

Antimicrobial Effect of Melittin Isolated from Syrian Honeybee (Apismellifera) Venom and its Wound Healing Potential

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Accepted on: 22-05-2013; Finalized on: 30-06-2013.

ABSTRACT

In this study, bee venom (BV) was collected from 12 Syrian beehives during June and July 2011 using electric shock method. Melittin, the major component of BV, was isolated and identified using RP-HPLC C18 column and MALDI-TOF-MS analysis. The obtained melittin exhibited a potent antibacterial activity particularly against Gram-positive bacteria as its MIC was 12.5µg/ml for *Listeria monocytogenes* compared with 200µg/ml for *Yersinia kristensenii* (a Gram-negative bacterium) indicating that melittin has significant antibacterial effects. Additionally, melittin treatment was found to significantly accelerate wound contraction and re-epithelialisation as wound sizes decreased dramatically and healed within 5 days in all melittin treatment for skin defects should be very effective in preventing and reducing the wound and scar sizes. However, further studies are needed to evaluate the precise mechanism of epithelial cell proliferation induced by melittin treatment.

Keywords: Melittin, AMPs, Antimicrobial Effect, Wound Healing.

INTRODUCTION

ee venom has a long history as a folk remedy in the treatment of various diseases including arthritis, angiocardiopathy, back pain, musculoskeletal pain, cancerous tumors, multiple sclerosis, healing wound, and skin diseases ¹⁻³. It is produced in the abdomen of worker bees from a mixture of acidic and basic secretions and stored in the venom sac⁴. Usually the venom is collected by an electrical shock method stimulating the honeybee to sting the surface of the collector glass sheet before being scraped and freeze dried.⁵ Honey bee venom consists of 88% water and the remaining 12% contains peptides (melittin, apamin, Mast Cell Deregulating (MCD) peptide, and promelittin), enzymes (hyaluronidase, and phospholipase A2), histamine, sinkaline, glycerol, noradrenaline, and aminoacids, carbohydrates, phospholipids, physiologically active amines and volatile ingredients which have diverse pharmacological properties and biological activities^{6, 7}. The most important active and prevalent substance which constitutes 50% of dry venom is melittin, a type of amphipathic, water soluble, linear peptide consisting of 26 amino acids with 6 positive charges, with a molecular weight 2849 Dalton (Da)^{8, 9}. This substance causes local pain via histamine release and enhances phospholipase A2 (PLA2) induced intravascular hemolysis as well as catecholamine release^{10, 11}. It has been reported that melittin has multiple effects, including antibacterial, antivirus, and anti-inflammation, in various cell types^{9, 12, 13}. Melittin can also induce cell cycle arrest, growth inhibition, and apoptosis in various tumor cells¹⁴⁻¹⁶.

Moreover, melittin is classified as one of the antimicrobial peptides (AMPs) found in many organisms including

bacteria, insects, fish, frogs, and mammals¹⁷. It is well documented that the extensive use of antibiotics over the past six decades has led to an increased prevalence of antibiotic resistance in both hospital- and communityacquired infections giving rise to a critical need for the development of new approaches for treatment of bacterial infections¹⁸. In recent years there has been considerable interest in the development of antimicrobial peptides (AMPs) as novel antibiotics ¹⁹. They are including melittin naturally occurring entities with potential as pharmaceutical candidates and/or food additives as exhibit broad specificity against both Gram-positive and Gram-negative bacteria²⁰ and may play a role in wound healing²¹. Contrary to organ regeneration which does not occur in nature, wound healing or tissue regeneration is a widespread event among all organisms. Wound healing in mammals is a multistep phenomenon that consists of an inflammatory phase involving scavengering of damaged cells by macrophages and phagocytes whose recruitments to the site is induced by cytokines secreted by cells of wounded tissues^{22, 23}. In the reparation phase, some differentiated cells (keratinocytes, granular cells, and fibroblasts) in the GO phase of the cell cycle dedifferentiate, returning, along with local stem cells, to G1 phase; start dividing to ensure the needed number of differentiated cells. This mitotic activity is also regulated by specific growth factors, and a host of regulatory molecules of the immune system. These cells seem to secret a network of collagen fibers which guide their migration. The healing phase involves the formation of keratinized and apoptosing cells, forming a deciduous crust. In human, normal wound healing requires 7-8 days; in articulated regions twice this time^{24, 2}



ISSN 0976 - 044X

Consequently and due to the recent advancement in biotechnology and to the medical use of bee venom components, new methods have been developed for identification, isolating and purifying Honeybee venom important components²⁶⁻²⁸.

