

THE USE OF CHICKEN IGY IN A DOUBLE ANTIBODY SANDWICH ELISA FOR THE QUANTIFICATION OF MELITTIN IN BEE VENOM AND BEE VENOM MELITTIN CONTENT IN COSMETICS

Lindsey Y. K. Suh¹
Tayabaa Kartoon¹
Naiyana Gujral¹

Youngmee Yoon¹
Joo Won Suh²
Hoon Sunwoo^{1*}

¹Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2E1

²Center for Nutraceutical and Pharmaceutical Materials, Myongji University, Cheoin-gu, Yongin, Gyeonggi-Do 449-728, Republic of Korea

*corresponding author: hsunwoo@ualberta.ca

Received 12 November 2014; accepted 30 April 2015

Abstract

Two enzyme-linked immunosorbent assay (ELISA) - based detection systems: indirect competitive ELISA and biotinylated double antibody sandwich ELISA (DAS-ELISA) were developed to determine the melittin concentration in honeybee (*Apis mellifera*) venom and the melittin concentration in cosmetics which contain bee venom. The indirect competitive ELISA employed chicken anti-melittin IgY. The biotinylated DAS-ELISA employed anti-melittin monoclonal antibody (MAb) and biotinylated anti-melittin IgY. To produce anti-melittin IgY; Sigma melittin was emulsified with Freund's incomplete adjuvant and immunised to Leghorn laying chickens intramuscularly at four different sites (50 µg/mL, 0.25 mL per site) of the breast muscles. After 5 to 8 weeks of the immunisation, anti-melittin IgY was extracted and analysed by ELISA. The anti-melittin IgY antibody produced was highly specific to melittin and did not cross-react with other bee venom proteins, as examined by ELISA and a western-blot assay. Indirect competitive ELISA demonstrated a higher range of melittin detection (2.5 to 80 µg/mL). Double antibody sandwich ELISA using MAb as the capture antibody and biotinylated polyclonal IgY as the detection antibody, provided a lower range of detection (2.5 - 40 ng/mL), which has a 1000 times higher sensitivity than that of indirect competitive ELISA. Therefore, indirect competitive ELISA is a useful tool to measure the concentration of melittin in bee venom as a raw material. Biotinylated DAS-ELISA, on the other hand, is more suitable for nanoscale quantification of melittin in commercial products.

Keywords: bee venom, DAS-ELISA, immunoglobulin Y, indirect competitive ELISA, melittin, monoclonal antibody.

INTRODUCTION

Bee venom, also known as apitoxin, is a blend of active compounds composed of enzymes, peptides, phospholipids, sugars, amines, and minerals. Melittin is the principal toxic component of bee venom that accounts for 50% of the bee venom dry weight. Melittin is a 26 amino acid long peptide (2.8 kDa) and has the chemical formula $C_{131}H_{228}N_{38}O_{32}$, and the amino acid sequence of GIGAVLKVLTTGLPALISWIKRKRQQ-NH₂ (Habermann, 1972; Gauldie et al., 1978).

Several researches have reported that melittin is a potent therapeutic agent in the treatment of

cancerous tumors, arthritis, and rheumatism (Park et al., 2004; Gajski and Garaj-Vrhovac, 2013; Koh et al., 2013). Melittin is most extensively studied for its antibacterial property. This water-soluble amphipathic peptide spontaneously associates with and partitions into lipid bilayer, in which it adopts a bent α -helical structure. At low concentrations, melittin causes transient permeabilisation of the cytoplasmic membrane and collapses the electrochemical gradient. Stable pore formation is observed at higher concentrations of melittin, resulting in size-independent leakage of macromolecules and cell lysis (Terwilliger et al., 1982; Raghuraman and Chattopadhyay, 2007; Lee et al., 2013).

Melittin has also been reported to suppress the expression of pro-inflammatory cytokines triggered by *Propionibacterium acnes* (*P. acnes*) through regulation of the NF- κ B and MAPK signaling pathways (Lee et al., 2014a). *Propionibacterium acnes* is a gram-positive skin resident bacteria that causes inflammatory acne vulgaris; the most common skin disease affecting 80% of individuals (Leyden, 2001; Bojar and Holland, 2004).

The anti-microbial and anti-inflammatory activities of melittin have raised a commercial interest in treating bacteria-caused skin problems and diseases. However, great care needs to be taken over the amount incorporated into commercial products due to the cytotoxicity of melittin (Jamasbi et al., 2014). Reliable quantification assays are needed for melittin which specify the endogenous levels in bee venom, and which specify the supplemental levels of melittin used in cosmetic formulations. A recently used method of melittin quantification is High Performance Liquid Chromatography (HPLC). This method requires a laborious sample preparation, costly reagents, and great expertise (Haghi et al., 2013). Although it can determine a concentration with satisfactory accuracy, HPLC is less practical when quantifying a bulk mixture of proteins. This is particularly true when quantifying bee venom and cosmetic products in the presence of molecules with similar molecular weight. Quantitative ELISA becomes a competitive option in terms of time consumption, cost-effectiveness, and detection of specific protein content, in a mixture with molecular size similarity. However, there is no report on the quantification of melittin using ELISA. The use of chicken IgY in the ELISA system has proven to be economical and efficient at detecting a wide range of proteins in serum and biological samples (Larsson et al., 1991; Schade et al., 2005).

The present study was undertaken to develop an ELISA system based on polyclonal anti-melittin IgY obtained from chicken egg yolks for detection of melittin in bee venom. The detection systems were further validated for the testing of melittin in commercially available cosmetics which contain bee venom.

