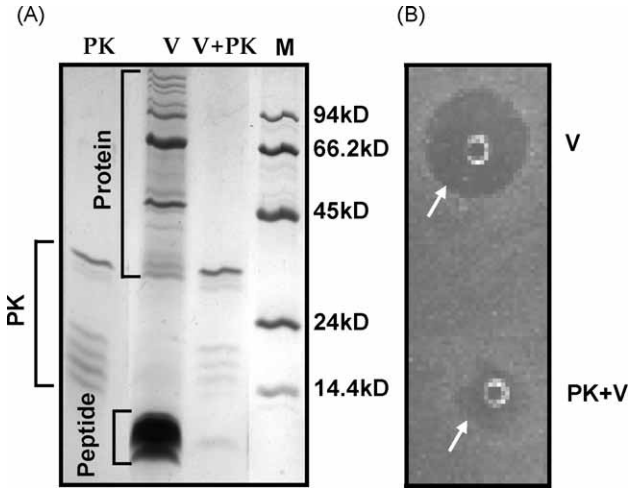

Fig. 2 – Effect of protease K digestion on antibacterial activity of *B. martensii* venom. 5 mg/ml venom was used. (A) Detection of digestion efficiency of protease K by SDS-PAGE and (B) Evaluation of antibacterial activity of digested and native venoms by inhibition zone assay on the plate of *M. luteus*. PK: protease K; V: venom; PK + V: venom digested by protease K; M: protein marker. Arrow labels the edge of inhibition zone.

species, we want to know whether peptides are key players involved in antibacterial response of the venom. To do this, we digested the venom using protease K at 37 °C overnight. SDS-PAGE revealed that proteins up 20 kD completely disappeared and only few peptides under 10 kD remained (Fig. 2A). Correspondingly, in the inhibition zone assay, the diameter of the inhibition zone dramatically reduced in comparison with the control (venom not treated with protease K) (Fig. 2B). Because only the digestion-resistant peptide component was present in the digested venom, it is reasonable to infer it is these peptides which conferred the observed antibacterial activity of the digested venom (Fig. 2B).

To determine whether there is any change in antibacterial activity of venoms before and after bacterial challenge, we established an infection model of *B. martensii* venom gland (Fig. 3A). After scorpions were infected 10 h, venoms were collected using an electric stimulation method (Fig. 3B). The sizes of the inhibition zones were compared between non-injected and injected scorpions. As shown in Fig. 3C, the challenged venoms resulted in larger inhibition zones than the control in the *M. luteus* agar plate with a concentration-dependent manner, suggesting that a possible inducible mechanism regulates the antibacterial response of scorpion venom gland. Remarkably, the increase in venom antibacterial activity after challenge appears to be related to an increase of peptide components under 10 kD, as detected by SDS-PAGE (Fig. 3D). In this case, most of protein components did not change except those with about 66 kD decreased.

To investigate whether the increase of antibacterial activity after venom gland infected is related to the up-regulation of ABP genes, we adopted semi-quantitative RT-PCR to detect change of *BmKb1* at the transcriptional level, a known ABP gene isolated from the *B. martensii* venom gland. The result revealed a significant increase of *BmKb1* transcripts in the challenged venom gland identified by the appearance of PCR product after 25 cycles whereas for the non-challenged template only after 32 cycles the PCR product can be seen (Fig. 3E).

Up-regulation of antibacterial peptide gene expression after infection is a common defense strategy which is controlled by some immune response elements in the upstream regulation region of a promoter [9]. To confirm whether there are such elements in the *BmKb1* promoter, we isolated its promoter region by a TAIL-PCR strategy. After tertiary amplification had been performed, one ~1 kb fragment was recovered and sequenced. Because *BmKb1* was isolated from a venom gland cDNA library, its 5′-end does not probably represent the real transcription start site (TSS) due to partial RNA degradation. To define such site we performed

