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Chunsheng Hou
College of Food Science, South China Agricultural University, 482 Wushan Street, Guangzhou, China.
Email: houfei2701@163.com

Liqiong Guo
College of Food Science, South China Agricultural University, 482 Wushan Street, Guangzhou, China.
Email: guolq@scau.edu.cn

Wuhua Wu
College of Food Science, South China Agricultural University, 482 Wushan Street, Guangzhou, China.
Email: 396015938@qq.com

Teng Wang
College of Food Science, South China Agricultural University, 482 Wushan Street, Guangzhou, China.
Email: 379600832@qq.com

Junfang Lin
College of Food Science, South China Agricultural University, 482 Wushan Street, Guangzhou, China.
Email: linjf@scau.edu.cn.

Correspondence:
Junfang Lin
College of Food Science, South China Agricultural University, 482 Wushan Street, Guangzhou, China.
Email: linjf@scau.edu.cn.
Tel:+86-20-38902762.

Molecular Cloning and Sequence Analysis of Two Peptides from Honeybee (*Apis mellifera* spp) Venom

Chunsheng Hou, Liqiong Guo, Wuhua Wu, Teng Wang, Junfang Lin

ABSTRACT

Honeybee is a major pollinator for agricultural crops and wild flower plants that supply food for human. *Apis mellifera ssp* is one of the most important honeybee species not only in Northeast China, but also in the world, and is one of subspecies of Italic honeybees with a stronger foraging and higher stress-resistance capability compared to other honeybee species. Two genes, melittin and secapin, from the venom of *A. mellifera ssp* were cloned and identified by RT-PCR for the first time. Compared of these two peptides amino acid sequence with those of the western and eastern honeybee revealed that they had highly similarities in the compositions of amino acid. The phylogenetic trees show that the two genes were more closely related to the westerner honeybee than the eastern honeybee. These results indicate that the amino acid sequences of melittin and secapin are different from those reported from *A. mellifera ligustica,* although they all belong to the same family. Furthermore, those results support the evidence to preserve and explore resources from *A. mellifera ssp.*

Keywords: Melittin, Secapin, Venom, Molecular cloning, *Apis mellifera ssp.*

1. Introduction

Honeybee venom is composed of enzymes, peptides and a large number of low molecular substances including melittin and secapin, which was widely applied to medicine treatment due to their physiological functions, such as anti-inflammatory, immunity-promoting, antibacterial, protecting hepatocytes and reducing postherpetic neuralgia[1, 2, 3, 4, 5, 6]. The full-gene genome sequence from western honeybee (*Apis mellifera ligustica*) had been published [7] to provide great convenience for research on other honeybee species. Some peptides had also been cloned and identified from the honeybee venom[8], and shown different physiological functions such as beneficial to relieve pressure, low tachycardic[9], antimicrobial[10], increased level of various cytokines and inhibited tumor cell[11]. The honeybee venom peptides, including melittin, secapin, apamin and mast cell degranulating peptide (MCDP), are the critical elements of the honey bee immune system, and more honey bee venom peptides were characterized by means of various molecular and isolated in recent years[9, 12, 13, 14].

Melittin and secapin account for more than 50% of the venom dry weight, they own the strongest antibacterial and hemolytic activity, although other components also have similar effects including phospholipase A_2 (PLA2) and MCDP[15]. More recently, a novel antimicrobial peptide named halictines was isolated and characterized from the eusocial bee *Halictus sexcinctus,* which has a noticeable hemolytic activity[16]. Another two peptides, bombolitin and osmin, were isolated from the venom of bumblebee and leafcutter bee and displayed that they possessed strong antibacterial and anti-fungal effects[17, 18]. Genes encoding melittin[19, 20] and secapin[21, 22] from the venom glands of worker bees of *A. mellifera ligustica* and *Apis cerana cerana* had been reported. And some similar venom peptides from other species were also obtained[23]. It is one of the efficient ways of the expanding research field to exploit venom molecules of insect origin by studying on bee venom peptides[24, 25], such as isolated a peptide named icarapin from *A. mellifera carnica* venom gland, which was unstable and allergic. However, little is known about the characteristics of venom from *A. mellifera ssp.*

A. mellifera ssp is one of the most important honey bee species in Northeast China. It possesses some superior characters including strong foraging ability and tolerant to low temperature[26].