MATERIAL AND METHODS

Chemicals

The following: 97% purity melittin, Freund's incomplete adjuvant, purified chicken immunoglobulin G (IgG), rabbit anti-chicken IgG, horseradish peroxidase (HRP) conjugated rabbit anti-chicken

IgG, streptavidin-HRP, and tetramethylbenzidine (TMB) liquid substrate system for membranes were purchased from Sigma (St. Louis, MO, USA). Biotin-naimdohexanoic acid-3-sumelittino-N-hydroxysuccinimide ester (NHS-PEO₄-Biotin) was from Fisher Scientific Canada (Ontario, Canada). Tetramethylbenzidine substrate was from KPL (Gaithersburg, MD, USA). Anti-melittin MAb was donated by Myung University (Yong In, Korea). A Bio-Rad protein assay kit was purchased from Bio-Rad Laboratory (Ontario, Canada). Lyophilized *Apis mellifera* bee venom was donated by South Korean honeybee farms. Two over-the-counter cosmetic creams containing purified bee venom were purchased from a local store.

Instrumentation

Sephacryl S-300 and S-100 gel filtration column were from GE Healthcare (Quebec, Canada). Microtiter 96-well plates were from Costar Inc. (Cambridge, MA, USA). The ELISA V_{max} kinetic microplate reader was from Molecular Devices Corp (Sunnyvale, CA, USA). Slide-A-Lyzer[®] was purchased from Thermo Scientific Pierce Protein Research Products (Ontario, Canada). The BioLogic DuoFlow chromatography system was from Bio-Rad (Ontario, Canada).

Separation of melittin from bee venom

Size exclusion chromatography

The components of bee venom were separated using Sephacryl S-100 column (1×100 cm). The column was equilibrated with phosphate buffered saline (PBS) on Bio-Rad BioLogic DuoFlow chromatography systems. The bee venom was homogenised in PBS (100 mg/mL) and centrifuged at 4000 rpm for 5 min. Supernatants were filtered through a 0.45 μ m filter and applied on the gel filtration column at a consistent flow rate of 4.2 mL/h at 4°C. One mL of fraction was collected per tube for 24 hours. The column effluent was monitored with a UV detector set at 280 nm.

SDS-PAGE

Each fraction obtained by size exclusion chromatography was further analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions. Each fraction was mixed with a sample buffer (pH 6.8, 0.0625 M Tris-HCl, 2% SDS, 10% glycerol, and 0.002% bromophenol blue) at a 1:1 ratio. Electrophoresis was performed at 110 V for 1.5 h, in Tris-Glycine running buffer (pH 8.3, 0.025 M Tris, 0.192 M glycine, 0.1% SDS) on a 10% acrylamide separating gel.

Production, purification, concentration, and specificity of anti-melittin polyclonal IgY

Production of anti-melittin polyclonal IgY

Production of polyclonal IgY directed against melittin was carried out as previously described by Sunwoo et al. (2011). Sigma melittin was suspended in sterilised PBS (0.14 M NaCl, 0.0015 M KH_2PO_4 , 0.0081 M Na_2HPO_4 , 0.0027 M KCl, pH 7.2) at a concentration of 100 $\mu\text{g}/\text{mL}$ and emulsified with an equal volume of Freund's incomplete adjuvant. Twelve, 23-week-old, Single Comb White Leghorn chickens were intramuscularly injected with the emulsified saline with or without melittin at four different sites (0.25 mL per site) of breast muscles (two sites per left or right breast muscle). A booster immunisation was given after two weeks of the initial immunisation. Eggs were collected daily for 8 weeks and stored at 4°C until the antibody extraction.

Purification of IgY

The egg yolk from immunised hens was manually separated from the egg white. The egg yolk was then diluted 1:6 by gently mixing with cold distilled water (pH adjusted to 4.0 with 0.1 M HCl) and further diluted to a final dilution of 1:10 with cold acidified distilled water (pH 2.0), adjusted to a final pH of 5.4, and incubated for 12 h at 4°C. The lipid granules of the yolk were sedimented by centrifugation (3,125 \times g for 20 min at 4°C), and the IgY-rich water-soluble fraction (WSF) was collected. Anti-melittin IgY antibodies in the WSF were further purified by ammonium sulfate (60%) followed by a Sephacryl S-300 gel filtration column. Purified IgY was freeze-dried for further use.

Titer of anti-melittin polyclonal IgY in egg yolks using indirect ELISA

Unless otherwise stated, the microtiter plate was washed four times with PBS containing 0.05% Tween 20 (PBST), between each step. A microtiter plate which had 96-wells was coated with Sigma melittin (100 μL per well), in carbonate-bicarbonate buffer (0.05 M, pH 9.6) at a concentration of 10 $\mu\text{g}/\text{mL}$, and incubated for 1 h at 37°C. Unbound sites were blocked with 100 μL of 3% bovine serum albumin (BSA) in PBST by incubating at 37°C for 1 h. After washing, the IgY-rich WSF (diluted 1:1000 in PBST) from egg yolks of melittin-immunized hens, or from egg yolks of the non-immunised control hens, was added (100 μL per well), and then incubated at 37°C for 1 h. After washing, 100 μL of rabbit anti-chicken IgG conjugated with horseradish peroxidase (HRP diluted 1:5000 in PBST) was added and incubated

at 37°C for 1 h. Color was developed with 100 μL of freshly prepared tetramethylbenzidine (TMB) substrate for 30 min, and absorbance was taken at OD_{650} on the ELISA V_{max} kinetic microplate reader. A determination was made of the OD value of anti-melittin IgY, by subtracting the value of the control antibody from that of melittin-specific antibody.

Total IgY concentration

To obtain the total IgY concentration, indirect ELISA was performed as above with some modifications. The enzyme-linked immunosorbent assay (ELISA) plate was coated with 100 μL per well of rabbit anti-chicken IgG, at a final concentration of 2 μg . Two-fold serial dilutions of sigma chicken IgG in PBS (0.5 to 0.031 $\mu\text{g}/\text{mL}$) were used as the reference antibody to prepare a standard curve. Freeze-dried samples of IgY were reconstituted in PBS and added to wells on the same plate. The total IgY concentration was determined using a standard curve.