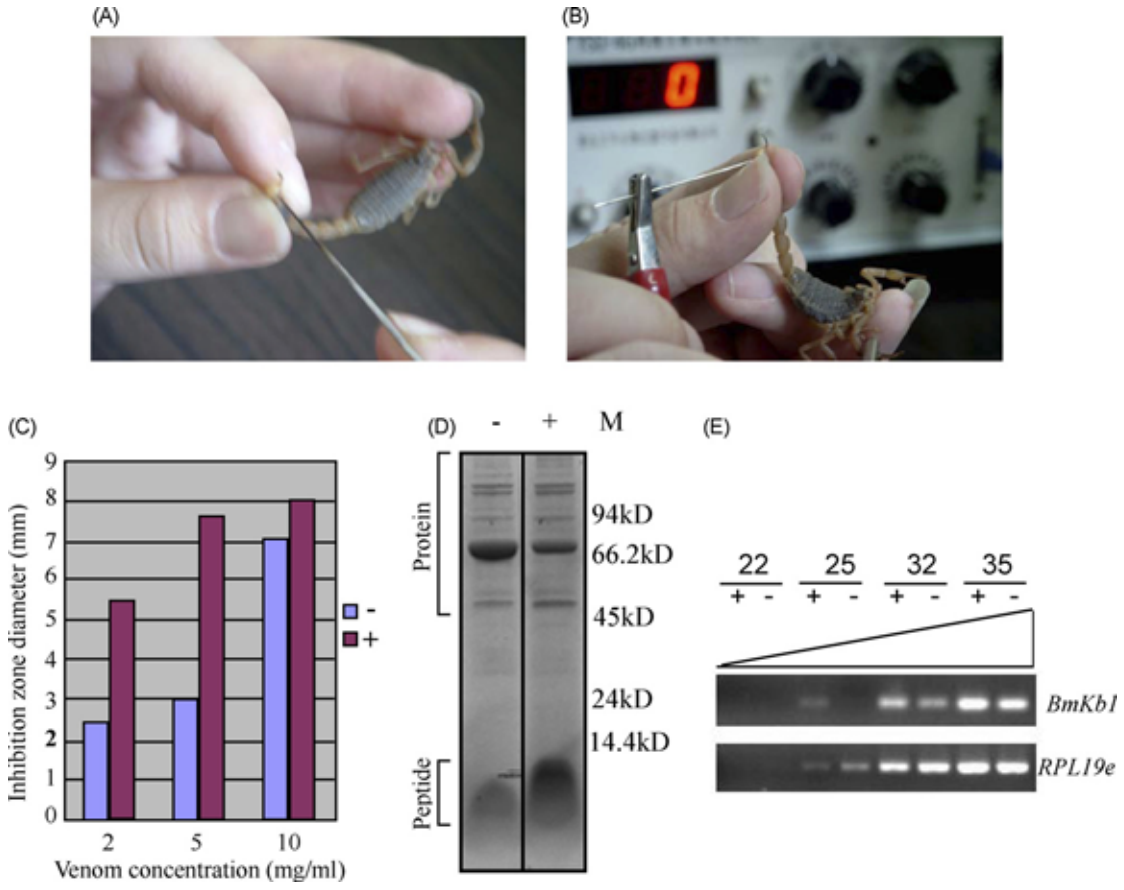

Fig. 3 – Evidence for an inducible antibacterial response of *B. martensii* venom gland: (+) challenged and (−) non-challenged. (A) Venom gland was challenged by bacteria; (B) extraction of scorpion venom under electric stimulation of telson; (C) antibacterial activity change before and after bacterial challenge; (D) venom protein/peptide component change before and after bacterial challenge; (E) semi-quantitative PCR detecting up-regulation of *BmKb1* before and after bacterial challenge. M: protein marker.

5′RACE which extended seven nucleotides (AGTTTCC) at the 5′-end. When direct mapping of cDNA obtained by 5′RACE into the genomic sequence, we found that the putative transcriptional start sequence TCA^{+1}GTTTC of *BmKb1* (where A^{+1} is the base at which transcription starts) exactly matches the eukaryotic initiator element consensus [(TC)CA^{+1}N(TA)(TC)(TC)(TC)] (N is any of the four bases). The initiator is thought to be an alternative promoter element which is capable of replacing the function of the TATA box, one of the most prominent core elements in eukaryotic as well as prokaryotic promoter. Its presence explains the lack of a TATA element at the 30 nucleotides upstream of the transcriptional initiation site of *BmKb1* promoter. In addition to the initiator element, P-Match program in combination of the known consensus motif information [9] identified additional 18 putative transcriptional factor binding sites with ≥95% reliability (Fig. 4A), of which many sites are homologous to response elements for transcription factors described in the promoters of insect immune responsive genes and mammalian acute-phase protein genes, including putative binding sites for nuclear factor κB (NF-κB), GATA factors, interferon consensus response elements (ICRE), nuclear factor endothelial leucocyte adhesion molecules (NF-ELAM 1) [9]. Given the role of these elements in regulating the expression of many immune-related genes, it is likely that these elements also are responsible for the antibacterial response of *BmKb1* after challenge. In addition to these putative regulatory motifs for the binding of transcriptional factors, a long hairpin was predicted to exist in the promoter region of *BmKb1* with $\Delta G_0 = -69.2$ kcal/mol at 37 °C (Fig. 4B). This hairpin is composed of 192 nucleotides (nt) with a 11 nt loop comprising the NF-κB motif. The location of this motif in a loop might facilitate its binding with the corresponding transcriptional factor.