However, so far functional peptides genes all known were cloned from the honeybee species, *A. mellifera ligustica,* and no information about genes of *A. mellifera ssp* venom were reported. In this study, we reported the nucleotide sequences of melittin and secapin from *A. mellifera ssp* (The Genbank accession numbers are JQ900378 and JQ900379, respectively.), and constructed phylogenetic tree for them from related species based on amino acid and elucidated their evolution relation.

2. Materials and Methods

2.1 Experiment Materials.

Honeybee (*A. mellifera ssp*) workers were obtained from the apiary of Institute of Heilongjiang Apiculture in China and raised under natural conditions. The stingers were collected from the worker bees' venom gland of *A. mellifera ssp*, and immediately placed in liquid nitrogen for storage until use.

Bacterial strain, *Escherichia coli (E. coli)* DH5α was kept in the Laboratory of Biomass Energy of College of Food Science of South China Agricultural University. Restriction endonucleases (*Bam* HI, *Xho* I), Taq polymerase, X-gal and DL-DNA 4500 marker were purchased from the Takara Company. A First-strand cDNA synthesis Kit was obtained from Shanghai Generay Biotechnology Company, and a pGEM T-easy Vector was purchased from Promega Company. All other chemical reagents were available in the Laboratory of Biomass Energy of College of Food Science of South China Agricultural University.

2.2 Experiment Procedures.

Two pairs of PCR primers were designed based on the sequence of melittin and secapin from *A. mellifera ligustica*[27,28]. The forward and reverse primers of the two sequences are as follows: the sequence of forward primer of melittin was GAAGCGATCGGAGAAATCAT(5'-3'), and the reverse primer was GATAGGTCGTAAATCGGCAT(5'-3'); the sequence of forward primer of secapin was AGAAGAATTATGAAGAACT(5'-3'), and the reverse primer was AAGTTCATACGAATTTAAG(5'-3'). Total RNA was isolated from venom glands of 50 live worker bees of *A. mellifera ssp*, and frozen in liquid nitrogen, ground into fine powder using a mortar and pestle. Total RNA was extracted using an RNA Kit (Promega) according to the manufacturer's instructions. The first strands were synthesized from total RNA by a Reverse Transcriptase Kit following the manufacturer's protocol.

PCR amplification was performed in a 50 μL volume containing 5 μL of 10×Taq buffer (Mg^{2+} plus), 0.2 mM deoxyribonucleotide triphosphate (dNTPs), 10μM each primer, 2.5 units of Taq DNA polymerase, and 100 ng of the template genomic DNA from stinger of *A. mellifera ssp*. The PCR of the melittin was carried out as follows: 1.5 min at 94 °C; 33 successive cycles of 40s at 94 °C, 40 s at 51 °C and 40 s at 72 °C, and a final extension of 6 min at 72 °C. The PCR of the secapin was carried out as follows: 1.5 min at 94 °C; 33 cycles of 40 s at 94 °C, 40 s at 55 °C, and 1 min at 72 °C, and a final extension of 8 min at 72 °C. The PCR products were examined by electrophoresis in 1.5% (w/v) agarose gels with ethidium bromide staining. The PCR products purified by gel Purification Kits were ligated into the pGEM T-easy vector. And the ligation products were transformed into component cells wit, and then grown on LB-agar plates containing 100 μg/mL ampicillin, 80 μg/mL X-gal, and 80 μg/mL IPTG. Subsequently, white colonies selected were cultured in a 3 mL medium with ampicillin. Plasmid DNA containing target genes were extracted and identified by *Bam* HI and *Xho* I.

2.3 Sequence Analysis.

The positive recombinant plasmid for melittin and secapin from transformants were sequenced by BGI Company (Shenzhen, China). Sequence analysis was performed using DNAman (Version 5.0) program. Their biological characters were analyzed using on-line tools (http://www.cbs.dtu.dk/services/, and http://web.expasy.org/cgi-bin/protscale/protscale.pl?1). The amino acid sequences were deduced using the NCBI (http://www.ncbi.nlm.nih.gov) database and DNAman 5.0 program. The full-length amino acid sequences were aligned using Clustal X1.83 program with those of related species from vertebrates and invertebrates obtained from NCBI database, and the phylogenetic tree was generated using MEGA 5.0 software based on the neighbor-joining method. The protein sequences of the representative species, *A. mellifera ligustica* and *A. cerana cerana,* were obtained from published reports.