Western Blot

As described above, a SDS-PAGE of purified (fraction III) and Sigma melittin was performed. The proteins were electrophoretically transferred from gel onto a nitrocellulose membrane at 100 V for 1 h in transfer buffer (pH 8.5, 120 mM glycine, 120 mM Tris). Unless otherwise stated, the membrane was washed four times with Tris buffer containing 0.05% Tween-20 (Tris-T) between each step. Non-specific binding was blocked with 3% BSA in Tris buffer (pH 8.0, 5 mM Tris-HCl, 0.15 M NaCl) at room temperature for 1 h. After blocking, the membrane was incubated with anti-melittin IgY (10 mg/mL, 1:1,000 dilution in Tris-T) at room temperature for 2 h, followed by 1 h of incubation with HRP-conjugated rabbit anti-chicken IgG (1:2000 dilution in Tris-T). After washing, the membrane was treated with a TMB liquid substrate system for color development.

Specificity of anti-melittin IgY

The cross-reactivity between IgY towards other bee venom proteins in fraction I, II, and IV obtained from size-exclusion chromatography, was determined by indirect ELISA with some modifications. The enzyme-linked immunosorbent assay plate was coated with 100 μL triplicates of each fraction and purified melittin (fraction III) in carbonate-bicarbonate buffer (10 μg of protein/mL). Freeze-dried anti-melittin IgY powder was reconstituted with PBST at a dilution of 1:1,000 and 100 μL was added per well. The reactivity of anti-melittin IgY to each fraction was determined by a comparison to the reactivity against purified melittin.

Development of the ELISA detection method for quantification of melittin in bee venom and for quantification of melittin in cosmetic products which contain bee venom

Indirect competitive ELISA

In test tubes, anti-melittin IgY reconstituted in PBS (1 µg/mL) was pre-incubated (4°C overnight) with a two-fold serial dilution of purified melittin (0 to 160 µg/mL) for standard, and lyophilised whole bee venom powder diluted in PBS (100 µg/mL). Unless otherwise stated, there were four washings with PBST conducted between each step of ELISA. The microtiter plate was coated with 100 µL per well of purified melittin (10 µg/mL in PBS) by incubating at 4°C overnight. The following day, the wells were blocked with 120 µL of 3% BSA (37°C for 45 min). The pre-incubated mixtures of melittin and IgY were added to each well (100 µL) and incubated at 37°C for 1 h. Horseradish peroxidase conjugated rabbit anti-chicken IgG at a 1:3,000 dilution was added to 100 µL per well and incubated at 37°C for 1 h. Color was developed with 100 µL of TMB for 5 min. Absorbance was measured at 650 nm using the ELISA V_{max} kinetic microplate reader. The concentration of melittin in bee venom was determined using a standard curve.

Biotinylation of anti-melittin IgY

Biotin conjugation was carried out according to the manufacturer's instruction. One mg of anti-melittin IgY in PBS (pH 7.2) was incubated with 20 µL of long arm NHS-PEO₄-Biotin (3 mg/mL) at room temperature for 1 h. The solution was mixed with 10 µL glycine (100 µg/µL) by gently shaking the mixture for 10 min, followed by dialysis in a Slide-A-Lyzer against PBS (pH 7.2) at 4°C overnight. To test the conjugation of biotin to IgY antibodies, the biotinylated antibodies were diluted 1:10, 1:100, and 1:1,000 and blotted onto a nitrocellulose membrane, followed by a 1 h incubation at room temperature. The membrane was washed three times with PBS (pH 7.2) and blocked with 5% BSA for 2 h, followed by incubation with streptavidin-HRP (1:1,000) at room temperature for 1 h. After washing, a TMB substrate for membranes was added for color development.

Biotinylated Double Antibody Sandwich (DAS) ELISA

Unless otherwise stated, washing was done four times with PBST between each step. A microtiter plate was coated with 100 µL of anti-melittin MAb

(1 µg/mL) in 0.05M carbonate buffer (pH 9.6) at 4°C overnight. Nonspecific binding sites were blocked with 120 µL of 3% BSA for 45 min. After blocking, 100 µL of purified melittin standard in a two-fold serial dilution (0 to 160 ng/mL), lyophilised whole bee venom powder (0 - 500 ng/mL), and two melittin containing cosmetic samples (1:2000, 1:4000, and 1:8000) in PBST were added and incubated at 37°C for 1 h. After washing, 100 µL of the biotinylated anti-melittin IgY (1 µg/mL) was added to each well and incubated at 37°C for 1 h. Streptavidin-HRP (1:1000) was added and incubated at 37°C for 45 min. After 10 min of color development with TMB, absorbance was taken at OD₆₅₀ using an ELISA V_{max} kinetic microplate reader. A determination of the ELISA value of antibody activity was done by subtracting the value of the control antibody from that of specific antibody. The concentration of melittin in bee venom and of melittin in those cosmetic samples which contain bee venom was determined by a standard curve.

Validation

The limit of quantification (LOQ) was calculated from 10 times the standard error of the predicted OD value for each standard melittin concentration in a regression divided by the slope.

Assay precision was determined by intra-assay (within plate; n = 10) and inter-assay (between plates; n = 8) variation.

Statistical analysis

Results were subjected to the analysis of variance using the SAS Software (SAS Institute, Inc., Cary, NC) and statistical significance of differences (p<0.05) was evaluated by the least significant difference procedure. The student t-test (one-tailed t-test) was used to analyse the significant difference (p<0.05) between the groups.

RESULTS

Purification of melittin from bee venom by size exclusion chromatography

Figure 1a illustrates four Sephacryl chromatography separated fractions of bee venom components (I - IV) based on the molecular weight. Each fraction was electrophoresed and protein presence was observed in fraction I (30 - 250 kDa), II (10 - 25 kDa), and III (2 - 15 kDa) (Fig. 1b). The band corresponding to the molecular weight of melittin (2.8 kDa) suggests melittin presence in fraction III.