4. Discussion

Studies have reported that venoms from snake, wasp and spider possess antibacterial activities that may be related to their protection role to venom gland tissues [1,8,10,13]. However, whether it is a similar case for scorpion venoms remains unknown at present. We initiated this work based on the following observations in terms of immunological roles of scorpion venoms: (1) Scorpion venom glands, as a venom-secreted organ, are exposed to the exterior through the envenomation apparatus at the end of telson and frequently directly contact some microorganisms present in their preys and survival entertainment, however, the glands themselves are not infected; (2) The *B. martensii* scorpion sprays its venom to the injured body (Gao and Zhu, unpublished observation) and some scorpion species often spray the venom on their own bodies to clean microorganisms [23]. Supported by the above facts, it is reasonable to infer that scorpion venoms may play multiple roles in protecting the venom gland and other parts of the scorpion from microbial infection.

Interestingly, antibacterial activity of *B. martensii* venom was detected only against gram-positive bacteria but not against gram-negative bacteria under the condition we tested. This is sharply different from endoparasitic wasp (*Pimpla hypochondriaca*) venom that was active against gram-negative bacteria but not against gram-positive bacteria [8]. The precise mechanism behind this phenomenon is still unclear.

Increase in both peptide components and antibacterial activity of venoms after challenge provided clues for some antibacterial peptide genes participating in inducible antibacterial response of *B. martensii* venom gland against bacterial infection. This has been confirmed by the PCR detecting the *BmKb1* transcript level of the venom gland after challenge.

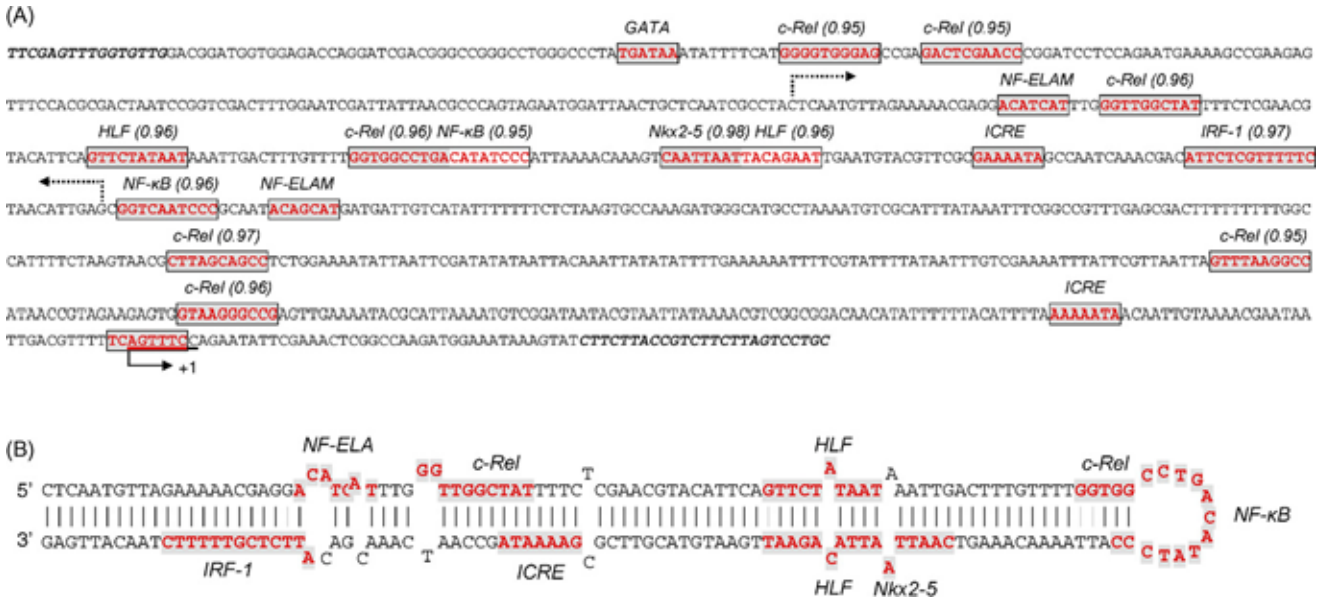

Fig. 4 – Promoter sequence (A) and the long hairpin of the *BmKb1* gene (B). Primer regions for TAIL-PCR are boldfaced and italicized. Dotted arrows label the region of the hairpin. Upstream immune response elements of the *BmKb1* gene are boxed and indicated by red letters, in which those with reliability values were predicted by P-Match and others from the consensus motif information [9]. 5′-End sequence determined by 5′RACE is underlined once. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