3. Results

Two peptides' cDNA were amplified by RT-PCR using total RNA extracted from the venom glands of *A. mellifera ssp*. The result of the agarose electrophoresis of PCR products amplified from venom cDNA of *A. mellifera ssp* was shown in Fig.1. The fragment size of the melittin was consistent with that of *A. mellifera ligustica* reported by[27], but the fragment size of the secapin was not consistent with those of *A. mellifera ligustica* and *A. cerana cerana* reported by Vlasak and Kreil (1984) (Fig.1A). The PCR products purified were cloned into the pGEM T-easy vector, and then the recombinant plasmids were identified by *Bam* HI and *Xho* I (Fig.1B) and shown that the fragment were in line with expected size.

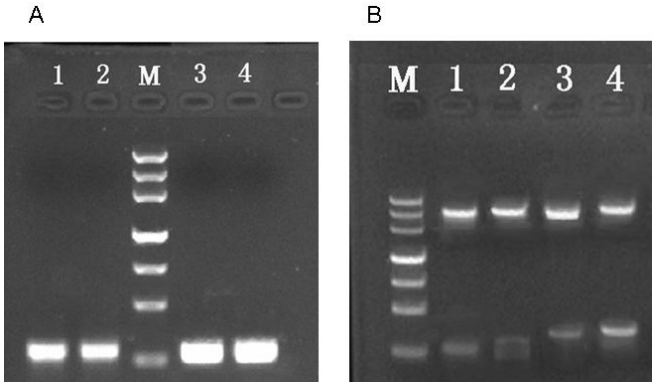

Fig 1: Identification of PCR products and digestion of the two genes from *Apis mellifera ssp.* M: molecular weight marker (DL4500); Lane 1 and 2: melittin; Lane 3 and 4: secapin.

The melittin and secapin fragments amplified were 213bp and 234bp in length, which encoded 71 and 78 amino acid, respectively. The multiple alignments demonstrated that the melittin and secapin genes shared more than 95% homology with those of *A. mellifera ligustica* and *A. cerana cerana* in terms of nucleotide sequences. The two peptides molecular weight were evaluated to be 7.77 kDa and 8.95 kDa by the Bioedit program. The result of multiple alignment (Fig 2) shown that the amino acid of melittin from *A. mellifera ssp* shared a high degree of homologues with that of *A. mellifera ligustica* (the GenBank accession number: NP_001011607.1), and significant difference from that of *A. cerana cerana* (the GenBank accession number:

Q8LW54.1). While the secapin differed from that of *A. mellifera ligustica* in many positions, but has high homologues with that of *A. cerana cerana* (Fig.3).

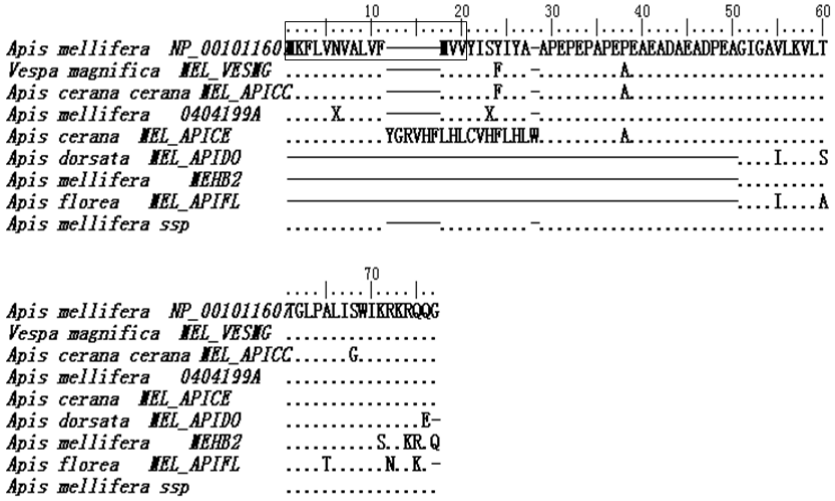

Fig 2: Alignment of the deduced amino acid sequences of the melittin. *Sequare* represents signal peptide.