Production of IgY antibody against melittin

The anti-melittin IgY antibodies obtained from egg yolks of immunised chickens were pooled weekly and titrated by the indirect ELISA (Fig. 2). The titer of anti-melittin IgY was undetectable on day 0, rapidly increased from week 2 to week 4 ($p < 0.05$), and thereafter remained relatively constant for up to 8 weeks. The eggs obtained from 5 to 8 weeks were found to have high levels of anti-melittin IgY activity. The total IgY content in the egg yolk was constant (46.85 ± 1.48 mg/mL) among the melittin-immunised chickens during weeks 5 to 8. There was no significant difference in the total IgY amount compared to the non-immunised eggs ($42.07 \pm$

1.34 mg/mL). Indirect ELISA results demonstrated 11.7% of anti-melittin IgY concentration in total IgY in the immunised group (5.48 ± 0.62 mg/mL), whereas the melittin-specific IgY was not detected in the control group.

Specificity of anti-melittin polyclonal IgY

The presence of melittin in fraction III as well as the reactivity of purified anti-melittin IgY against melittin was confirmed by western blot assay. The band at 2 - 10 kDa in fraction III has the same molecular weight and band pattern as that of Sigma melittin (Fig. 3). Therefore, fraction III was used as purified melittin for further experiments. Western blot results showed that anti-melittin IgY antibody

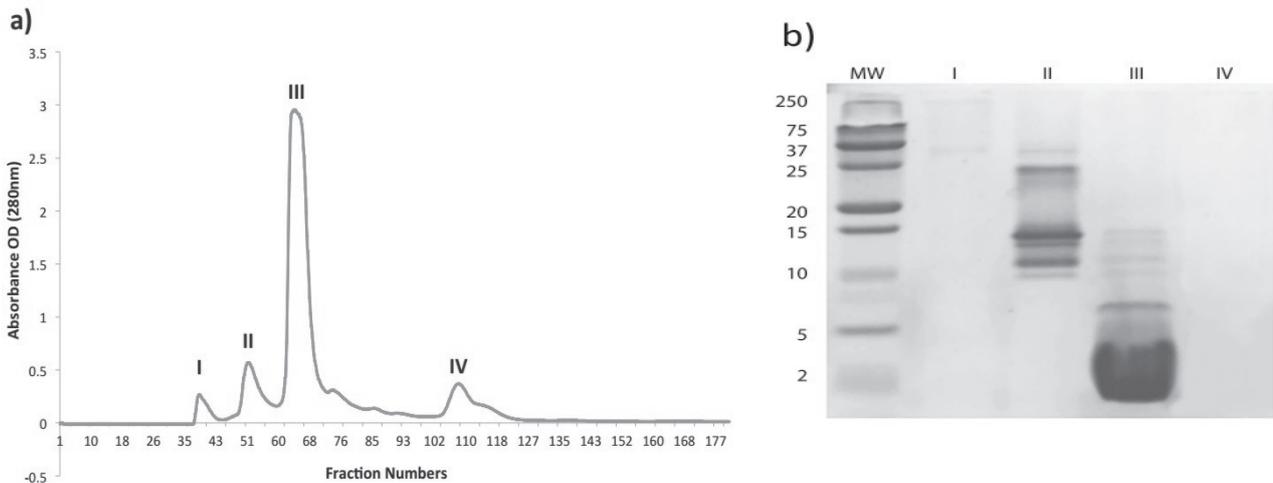


Fig. 1. (a) Size-exclusion chromatography of crude bee venom showing four main peaks. Purified bee venom was filtered through Sephacryl S-100 column at a flow rate of 4.2 mL/h at 4°C for 24 hours. Absorbance was taken at OD = 280 nm. (b) SDS-PAGE of size-exclusion chromatography fractions. Fractions I to IV were run on a non-reducing gel. MW - Molecular weight.

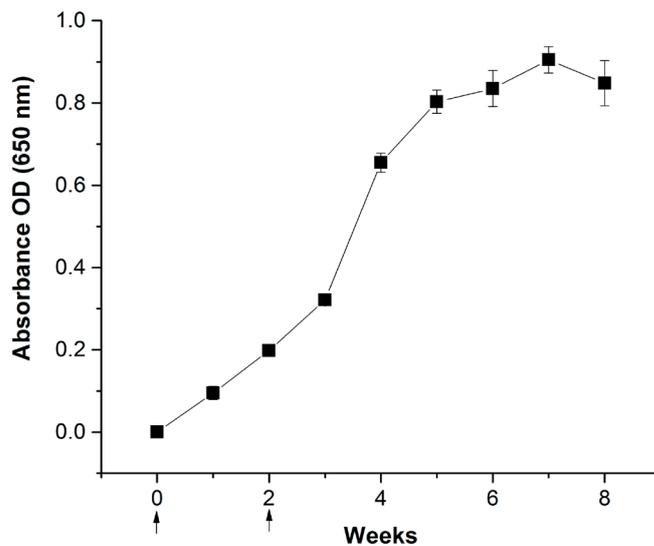


Fig. 2. The anti-melittin IgY activity during the 8 weeks of immunisation period. Antibody activities in the purified IgY solution (at 1:1,000 dilution) were measured by indirect ELISA and expressed as absorbance at 650 nm. Values are the mean of quadruple samples. Vertical bars indicate the standard deviation. Arrows indicate the week of immunisation.