However, synthesized BmKb1 showed only weak inhibition effect on gram-positive bacterium *M. luteus*, which appears to be inconsistent with the increase of antibacterial activity of the crude venom after challenge. Such inconsistency could be explained by the possible allosteric effect of BmKb1 which might enhance antibacterial activity of other components in scorpion venom. For example, some weakly toxic polypeptides in scorpion venoms have been found to have ability to enhance the effect of other active neurotoxins [4]. As an important component of animal innate immunity, ABPs are often constitutively expressed or induced by microorganisms or their products. To the best of our knowledge, the discovery of inducible mechanism involved in antibacterial response of *BmKb1* is the first work in venom ABPs.

In comparison with the promoters of two previously cloned scorpion venom peptide genes (*opiscorpine* and *AaHI′*) [8,28], *BmKb1* promoter is unique in the following aspects: (1) the lack of two distinct modules (A and B) separated by one spacer of about 200 bp which are conserved between *opiscorpine* and *AaHI′*; (2) the existence of some typical response elements (e.g. NF-kB motif) and a long hairpin which are lacked in *opiscorpine* and *AaHI′*. Whether these remarkable differences in promoter organization and regulatory elements reflect their expression regulation pattern differentials between *BmKb1* (inducible) and *opiscorpine/AaHI′* (constitutive) remains an open question. Given Toll signal pathway is the most crucial components controlled the Gram-positive bacterial attack in *Drosophila* [11], recognition of two NF-κB motifs hints that a similar signal pathway could also have been evolved in scorpion venom gland cells for the regulation of the expression of these antibacterial peptide genes in a similar manner. In fact, successful isolation of Toll-like receptor and serpin cDNA clones from *B. martensii* venom gland further strengthens our assumption (Zhu et al., unpublished data). If this is true, scorpion venom gland might represent an ideal model to study arachnida Toll pathway related to innate immunity from an evolutionary perspective.

Our work thus paves the way for further characterizing immune-related components involved in *B. martensii* venom gland antibacterial response at the molecular level, which will undoubtedly expand our understandings in innate immunity of exposed tissue of metazoans. Because of considerable amounts of polypeptide component's involvement in antibacterial response of venom gland, this offers clues for the search of new antibacterial template from scorpion resources for drug design.

Acknowledgements

Zhu would like to thank the support of Bairen Plan from the Chinese Academy of Sciences, China. This work was also granted by the National Natural Science Foundation of China (30621003).