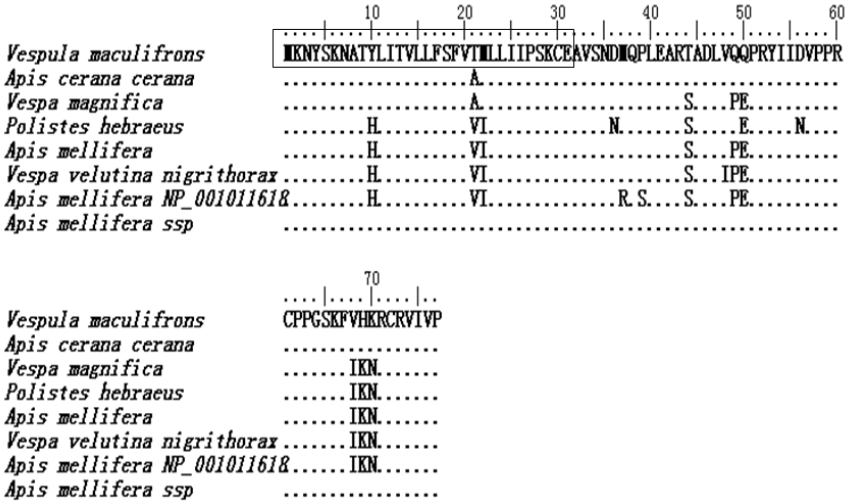

Fig 3: Alignment of the deduced amino acid sequences of the secapin. *Sequare* represents signal peptide.

Phylogenetic trees of the melittin and secapin were built based on the amino acid from different honey bee species and related species including eastern and western honeybee from China, India and American, which the complete sequences downloaded from the NCBI database.

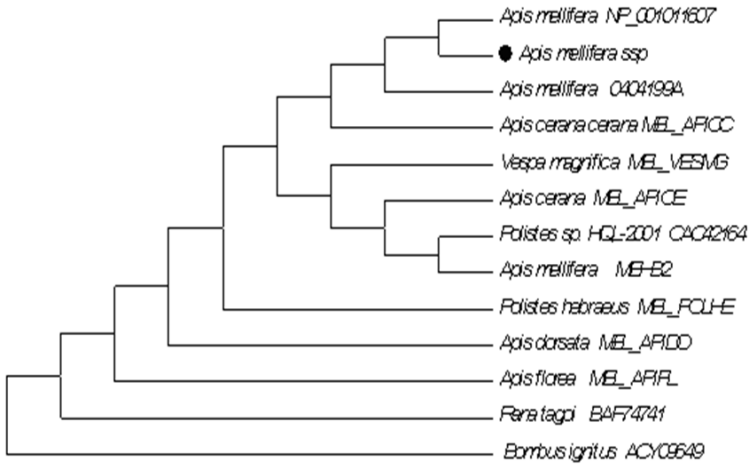

Fig 4: Phylogenetic relationships for *A. mellifera ssp* and related species constructed based on the alignment of the amino acid sequence of melittin homologues. Black dot represents the *A. mellifera ssp*

Phylogenetic analysis was performed based on the neighbor-joining method using the amino acid sequences obtained. Results show

that the two peptides from *A. mellifera ssp* are most closely related to that of A. *mellifera* ligustica. However, an interesting finding that melittin was at the same cluster with that of A. *mellifera* ligustica (Fig.4), while secapin was at the same cluster with that of *vespula*, although they all came from the *A. mellifer ssp* (Fig.5). And the trees illustrated the melittin have a relatively close relationship with that of *Apis mellifera,* suggesting the evolutionary relationship of different selected animals.

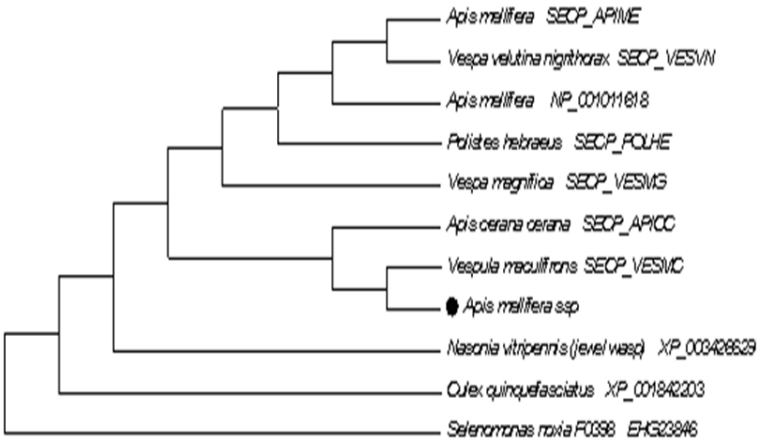

Fig 5: Phylogenetic tree constructed based on the alignment of the amino acid sequence of secapin homologs. Black dot represents *Apis mellifera ssp*.

4. Discussion

As social insect, *A. mellifera ssp* have formed their own traits that could reduce the impact of their large numbers of parasites and pathogens, and increase the capacity of innate immunity system. Each functional peptide takes responsibility for a different duty in the innate immune system of the honey bee. Melittin and secapin are the most mainly components of honey bee venom, which all have the ability on the anticoagulation.

