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Functional characterization of naturally occurring melittin peptide isoforms in two honey bee species, *Apis mellifera* and *Apis cerana*

Doori Park[a], Je Won Jung[a], Mi Ok Lee[c], Si Young Lee[a], Boyun Kim[a], Hye Jun Jin[b], Jiyoung Kim[a], Young-Joon Ahn[a], Ki Won Lee[a], Yong Sang Song[a], Seunghun Hong[b], James E. Womack[c], Hyung Wook Kwon[a,*]

[a] *WCU Biomodulation Major, Department of Agricultural Biotechnology, College of Agriculture & Life Sciences, Seoul National University, Seoul 151-921, Republic of Korea*
[b] *Department of Physics and Astronomy, Seoul National University, Seoul 151-747, Republic of Korea*
[c] *Department of Veterinary Pathobiology, Texas A&M University, College Station, TX 77843, United States*

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ABSTRACT

Insect-derived antimicrobial peptides (AMPs) have diverse effects on antimicrobial properties and pharmacological activities such as anti-inflammation and anticancer properties. Naturally occurring genetic polymorphism have a direct and/or indirect influence on pharmacological effect of AMPs, therefore information on single nucleotide polymorphism (SNP) occurring in natural AMPs provides an important clue to therapeutic applications. Here we identified nucleotide polymorphisms in melittin gene of honey bee populations, which is one of the potent AMP in bee venoms. We found that the novel SNP of melittin gene exists in these two honey bee species, *Apis mellifera* and *Apis cerana*. Nine polymorphisms were identified within the coding region of the melittin gene, of which one polymorphism that resulted in serine (Ser) to asparagine (Asp) substitution that can potentially effect on biological activities of melittin peptide. Serine-substituted melittin (Mel-S) showed more cytotoxic effect than asparagine-substituted melittin (Mel-N) against *E. coli*. Also, Mel-N and Mel-S had different inhibitory effects on the production of inflammatory factors such as IL-6 and TNF-α in BV-2 cells. Moreover, Mel-S showed stronger cytotoxic activities than Mel-N peptide against two human ovarian cancer cell lines. Using carbon nanotube-based transistor, we here characterized that Mel-S interacted with small unilamellar liposomes more strongly than Mel-N. Taken together, our present study demonstrates that there exist different characteristics of the gene frequency and the biological activities of the melittin peptide in two honey bee species, *Apis mellifera* and *A. cerana*.

1. Introduction

The honey bee is a valuable organism both in agricultural and also in biological research, especially as a social insect [9]. The European honey bee, *Apis mellifera*, by far the most common domesticated honey bee species, is widely used in apiculture around the world and is an important pollinator. Though *A. mellifera* is attractive to beekeepers, it is susceptible to several infection diseases [11]. In contrast, the Asian honey bee, *Apis cerana*, from eastern and southern parts of Asia, is known to possess an effective defense against mites, wasps, and some pathogenic diseases and have an enhanced ability to adapt to extreme weather conditions [45,50]. However, *A. cerana* is extremely sensitive to certain environments, which makes it difficult to domesticate. Nevertheless, the economic value and pharmacological potential of *A. cerana* have become noteworthy [47,51].

Some eusocial insects, including several ants and honey bees, have evolved unique defense systems, such as individual molecular defense mechanisms and social (or behavioral) immunity [10,12]. They make strong antimicrobial peptides (AMPs) which are known to be expressed in an innate immune system and venom system [49]. AMPs presented in hemolymph, cuticles, or venom glands have antimicrobial functions and serve as weapons against potential predators [4,7,21]. Melittin, one of the well-known AMPs identified in *Apis mellifera*, is a major toxic component of bee venom, which accounts for 40–60% of the dried venom [16]. This peptide is mainly composed of a hydrophobic amino-terminal region (residue 1–20) and an entirely hydrophilic carboxyl-terminal region (residue 21–26). Because the asymmetric distribution of polar and non-polar side chains, melittin has a strong

* Corresponding author at: WCU Biomodulation Major, Department of Agricultural Biotechnology, Seoul National University, Seoul 151-921, Republic of Korea. Tel.: +82 2 880 4915.
E-mail addresses: biomodeling@snu.ac.kr, biomodels@gmail.com (H.W. Kwon).

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binding affinity with membranes [35]. Thus, this characteristics of melittin has received much attention to therapeutic potential for various types of bacterial and viral diseases [1,13,25,43], inflammatory diseases [30–32], and also tumorous diseases [28,29,48].

Recently, there has been growing interests in the role of AMPs regarding immunomodulatory properties associated with genetic variation. It has been reported that the allelic variation in AMPs, with single nucleotide polymorphisms (SNPs) as the most abundant genetic variation [41], have an influence on susceptibility to certain diseases by altering their own functional properties [8,41]. Depending on location, a SNP may affect transcriptional regulation, alter the functional property of the translated proteins, and even modify phenotypic diversity [42]. To date, considerable information on genetic polymorphisms has been reported on mammalian AMPs, especially in domestic animals and humans in terms of diagnostic purposes, pharmacogenetic analysis, and evolutionary studies [8,24,40]. However, despite the strong immunomodulatory effects of many AMPs produced in insects, the significance and differential influence of polymorphisms in insect AMPs have not yet been well-studied [34].

In this regard, we here identify nucleotide variations in melittin genes derived from two different honey bee species. This study is the first report of a minor form of melittin in *A. mellifera*. The goal of our study was to compare the functional properties of two melittin peptides resulting from non-synonymous SNPs in the coding region of two major honey bee species, *A. mellifera* and *A. cerana*.