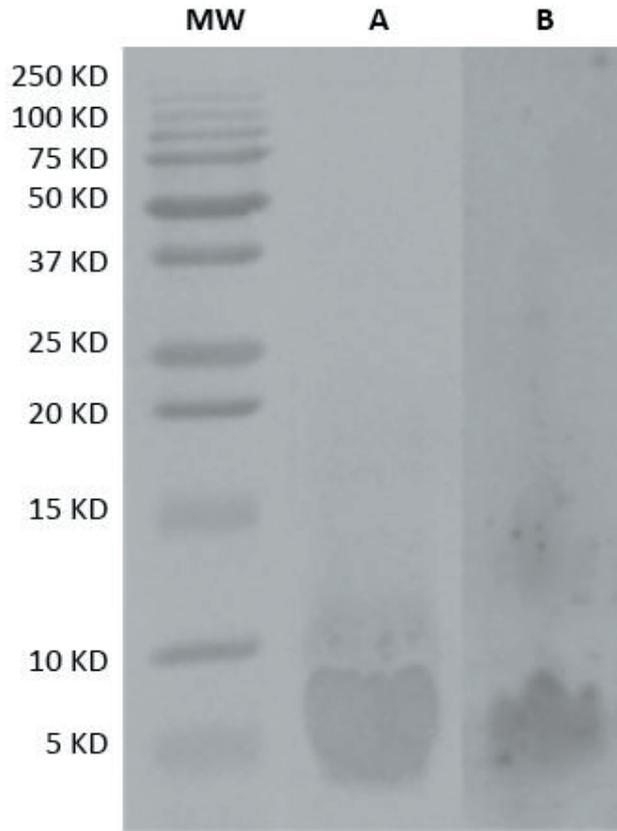


Fig. 3. Western blot of Sephacryl S-100 column size excluded bee venom protein fraction III (A) and Sigma melittin (B) with anti-melittin IgY. MW - Molecular weight.

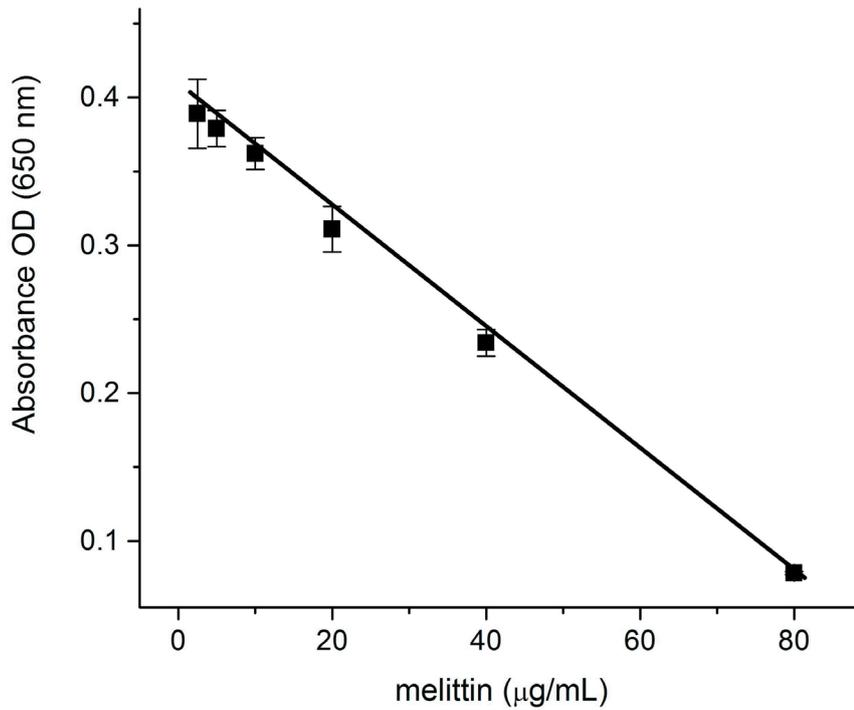


Fig. 4. The standard curve of indirect competitive ELISA for the melittin quantification with a working linear range of 2.5 to 80 µg/mL. For each point, the concentration was calculated from the mean of quadruplicate determinations. Vertical bars indicate the standard deviation.

reacted with both purified and Sigma melittin (Fig. 3), indicating a satisfactory reactivity of the anti-melittin IgY polyclonal antibodies. The specificity of the anti-melittin IgY was further investigated by evaluation of the cross-reactivity between IgY and other bee venom proteins. Indirect ELISA results indicated no cross-reactivity between anti-melittin IgY and other bee venom proteins (data not shown).

Indirect Competitive ELISA

A linear standard curve with a correlation coefficient of 0.95 was achieved with serial dilutions of melittin in the range of 2.5 to 80 µg/mL (Fig. 4). No detection was observed when non-specific IgY was used at the same concentration. The validity was further examined by precision tests. The intra-assay coefficient of variation (intra-assay CV) in 10

replicates was 8.1% and the inter-assay coefficient of variation (inter-assay CV) in 8 replicates was 9.5%. The amount of melittin measured in the bee venom was 54.75 ± 8.55 µg/mL at a dilution of 1:1,000.

Biotinylated DAS ELISA

A double-antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) with biotinylated polyclonal IgY showed a detection range of 2.5 - 40 ng/mL (Fig. 5) with a correlation coefficient of 0.98. The validity of the system was further examined by precision tests. The intra-assay CV determined by replicate measurements (n = 10) of serially diluted melittin was 6.8%, and the inter-assay CV determined by eight independent measurements was 9.6%. The amount of melittin in the bee venom samples (n = 6) measured by the system was