In this study, we described the basic sequence analysis of two genes cloned, which were derived from bee venom. These two genes were cloned, identified and analyzed in the first time here. One of the two interesting findings in the present study was that melittin from the venom gland of *A. mellifera ssp* contained the completely same size in length and nucleotide in cDNA sequences with that of A. *mellifera* ligustica, and it suggested that they could have the same biological characteristics based on the same structure. While secapin contained difference size cDNA sequence in length with those of A. *mellifera* ligustica and *A. cerana cerana*. The other discovery was that the amino acid residues of the signal peptide of secapin of *A. mellifera ssp* had highly identity with that of *A. cerana cerana* (Fig 3), although they belonged to different family. The range of signal peptide of secapin was from 1 to 32, and cleavage position may be located between 32 and 33 (Fig.3), and was consist with that of queen bee venom (position 32and 33) [28]. Then we analyzed the hydrophobicity of the amino acid of melittin and secapin using online Protscale Program, which revealed that they all belonged to hydrophobic amino acids.

The isoelectric point (pIs) of the melittin and secapin was 4.69 and 9.45, and belonged to the acidic and alkaline amino acid, respectively. The component of secondary structure predicted of melittin is α-helix (50.7%), extended strand (15.49%), beta turn (14.08%), and random coil (19.72%). While the secondary structure of secapin is α-helix (44.16%), extended strand (19.48%), beta turn (5.19%), and random coil (31.17%).

These results are consistent with those of several recent reports [2, 22, 27]. In order to understand the genetic roots and evolutionary relationships of melittin and secapin from A. *mellifera ssp*, a phylogenetic tree was constructed based on the deduced amino acid sequences of related species. The trees revealed melittin and secapin were clustered into two distinct groups. As expected, based on genetic sequences, the melittin of A. *mellifera ssp* and A. *mellifera.* linguistic were clustered together in a single group. However, the secapin of A. *mellifera ssp* was cluster with that of *vespula*, which was one of the wasps. And this group also has another bee species, A. *cerana cerana*. So we concluded that this group of secapin is very closely related. These phylogenetic trees suggested that they may be share the geographical similarity. And also shown that the relationships between the honeybee and other species, included some vertebrates, involved the conservation and the variation of these genes during long-term evolution.

The traits of melittin and secapin could be explained by their structures. The conformation of the peptides in different conditions might be different, although the structures of all peptides within membranes were thought to be α-helical[29]. The rigidity of membrane has great impact on melittin-membrane interaction[30]. There are two phosphorylation sites in secapin at position 6 and 36, but not in melittin. Then, amino acid sequences of melittin from *A. mellifera ssp* had many sites different from that reported of *A. cerana cerana*; whereas secapin of *A. mellifera ssp* had only one position different from that of *A. cerana cerana,* although *A. mellifera ssp* and *A. cerana cerana* belonged to different genus. These structural characteristics suggested that a new method is likely to be developed to isolate melittin, secapin and PLA_2 from honey bee venom due to their similar biological characters. At present, there has not been effective method to isolate them from bee venom yet, although some studies had been performed[31].

Melittin and secapin are the two main components of honey bee venom, which play an important role in pharmacological effect, such as anti-inflammatory and hemolytic action[1,3]. When it comes to histamine released, this affects nearly 20% of the population [32, 2]. But studies of structure and functions of melittin and secapin were also limited to date.

The main peptides and proteins of bee venom have been widely studied and used in many fields. Especially, melittin has been applied for studying the interactions of lipid-protein as propriety model peptide[12]. Melittin is a water soluble toxic peptide, and

could induce many effects on living cells due to its disturbance of cell membranes. For instance, it can modulate mutual function when melittin interacts with phospholipases and calmodulin[33]. Melittin from the western honey bee venom contains a praline at position 14, which is highly conserved in related peptides of various bee venoms, and it influences indirectly the electrostatic properties of the natural peptide[34]. However, the charge of membrane can also modify and regulate the properties of conformation and dynamics of peptides[35]. These results indicate that melittin could directly activate small and medium-sized sensory neurons, PLA2-COXs/LOXs cascade pathways[36], which inhibits the activation of the transcription factors[37].

Although the genes of melittin and secapin from the *A. mellifera ssp* venom were cloned and analyzed, there are many questions need to further to answer, such as the difference of biological activity between bee species. Our further studies will be focused on their biological activities of these two peptides.

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