2. Materials and methods

2.1. Sample collection and DNA extraction

Bees were taken from 3 different colonies in College of Agriculture and Life Sciences, Seoul National University (SNU), Seoul, Korea during the summer season. While we conducted this experiment, the queen bee was not changed genetically. Worker bees were captured and directly displaced in liquid N_2, and stored at −80 °C. Genomic DNA was isolated from individual bee's abdomen using AccuPrep Genomic DNA Extraction Kit (BIONEER, Cat No. K-3032), according to manufacturer's instructions.

2.2. PCR primer design and SNP analysis

NCBI Ref sequence (NM_001011607.1) was selected for primer design the melittin gene. The primer pairs were selected by Primer 3. PCR was conducted using 2X Premix-MG Taq (Macrogen, Cat No. MP018S) following the manufacturer's instructions and reaction was performed under the following conditions: pre-denaturation step at 95 °C for 3 min, 30 amplification cycles with denaturation – 95 °C, 30 s; annealing – 50 °C, 30 s; and an elongation step – 72 °C, 30 s; respectively, followed by elongation again at 72 °C for 5 min. To confirm the single nucleotide polymorphisms in melittin gene, PCR products were sent to the sequencing company (Biomedic Co., Ltd., Seoul, Korea). The sequencing results were analyzed using ClustalW and FinchTV program.

2.3. Peptide synthesis

Two different melittin peptides were designed differing by Ser-18 and Asp-18 at the N-terminal of the melittin α-chain (Fig. 1). Peptides were purified to >95% purity grade through reverse-phase HPLC. Peptides dissolved in phosphate buffer (PBS, pH 7.2) were stored at −20 °C before use.

2.4. Anti-bacterial assay

Gram-negative bacteria, *Escherichia coli* ATCC25922 was purchased from Korean Collection for Type Culture and tested against each peptide. Overnight cultures of bacteria were subcultured for an additional 2–3 h at 37 °C to a mid-logarithmic phase and suspended to 5×10^6 colony – forming unit (CFU)/ml in the same buffer. Bacteria suspensions (90 μl) were placed into 96 well plates, followed by the addition of 10 μl of serial diluted melittin peptides (final 0, 0.1, 0.25, 0.5, 1 μM) in triplicate. After 2 h incubation at 37 °C, equal volume of BacTiter-Glo™ Reagent (Promega) was added and incubated for five minutes after which luminescence was measured with GLOMAX multi detection system (Promega).

2.5. Confocal laser-scanning microscopic analysis

Escherichia coli cells in mid-logarithmic phase were prepared as described in the antibacterial assay section. *E. coli* cells (6.5×10^6 CFU) in 0.85% NaCl were incubated with 2.5 μM peptide (final 0.25 μM). After incubation, the LIVE/DEAD® *Bac*light™ Bacterial Viability Kit (Invitrogen, Eugene, OR) was used to stain the adhered cells. Cells were then observed by using confocal laser scanning microscopy (CLSM) (Zeiss, Thornwood, NY). More than thirty images were collected in each experiment. Experiments were repeated three times using independent bacterial cultures. Images were processed with Zeiss Zen 2009 software (Carl Zeiss Microscopy, Germany).

2.6. BV-2 Cell culture and cytokine measurement

The murine microglial BV-2 cells were a gift from Dr. Sunyoung Kim (Seoul National University, Seoul, Korea). BV-2 cells were grown in DMEM supplemented with 10% FBS and 0.1% penicillin/streptomycin and cultured at 37 °C in a humidified atmosphere of 5% CO_2. BV-2 cells were seeded in 6-cm dishes at a density of 1.0×10^6 cells per wells and treated with 0.1 or 1 μM melittin peptides in 10% FBS DMEM for 12 h, followed by 0.1 μg/ml LPS in serum-free DMEM for 24 h. The level of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and interleukin (IL)-6 in culture supernatants was measured by enzyme-linked immunosorbent assay (ELISA) (Thermo Fisher Scientific, Waltham, MA, USA), according to manufacturer's instructions.

2.7. Cancer cell cytotoxicity assay

SKOV-3 (human ovarian adenocarcinoma cancer cell, ATCC® HTB-77™) and PA-1 (human ovarian teratocarcinoma cancer cell, ATCC® CRL-1572™) cells were plated into 96-well plates at density of each 2.0×10^3 and 5.0×10^3 cells per well. The cells were cultured for 24 and 48 h in the presence of various concentrations of melittin peptide (0, 0.1, 0.25, 0.5, 1.0, 2.5 or 5 μM) dissolved in distilled water. SKOV-3 and PA-1 cells were incubated with 50 μl MTT solution for 4 h at 37 °C. After MTT was removed, the cells were solubilized in 200 μl dimethyl sulfoxide (DMSO) for 30 min. The optical density at 540 nm was determined by a spectrophotometer, Lab system Multiskan (Labsystem, Helsinki, Finland).

2.8. Fabrication of swCNT transistors

2.5 mg of single-walled carbon nanotubes (swCNTs) (Hanwha, Korea) were dispersed in 1,2-dichlorobenzene (50 ml) by applying ultrasonic vibration for 20 min. The concentration of swCNT suspensions was 0.05 mg/ml. For swCNT assembly, an octadecyltrichlorosilane (OTS) self-assembled monolayer (SAM) with non-polar terminal groups was first patterned on a SiO_2 (1000 Å) substrate *via* photolithography as reported previously [23]. A

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