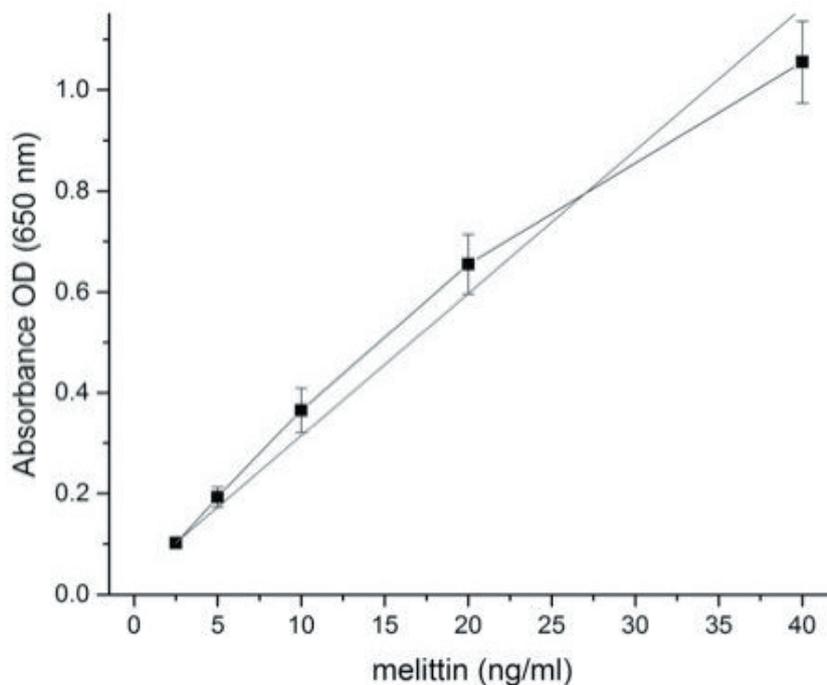


Fig. 5. The standard curve of double antibody sandwich ELISA for the melittin quantification with a working linear range of 2.5 - 40 ng/mL. For each point, the concentration was calculated from the mean of quadruplicate determinations. Vertical bars indicate the standard deviation.

Table 1.

The melittin content in cosmetic products which contain bee venom (dilutions 1:2,000 to 1:8,000). The concentration of melittin in each product was calculated by a reference to the standard curve and expressed as micrograms of melittin per milliliter of product

Products	Dilutions			Mean
	1:2,000	1:4,000	1:8,000	
Cream 1	4.33 ± 0.30	4.69 ± 0.74	4.04 ± 0.98	4.35 ± 0.74
Cream 2	6.82 ± 0.44	6.58 ± 0.91	6.29 ± 0.98	6.56 ± 0.74

26.92 ± 2.11 µg/mL at a dilution of 1:1,000,000. Melittin content in the two cosmetic products containing purified bee venom was measured in different dilutions. Dilutions at 1:2,000, 1:4,000, and 1:8,000 were appropriate for the standard curve for both products, with the mean of 4.53 µg/mL and 6.56 µg/mL each (Tab. 1). The amount of melittin was not disclosed on the product label.

DISCUSSION

Bee venom acupuncture has been widely used in traditional medicine to relieve pain and treat arthritis (Park et al., 2004). The pharmacological effects of bee venom, such as anti-bacterial, anti-viral, anti-tumor, anti-inflammatory, are attributed to the bioactive compounds of the bee venom, which were separated by Sephacryl chromatography (Fig. 1a) (Juvvadi et al., 1996; Falco et al., 2013; Gajski and Garaj-Vrhovac, 2013; Lee et al., 2014b). The fractions I-IV were further electrophoresed to determine the proteins present in each fraction (Fig. 1b). Large enzymes, including phosphatase and hyaluronidase, were found in fraction I, phospholipase A2 was found within fraction II, and low molecular weight peptides such as apamine, adolapine, MCD peptides, and melittin were found within fraction III (Banks and Shipolini, 1986; de Lima and Brochetto-Braga, 2003; Matysiak et al., 2011). Melittin is the major peptide in bee venom that constitutes 50% of bee venom dry weight, as noted by the high OD value and band thickness in SDS-PAGE. The broad range of molecular weight (2-10 kDa) shows spontaneous aggregation of melittin monomers into tetramers in aqueous solutions. (Qiu et al., 2005).

In dermatology, melittin has been utilised for its anti-microbial activity and anti-inflammatory activity on skin-resident microorganisms such as *P. acnes* (Dosler and Gerceker, 2012; Lee et al., 2014a). Topical cosmetic products containing bee venom are gaining popularity for the treatment of acne and skin diseases. However, the cytotoxicity of melittin limits the amount of melittin that can be used in cosmetic formulations (Han et al., 2013; Jamasbi et al., 2014). Commercial cosmetic products compromise cytotoxicity simply by reducing the amount of melittin which also leads to an input cost reduction. However, there is no sensitive method to detect melittin in bee venom and melittin in those cosmetic products which contain bee venom. The reliable, accurate technique, HPLC, quantifies a protein, but the previously reported LOQ for melittin (3.2 µg/mL) is inadequate for a concentration measurement in cosmetic samples (Haghi et al., 2013). Such samples

typically keep melittin levels below 0.05% (unpubl. data). On the other hand, immunoassay is a cost-effective quantification method in which higher sensitivity may be achieved.

The chicken polyclonal IgY based ELISA system offers a couple of advantages over the use of monoclonal antibody. Due to a high phylogenetic difference, chickens produce a high titer of antibody against a wide range of proteins including highly conserved mammalian proteins (Sunwoo et al., 2011). It was possible to obtain IgY egg yolk antibody without sacrificing or bleeding the animal but by simply collecting eggs on a daily basis. The yield of IgY antibody is higher than that of IgG antibodies obtained by conventional immunisation methods; i.e. 200 mg of sera IgG can be obtained once, with approximately 5% constituting the specific IgG, whereas in chickens, approximately 3000 mg of IgY can be harvested from egg yolks each month, with 5 to 10% constituting the specific IgY (Gujral et al., 2012a, b). The proportion of bovine proteoglycan-specific IgY in the total IgY was 9.0% (Li et al., 1998). Similarly, the proportions of bovine and human lactoferrin-specific IgY in the total IgY were 8.7% and 9.2%, respectively (Sunwoo et al., 2011). Our results indicate that titer of anti-melittin polyclonal IgY antibody produced from chicken is higher (11.7%) than antibody against typical mammalian proteins. This higher antibody production is due to the high immunogenicity of melittin in chicken.