Consequently, aims of the current study are: Collecting bee venom from Syrian honeybee and melittin isolation using HPLC-semi preparative system; Melittin identification using Matrix Assisted Lazer Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS); Testing melittin antibacterial activity against Gram positive and Negative bacteria and investigating its wound healing potential on rats.

MATERIALS AND METHODS

Sample collection

Twelve Syrian beehives *Apismellifrea* were obtained from the Ministry of Agriculture and agrarian reform, silk and bee division. Bee venom samples were collected according to Benton protocol⁵ in June and July 2011 using bee venom collector as instructed by the manufacturer (Chung Jin biotech Co., Ltd, Korea).

Samples preparation

30mg of crude venom was dissolved in 2ml of ddH₂O and centrifuged at 12,000 rpm for 15 min at 4°C. Soluble supernatant was re-centrifuged at the same conditions, filtered through 0.2 μ m membrane filter, lyophilized and stored at -20°C in the dark until needed²⁸.

LC-DAD semi preparative isolation and purification

HPLC semi preparative system (Jasco) consisting of a binary pump (pu-2087plus), an auto sampler (AS-2050), a column compartment (Co-2060plus), DAD (MD2010/2015) and a fraction collector microcomputer controlled CHF (122SC) was used in this work. System operation, data acquisition, and analysis were controlled and processed by chromNAV software. Chemicals such as TFA, acetonitrile, and Licrosolv water were HPLC grade and supplied by Merk (Germany). Lyophilized bee venom Apismellifera (SBV) and melittin were supplied by Sigma (USA) and working concentrations were adjusted to 8mgl/ml,0.08mg/ml respectively of deionised water. According to ²⁹ chromatographic isolation was performed with RP C18 column 4.6 mm x 25 cm 3.5µl (Tecnokroma, Spain). Stepwise gradient elution applied was 5% B rising to 80% in 40 min and the initial conditions were restored until 41 min and retained 43 min as a reconditioning step for the next run. Where A was 0.1% TFA in water, B was 0.1% TFA in acetonitrile: water (80:20, V: V). Isolation was carried out at 30°C, 80µl was the injected volume for local bee venom (LBV) with concentration of 8 mg/ml sample with a mobile phase flow rate at 0.7 ml/min. The column was cleaned before each bee venom booster with pure water samples injected at the same conditions. Finally the target fraction was collected several times, concentrated by a speed vacuum, re-injected (analyzed) at the same HPLC conditions mentioned above, photo diode array (PDA) detector scanning from 200 to 500 nm ,then lyophilized and the isolated peptide was quantified by its dry weight.

Protein quantification

Protein concentration within collected venom samples and its isolated fraction was determined using Brad-ford assay ³⁰ and bovine serum albumin (Sigma, USA) was used as a standard reference.

Tricine-SDS-PAGE

LBVand the isolated fraction were resolved on 16% Tricine-SDS-PAGE according to Schägger³¹ and a low molecular marker (Sigma, 26.6-1.7Da) was used. Gels were visualized by Coomassie Blue G-250 staining (Serva, Germany) and distained overnight before they were analyzed.