REFERENCES

[1] Blaylocks RS. Antibacterial properties of KwaZulu natal snake venoms. Toxicon 2000;38:1529–34.
[2] Bradford MM. A rapid sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 1976;72:248–54.
[3] Chekmenev DS, Haid C, Kel AE. P-Match: transcription factor binding site search by combining patterns and weight matrices. Nucleic Acids Res 2005;33:W432–7. Web Server issue.
[4] Cohen L, Lipstein N, Gordon D. Allosteric interactions between scorpion toxin receptor sites on voltage-gated Na channels imply a novel role for weakly active components in arthropod venom. FASEB J 2006;20:1933–5.
[5] Conde R, Zamudio FZ, Rodriguez MH, Possani LD. Scorpine, an anti-malaria and anti-bacterial agent purified from scorpion venom. FEBS Lett 2000;471:165–8.
[6] Corzo G, Escoubas P, Villegas E, Barnham KJ, He W, Norton RS, Nakajima T. Characterization of unique amphipathic antimicrobial peptides from venom of the scorpion *Pandinus imperator*. Biochem J 2001;359:35–45.
[7] Dai L, Corzo G, Naoki H, Andriantsiferana M, Nakajima T. Purification, structure-function analysis, and molecular characterization of novel linear peptides from scorpion *Opisthacanthus madagascariensis*. Biochem Biophys Res Commun 2002;293:1514–22.
[8] Dani MP, Richards EH, Isaac RE, Edwards JP. Antibacterial proteolytic activity in venom from the endoparasitic wasp *Pimpla hypochondriaca* (Hymenoptera: Ichneumonidae). J Insect Physiol 2003;49:945–54.
[9] Eggleston P, Lu W, Zhao Y. Genomic organization and immune regulation of the defensin gene from the mosquito, *Anopheles gambiae*. Insect Mol Biol 2000;9:481–90.
[10] Haeberli S, Kuhn-Nentwig L, Schaller J, Nentwig W. Characterisation of antibacterial activity of peptides isolated from the venom of the spider *Cupiennius aslei* (Araneae: Ctenidae). Toxicon 2000;38:373–80.
[11] Hoffmann JA. The immune response of *Drosophila*. Nature 2003;426:33–8.
[12] Hultmark D, Engström A, Andersson K, Steiner H, Bennich H, Boman HG. Insect immunity. Attacins, a family of antibacterial proteins from *Hyalophora cecropia*. EMBO J 1983;2:571–6.
[13] Kuhn-Nentwig L. Antibacterial and cytolytic peptides of venomous arthropods. Cell Mol Life Sci 2003;60:2651–68.
[14] Liu YG, Robert FW. Thermal asymmetric interlaced PCR: automatable amplification and sequencing of insert end fragments from PI and YAC clones for chromosome walking. Genomics 1995;25:674–81.
[15] Luo F, Zeng XC, Hahin R, Cao ZJ, Liu H, Li WX. Genomic organization of four novel nondisulfide-bridged peptides from scorpion *Mesobuthus martensii* Karsch: gaining insight into evolutionary mechanism. Peptides 2005;26:2427–33.
[16] McPherson MJ, Moller SG. PCR. Trowbridge: Cromwell Press; 2000.
[17] Moerman L, Bosteels S, Noppe W, Willems J, Clynen E, Schoofs L, Thevissen K, Tytgat J, Van Eldere J, van der Walt J, Verdonck F. Antibacterial and antifungal properties of α-helical, cationic peptides in the venom of scorpions from southern Africa. Eur J Biochem 2002;269:4799–810.
[18] Polis GA. The biology of scorpions. Stanford, CA: Stanford University Press; 1990.
[19] Possani LD, Becerril B, Delepierre M, Tytgat J. Scorpion toxins specific for Na^+-channels. Eur J Biochem 1999;264:287–300.
[20] Possani LD, Corona M, Zurita M, Rodriguez MH. From noxiustoxin to scorpine and possible transgenic mosquitoes resistant to malaria. Arch Med Res 2002;33:398–404.
[21] Possani LD, Merino E, Corona M, Bolivar F, Becerril B. Peptides and genes coding for scorpion toxins that affect ion-channels. Biochimie 2000;82:861–8.

[22] Shafer WM. Antibacterial peptide protocols. New Jersey: Humana Press; 1997.
[23] Torres-Larios A, Gurrola GB, Zamudio FZ, Possani LD. Hadrurin, a new antimicrobial peptide from the venom of the scorpion *Hadrurus aztecus*. Eur J Biochem 2002;267:5023–31.
[24] Tytgat J, Chandy KG, Garcia ML, Gutman GA, Martin-Eauclaire MF, van der Walt J, Possani LD. A unified nomenclature for short-chain peptides isolated from scorpion venoms: alpha-KTx molecular subfamilies. Trends Pharmacol Sci 1999;20:444–7.
[25] Tzou P, et al. Methods for studying infection and immunity in *Drosophila*. In: Sansonetti PJ, Zychlinsky A, editors. Methods in microbiology. Academic Press; 2002. p. 507–30.
[26] Zeng XC, Wang SX, Zhu Y, Zhu SY, Li WX. Identification and functional characterization of novel scorpion venom peptides with no disulfide bridge from *Buthus martensii* Karsch. Peptides 2004;25:143–50.
[27] Zhu S, Gao B, Tytgat J. Phylogenetic distribution, functional epitopes and evolution of the CS$\alpha\beta$ superfamily. Cell Mol Life Sci 2005;62:2257–69.
[28] Zhu S, Tytgat J. The scorpine family of defensins: gene structure, alternative polyadenylation and fold recognition. Cell Mol Life Sci 2004;61:1751–63.
[29] Zhu S, Darbon H, Dyason K, Verdonck F, Tytgat J. Evolutionary origin of inhibitor cystine knot peptides. FASEB J 2003;17:1765–7.