In the present study, we developed two different ELISA systems to detect melittin in bee venom and to detect the melittin in cosmetic products which contain bee venom. The indirect competitive ELISA was 1000 times less sensitive than biotinylated DAS ELISA when it came to detecting melittin, resulting in 2.5 µg/mL and 2.5 ng/mL of LOQ, respectively. The difference in sensitivity is mainly due to a strong covalent biotin-streptavidin interaction ($K_d = 10^{-15}$ M) between the detecting antibody and the enzyme of the DAS-ELISA system. Four biotin-binding sites per streptavidin molecule also offer greater sensitivity in the detection system (Green, 1990).

Indirect ELISA successfully measured melittin content in bee venom within close proximity to the previously measured concentration using HPLC-diode array detector-tandem mass spectrometry (HPLC-DAD-MS/MS), which is 46 - 53% (Zhou et al., 2010). However, the detection of melittin in the two cosmetic samples was unsuccessful. With a lower dilution factor, the cream was not fully dissolved, whereas with a higher dilution factor, the concentration was out of range to be quantified using the indirect competitive ELISA (linear detection

range of 2.5 to 80 µg/mL). Therefore, the broader range as well as a higher detection limit provided by this system are inappropriate for quantifying small amounts of melittin in the cosmetics which contain bee venom, but the range and detection limit are more useful to quantify the higher melittin content present in bee venom.

Our result shows that biotinylated DAS-ELISA successfully quantifies melittin in those commercially available cosmetic products which contain bee venom (Tab. 1). The Limit of Quantitation (LOQ) of this developed DAS ELISA was approximately 1000 times and 400 times more sensitive than those obtained from reverse-phase HPLC (3.2 µg/mL) and HPLC-DAD-MS/MS (1.0 µg/mL), respectively (Zhou et al., 2010; Haghi et al., 2013). The cream sample dilutions 1:2000, 1:4000, and 1:8000, were appropriate for the detection system. Consistency of the results at different dilutions suggests that the other ingredients in the cream do not interfere with the assay at dilutions higher than 1:2000. The narrow detection range and low LOQ is less preferable for detection of melittin in natural bee venom, but is appropriate for quantifying very low amounts in formulations. Therefore, depending on the presence of melittin in a sample, from microgram to nanogram levels, one of the two developed ELISA systems may be selected. Bee venom raw material suppliers and product manufacturers may use these detection systems to include the known amount of melittin as well as to declare the presence of melittin in their products.

CONCLUSION

Two cost-effective ELISA systems have been developed for melittin quantification using anti-melittin IgY from hyperimmunised chicken egg yolk. Indirect competitive ELISA has a higher concentration range of detection, which allows for the measurement of natural concentrations of melittin in bee venom. Biotinylated DAS-ELISA system is highly sensitive, providing a very low concentration detection range, which is suitable for the quality control of cosmetic or pharmaceutical formulations.

ACKNOWLEDGEMENT

This work was carried out with the support of the Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ01128901), Rural Development Administration, Republic of Korea. Authors acknowledge Dr. Suh as a co-corresponding author.

REFERENCES

- Banks B. E. C., Shipolini R. A. (1986) Chemistry and pharmacology of honey bee venom. In: Piek T. (Ed) Venoms of the Hymenoptera. Biochemical, pharmacological and behavioural aspects. Academic Press. University of Michigan. Michigan, USA: 329-416.
- Bojar R. A., Holland K. T. (2004) Acne and *Propionibacterium acnes*. Clinics in Dermatology 22(5): 375-379. DOI: 10.1016/j.clindermatol.2004.03.005
- de Lima P. R., Brochetto-Braga M. R. (2003) Hymenoptera venom review focusing on *Apis mellifera*. Journal of Venomous Animals and Toxins including Tropical Diseases 9(2): 149-162. DOI: 10.1590/S1678-91992003000200002
- Dosler S., Gerceker A. A. (2012) In vitro activities of antimicrobial cationic peptides; melittin and nisin, alone or in combination with antibiotics against Gram-positive bacteria. Journal of Chemotherapy 24(3): 137-143. DOI: 10.1179/1973947812y.0000000007
- Falco A., Barrajon-Catalan E., Menendez-Gutierrez M. P., Coll J., Micol V., Estepa A. (2013) Melittin-loaded immunoliposomes against viral surface proteins, a new approach to antiviral therapy. Antiviral Research 97(2): 218-221. DOI: 10.1016/j.antiviral.2012.12.004
- Gajski G., Garaj-Vrhovac V. (2013) Melittin: A lytic peptide with anticancer properties. Environmental Toxicology and Pharmacology 36(2): 697-705. DOI: 10.1016/j.etap.2013.06.009
- Gauldie J., Hanson J. M., Shipolini R. A., Vernon C. A. (1978) Structure of some peptides from bee venom. European Journal of Biochemistry 83(2): 405-410. DOI: 10.1111/j.1432-1033.1978.tb12106.x
- Green N. M. (1990) Avidin and Streptavidin. Methods in Enzymology. 184: 51-67.
- Gujral N., Suresh M. R., Sunwoo H. (2012a) Quantitative double antibody sandwich ELISA for the determination of gliadin. Journal of Immunoassay and Immunochemistry 33(4): 339-351. DOI: 10.1080/15321819.2012.655823
- Gujral N., Lobenberg R., Suresh M., Sunwoo H. (2012b) In-Vitro and In-Vivo Binding Activity of Chicken Egg Yolk Immunoglobulin Y (IgY) against Gliadin in Food Matrix. Journal of Agricultural and Food Chemistry 60(12): 3166-3172. DOI: 10.1021/jf205319s