In-gel digestion and MALDI-TOF mass spectrometry

Target protein band was excised manually from destained gels, digested and analyzed by MALDI-TOF-MS as described ³². Briefly, the excised gel containing the protein of interest was placed in a protein low binding tube (Eppendorf) containing 500 μ l of MilliQ-ddH₂O at 4ºC for 24 h. Water was discarded and 300 µl of 50 mM triethylammonium bicarbonate buffer (TEAB, Sigma) was added. The tubes were gently shaken at room temperature (RT) for 15 min before supernatant was replaced with 50 mM TEAB/50% CH3CN (acetonitrile) solution twice each for 15 min at RT with gentle agitation. Supernatant was removed, 100 µl of CH₃CN was added to rehydrate the protein band for 5 min at RT. Gel pieces were dried in a speed vacuum before they were reduced with 10 mM DTT, 50 mM TEAB for 1 h at 56°C and alkynated with 55 mM iodoacetamide, 50 mM TEAB for 45 min at RT in the dark. After this treatment, each gel piece was minced and lyophilized, then swollen at 37°C overnight in 50 mM TEAB containing 50 ng of modified trypsin (Promega, Madison, USA). Protein peptides were collected, and gels were washed with 0.1% TFA in 50% CH₃CN three times to collect the remaining peptides. Peptides were cleaned using C-18 resin ready packed tips and diluted into freshly prepared saturated sinapinic acid dissolved in 50% acetonitrile, 0.3% trifluoroacetic acid (TFA). 2 µl samples were spotted onto a stainless steel plate and spectra were collected by averaging three shots each for 200-300 laser shots. Samples were irradiated using Bruker Microflex MALDI/TOF mass spectrometer (Bruker Daltonics, Germany) with a 377-nm nitrogen laser, attenuated and focused on the sample target using the built-in software (Microflex package). lons were accelerated with a deflection voltage of 30 kV and differentiated according to their m/z using a time-of-flight mass analyzer.

Database searches

Peptide masses (mass list) generated from the peptide mass fingerprint (PMF) were used to search the NCBI database with the MASCOT search engine (Matrix



Science, UK) for protein identification. The search parameters were set according to the relevant literature³³. MASCOT uses a probability-based molecular weight search (Mowse) score to evaluate data obtained from tandem mass spectra. The Mowse score was reported as -10 x log (*p*) where *p* is the probability that the observed match between experimental data and the database sequence was a random event ³⁴. Mowse scores greater than 80 were considered statistically significant (*p*< 0.05).

Bacterial strains

Strains of Gram negative (*Salmonella enteric ATCC 7001*, *Yersinia kristensenii* ATCC 33639) and Gram positive bacteria (*Listeria monocytogenes* ATCC 19111, and *Staphylococcus aureus* ATCC 11632) were obtained (Microbiologics, France) and used in these experiments. Cultures of bacteria were prepared by inoculating of stock bacteria on nutrient agar plates at 37C° for 24h.

Antimicrobial Activity Assay

To assess antimicrobial activity of melittin, minimum inhibitory concentrations (MIC) were determined by broth-dilution method.³⁵ Briefly, single colony were inoculated into in 3ml Luria-Bertani Medium (LB broth) (BDH, UK), grown (37 °C, shaking at 150 rpm) until the OD at 600 nm was approximately 0.5-0.8 and matched with 0.5MacFarland standard (Fisher, UK), then bacterial working solution of mid-log phase (2×10^5 cfu/ml) was prepared. In a clear bottom 96-well plate, six 2-fold serial dilutions of mellitin were made and added to the bacterial working solution described above giving final concentrations of (12.5-200 $\mu g/ml$ melittin and 1 \times 10 $^{\circ}$ bacterial suspension cfu/ml), each concentration was in triplicate. The plate was incubated overnight (37 °C, 150 rpm) and the OD600 values were measured using a ELISA reader (Fisher, UK). MIC defined as the lowest final concentration of melittin that inhabits visible growth of bacteria was obtained.

Animals

Nine healthy adult male Wistar rats (France) weighting 170 ± 20 g maintained at the department of molecular biology and Biotechnology (AECS, Syria) were housed individually in polycarbonate cages that were maintained under a constant 12-h light: 12-h dark cycle and temperature of 22 ± 5 °C and relative humidity of $60\pm10\%$ throughout the experimental period. The rats were given free access to food and water.

Full-thickness excision-wound model

Rats were anaesthetized prior to the infliction of experimental wound by light ether. Surgical intervention was strictly carried out under sterile conditions after removing hair from the symmetrical lateral back (requested areas). On the same rat, two symmetrical homologous full thickness wounds (2cm x 1mm) were made, one wound was treated with Vaseline only (V, control) and the other one was treated with Vaseline and melittin (M, 1/1000 melittin /vasline w/w), A 3x3 cm AIDerm roll (Allshefa, Syria) was used to prevent losses of melittin or Vaseline. Wounds were monitored on days 2, 5 and 8.

RESULTS AND DISCUSSION

Separation and identification of Melittin

RP HPLC with a column packed with C18 material was used to fractionate the main components of Syrian bee venom obtained from 12 beehives in summer 2011. The best separation conditions were those of 5-80% B linear gradient elution at 0.7ml/min flow rate of a mobile phase and a temperature of 30°C. The accuracy and repeatability of isolation method were adequate. Under these optimized conditions, five fractions were seen for both LBV and SBV venom 8mg/ml samples (Figure 1). Isolated fraction number five (F5) thought to be melittin, the main component of bee venom because it showed a major peak. Assessment using reverse-phase under same HPLC isolation conditions for F5 was made; single peak was featured and the retention time of it corresponded to the retention time of a standard Sigma melittin sample (Figure 2). Isolated peptide purity was ~85% using3Dview of the PDA scanning chromatogram (data not shown). Additionally, F5 was loaded onto a 16% Tricine-SDS-PAGE and its molecular weight corresponded with the expected molecular weight of melittin (Figure 3). Furthermore, MALDI-TOF-MS was utilized to confirm the identity of F5. The protein band seen on Tricine-SDS-PAGE, was identified by MALDI-TOF mass spectrometry analysis and database search. Peptide mass values (Figure 3A) were used for identification using MASCOT peptide mass fingerprint (PMF) search that identified fraction 5 as melittin with a Mowse score of 90 (Figure 3B) considering that Mowse scores greater than 80 were statistically significant (p < 0.05).



Figure 1: HPLC separation profile of Syrian bee venom using Tecnokroma C18 packed column.

Sigma bee venom (blue), Syrian bee venom (Red, 8mg/ml).





Figure 2: Melittin identification using Tecnokroma C18 column.

Arrows indicate a standard Sigma melittin (800 μ g/ml, Red) and fraction 5 isolated from LBV (Blue).



Figure 3: Separation of LBV and Fraction 5 on 16% Tricine SDS-PAGE stained with Coomassie blue R-250

M: Protein marker (Sigma), F5: fraction five isolated by RP HPLC, LBV: Local bee venom.

Melittin Antimicrobial Activity

Broth dilution assays were used to examine the antimicrobial activity of melittin against two Grampositive bacteria species, *Staphylococcus aureus* and *Listeria monocytogenes*; and two Gram-negative species *Salmonella enterica*, *Yersinia kristensenii*. Melittin showed higher antibacterial activities towards Gram-positive bacteria (Table 1). The MIC was 12.5µg/mlfor *Listeria monocytogenes* compared with 200µg/ml for *Yersinia kristensenii* indicating that melittin has significant antibacterial effects. These results are in agreement with previous studies in which melittin was shown to exhibit a potent broad-spectrum antimicrobial activity against different bacteria^{13, 36-39}.

Generally, the action mechanism of melittin or any of these AMPs is not well understood. Several researchers suggested that for many of these peptides including melittin a possible target is the lipid bilayer of the membrane⁴⁰. Due to the AMPs positive charge they could interact more strongly with the highly negatively-charged membranes of bacteria as opposed to the nearly neutral plasma membranes of eukaryotic cells and their amphipathic secondary structure facilitates partitioning into the membrane bilayer^{40, 41}.



4A: Spectra generated for melittin using MALDI-TOF-MS.