- Habermann E. (1972) Bee wasp venoms. *Science* 177(4046): 314-322. DOI: 10.1126/science.177.4046.314
- Haghi G., Hatami A., Mehran M. (2013) Qualitative and Quantitative evaluation of melittin in honeybee venom and drug products containing honeybee venom. *Journal of Apicultural Science* 57(2): 37-44. DOI: 10.2478/jas-2013-0015
- Han S. M., Lee K. G., Park K. K., Park S. C. (2013) Skin sensitization study of bee venom (*Apis mellifera* L.) in guinea pigs and rats. *Cutaneous and Ocular Toxicology* 32(1): 27-30. DOI: 10.3109/15569527.2012.698402
- Jamasbi E., Batinovic S., Sharples R. A., Sani M., Robins B., Roy M., Wade J., Separovic F., Hossain M. A. (2014) Melittin peptides exhibit different activity on different cells and model membranes. *Amino Acids* 46(12): 2759-2766. DOI: 10.1007/s00726-014-1833-9
- Juvvadi P., Vunnam S., Merrifield R. B. (1996) Synthetic melittin, its enantio, retro, and retroenantio isomers, and selected chimeric analogs: Their antibacterial, hemolytic, and lipid bilayer action. *Journal of the American Chemical Society* 118(38): 8989-8997. DOI: 10.1021/ja9542911
- Koh P. S., Seo B. K., Cho N. S., Park H. S., Park D. S., Baek Y. H. (2013) Clinical effectiveness of bee venom acupuncture and physiotherapy in the treatment of adhesive capsulitis: a randomized controlled trial. *Journal of Shoulder and Elbow Surgery* 22(8): 1053-1062. DOI: 10.1016/j.jse.2012.10.045
- Larsson A., Karlssonparra A., Sjoquist J. (1991) Use of Chicken antibodies in enzyme immunoassays to avoid interference by rheumatoid factors. *Clinical Chemistry* 37(3): 411-414.
- Lee M. T., Sun T. L., Hung W. C., Huang H. W. (2013) Process of inducing pores in membranes by melittin. *Proceedings of the National Academy of Sciences of the United States of America* 110(35): 14243-14248. DOI: 10.1073/pnas.1307010110
- Lee W. R., Kim K. H., An H. J., Kim J. Y., Han S. M., Lee K. G., Park, K. K. (2014a) Protective effect of melittin against inflammation and apoptosis on *Propionibacterium acnes*-induced human THP-1 monocytic cell. *European Journal of Pharmacology* 740: 218-226. DOI: 10.1016/j.ejphar.2014.06.058
- Lee W. R., Kim K. H., An H. J., Kim J. Y., Chang Y. C., Chung H., Park Y. Y., Lee M. L., Park K. K. (2014b) The Protective Effects of Melittin on *Propionibacterium acnes*-Induced Inflammatory Responses In Vitro and In Vivo. *Journal of Investigative Dermatology* 134(7): 1922-1930. DOI: 10.1038/jid.2014.75
- Leyden J. J. (2001) The evolving role of *Propionibacterium acnes* in acne. *Seminars in Cutaneous Medicine and Surgery* 20(3): 139-143. DOI: 10.1053/sder.2001.28207
- Li X., Nakano T., Chae H. S., Sunwoo H., Sim J. S. (1998) Production of chicken egg yolk antibody (IgY) against bovine proteoglycan. *Canadian Journal of Animal Science* 78(3): 287-291.
- Matysiak J., Schmelzer C. E. H., Neubert R. H. H., Kokot Z. J. (2011) Characterization of honeybee venom by MALDI-TOF and nanoESI-QqTOF mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis* 54(2): 273-278. DOI: 10.1016/j.jpba.2010.08.020
- Park H. J., Lee S. H., Son D. J., Oh K. W., Kim K. H., Song H. S., Kim G. J., Oh G. T., Yoon D. Y., Hong J. T. (2004) Antiarthritic effect of bee venom - Inhibition of inflammation mediator generation by suppression of NF-kappa B through interaction with the p50 subunit. *Arthritis and Rheumatism* 50(11): 3504-3515. DOI: 10.1002/art.20626
- Qiu W. H., Zhang L. Y., Kao Y. T., Lu W. Y., Li T. P., Kim J., Sollenberger G. M., Wang L. J., Zhong D. P. (2005) Ultrafast hydration dynamics in melittin folding and aggregation: Helix formation and tetramer self-assembly. *Journal of Physical Chemistry B* 109(35): 16901-16910. DOI: 10.1021/jp0511754
- Raghuraman H., Chattopadhyay A. (2007) Melittin: A membrane-active peptide with diverse functions. *Bioscience Reports* 27(4-5): 189-223. DOI: 10.1007/s10540-006-9030-z
- SAS Version 9.1.3 (2002-2004) Help and Documentation. SAS Institute Inc. Cary, NC, USA.
- Schade R., Calzado E. G., Sarmiento R., Chacana P. A., Porankiewicz-Asplund J., Terzolo H. R. (2005) Chicken egg yolk antibodies (IgY-technology): A review of progress in production and use in research and human and veterinary medicine. *ATLA Alternatives to Laboratory Animals* 33(2): 129-154.

Sunwoo H., Gujral N., Suresh M. (2011) Competitive and double antibody sandwich ELISA for the quantification of lactoferrins by using monoclonal and chicken egg yolk IgY antibodies. *Journal of Immunoassay and Immunochemistry* 32(2): 79-92. DOI: 10.1080/15321819.2010.543218

Terwilliger T. C., Weissman L., Eisenberg D. (1982) The structure of melittin in the form I crystals and its implication for melittin's lytic and surface activities. *Biophysical Journal* 37(1): 353-361.

Zhou J. H., Zhao J., Zhang S. X., Shen J. Z., Qi Y. T., Xue X. F., Li Y., Wu L. M., Zhang J. Z., Chen F., Chen L. Z. (2010) Quantification of melittin and apamin in bee venom lyophilized powder from *Apis mellifera* by liquid chromatography-diode array detector-tandem mass spectrometry. *Analytical Biochemistry* 404(2): 171-178. DOI: 10.1016/j.ab.2010.05.014