4B: Mascot result identifying a significant homologue.

Figure 4: Melittin identification using MALDI-TOF-MS and Mascot data search.

Table 1: Melittin Minimal inhibitory concentrations (MIC) on different bacterial species

Bacterial species	Salmonella enterica	Yersinia kristensenii	Listeria monocytogenes	Staphylococcus aureus
Melittin MIC (µg/ml)	100	200	12.5	25

All experiments were performed in triplicate. Data were presented as mean p<0.05.



In addition, melittin is cationic peptide and its interaction with anionic phospholipids would provide a ready explanation for its specificity towards bacterial membranes also It adopts amphipathic α -helical structure, a single tryptophan residue that has been found to be critical for its activity; Removal or substitution of the tryptophan residue in melittin causes a decrease in antimicrobial activity^{42, 43}. The proline at position 14 and the polar residues 23-26 at the C-terminus are important for the lytic action of melittin. It adopts different locations, orientations, and association states within membranes under different conditions. In bilayer systems, melittin may occupy two locations; it either remains on the bilayer surface or takes up a transmembrane orientation. In different lipids and at two hydration levels (6% w/w and 30% w/w), infrared techniques have shown that the orientation of the melittin is with the α -helical segments oriented roughly perpendicular to the plane of the membrane⁴⁴⁻⁴⁸ Additionally, melittin was found to enhance PLA2 activity and its antibacterial effect because it lowers the surface tension of water at the level of the plasma membrane, acting mainly by its natural detergernt-like effect on the plasma membrane causing cell lysis, so by disrupts membranes and then phospholipase A 2 cleaves bonds in the fatty acid portion of the bilipid membrane layer ^{10, 49}.

Accelerated wound healing

We investigate the wound repair capability of melittin, a wounding experiment was conducted on rats and the size

and the timing of wound closure was monitored. Melittin treatment was found to significantly accelerate wound contraction and re-epithelialisation. The wound size decreased dramatically within 5 days in all melittin treated rats (M) compared with 8 days in control (C) wounds indicating that melittin showed an explicit reduction in wound size as compared with the control (Figure 5). The melittin effect was also clear in scar formulation as melittin treated scars were much smaller and less visible than those of the controls Figure 5).

These results are in agreement with other studies indicated that the effects of Honeybee venom on wound healing may involve biological mechanisms associated with the expressions of TGF-b1, fibronectin, VEGF and collagen-1^{50, 51}. These findings suggested that topical melittin treatment for skin defects should be very effective in preventing and reducing wound sizes. It is thought that melittin, as major component of BV, and one of the antimicrobial peptides which have been implicated as positive effectors of wound repair, induces membrane permeabilisation by reorganizing lipid assemblies through vesicularisation of multibilayers, fusion of small lipid vesicles and fragmentation into discs and micelles⁵² Meltittin demonstrated significant antinociceptive and anti-inflammatory effects, antibacterial actions and immunity boosting. Further study of the biological wound healing role of melittin and other BV small peptides and their structure may therefore lead to the development of new therapies for wound repair⁵⁵⁻⁵⁷.



Figure 5: Timing of wound healing in melittin treated (M) and Vaseline (control) treated wounds (C) on the same rat.

CONCLUSION

Melittin has promising antibacterial effects mainly against Gram-positive bacteria. It is suggested that the current melittin treatment for skin defects is very effective in preventing and reducing wound sizes. Healing wound experiments showed that melittin treatment has significantly accelerated the healing time in all rats compared with the control (Vaseline treated wounds). However, further studies are needed to evaluate the precise mechanism of epithelial cell proliferation induced by BV or its isolated peptides treatment and critical modifications are desired to reduce BV cytotoxicity to eukaryotic cells and thus enhance its usefulness.

Acknowledgments: The authors thank the Director General of the AECS and the head of the Molecular Biology and Biotechnology department for their support and Dr.EyadGhanem for his technical support.



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Source of Support: Nil, Conflict of Interest: None.

