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ORIGINAL ARTICLE

Detoxification of Bee Venom Increases Its Anti-inflammatory Activity and Decreases Its Cytotoxicity and Allergenic Activity

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Abstract

Bee venom is a medicinal product that is widely used in traditional therapies owing to its excellent anti-inflammatory activity. However, the use of bee venom has shown adverse effects. Therefore, there is a need for research that can remove the cytotoxicity of bee venom and enhance its efficacy. In this study, we hydrolyzed melittin, the main component of bee venom, and removed the other components to eliminate the toxicity of bee venom. To compare the efficacy of bee venom and detoxified bee venom, we examined their antioxidant effects using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. In addition, cytotoxicity was confirmed in MCF 10A and RAW 264.7 cells, using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay. Detoxified bee venom showed a strong antioxidant activity and decreased a cytotoxicity in MCF 10A and RAW 264.7 cells. The anti-inflammatory activity of detoxified bee venom and bee venom were assessed by comparison of the expression of inflammatory cytokine mRNA and phosphorylation of IκBα in RAW 264.7 cells. Degranulation in RBL-2H3 cells was analyzed through β-hexosaminidase release assay to confirm the allergenic activity of bee venom and detoxified bee venom. Treatment of the detoxified bee venom inhibited inflammatory cytokine mRNA expression, IκBα phosphorylation, and β-hexosaminidase release. Taken together, the results indicated that compared to bee venom, detoxified bee venom exhibited decreased cytotoxicity and allergenicity and increased anti-inflammatory activity. In conclusion, detoxification of bee venom efficiently decreases the adverse effects, making it suitable for medicinal applications.

Keywords Bee venom · Detoxification · Anti-inflammatory activity · Allergenic activity · Antioxidant activity

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Introduction

Bee venom is a poison that bees secrete from the tips of bee stings to protect their colonies. The main component of bee venom is melittin, and there are reports of its pharmacological effects [1–3]. Traditional therapy using bee venom has been used to prevent and treat various diseases in humans [4, 5]. Moreover, various studies have shown the antimicrobial and anticancer effects of bee venom, particularly its strong anti-inflammatory activity [6–10]. However, the ingredients of bee venom are also stimulants that cause an inflammatory reaction [11–13]. Serious side effects, such as anaphylaxis, which can lead to death in severe cases, limit the use of bee venom [14–16]. Despite the strong pharmacological effects of bee venom, studies on potential beneficial effects have not been conducted much, and studies are needed to overcome these limitations.

The inflammatory reaction rapidly removes pathogenic factors or regenerates damaged tissues, after harmful substances invade a living body or in metabolic disorders and various traumas [17, 18]. Depending on the duration, inflammation can be divided into acute inflammation, which occurs over several minutes, and chronic inflammation, which occurs over months to years [19]. When the inflammatory reaction is stimulated by trigger substances, phagocytes recognize the stimulus through surface receptors and activate signaling pathways through transcription factors, such as nuclear factor-kappa B (NF-κB), to secrete pro-inflammatory cytokines [20–22]. These pro-inflammatory cytokines activate immune cells involved in inflammation, play a role in increasing angiogenesis and vascular permeability, and participate in the activity of macrophages [23, 24]. Inflammatory reactions play an essential role in the survival of the body in an environment that threatens it. However, excessive inflammatory reactions in abnormal situations can cause various diseases such as cancer, vasculitis, and myocarditis [25–27]. In particular, cytokine storms have appeared in severely ill patients with COVID-19, a phenomenon in which normal cells are attacked due to excessive inflammatory reactions [28–30].

In this study, detoxified bee venom was prepared by removing toxicity while maintaining anti-inflammatory activity of bee venom. In addition, we confirmed the antioxidant effect, cytotoxicity, effect on inflammatory cytokine release, effect on phosphorylation of IκBα, and effect on β-hexosaminidase release of detoxified bee venom (Table 1).

Table 1 Inflammatory cytokine primers for qRT-PCR

Gene		Primer sequence (5′ to 3′)	Product size (bp)
β-actin	Forward	TCA CCC ACA CTG TGC CCA TCT ACG	295
	Reverse	CAG CGG AAC CGC TCA TTG CCA ATG	
TNF-α	Forward	AGG GTC TGG GCC ATA GAA CT	103
	Reverse	CCA CCA CGC TCT TCT GTC TAC	
IL-6	Forward	GTC CTT CAG AGA GAT ACA GAA ACT	113
	Reverse	AGC TTA TCT GTT AGG AGA GCA TTG	
iNOS	Forward	CAG CTG GGC TGT ACA AAC CTT	95
	Reverse	CAT TGG AAG TGA AGC GTT TCG	

Materials and Methods

Bee Venom

The bee venom used in this experiment was from Georgian bees and as purchased from New Technology Laboratory LTD, Georgia (https://beevenomgeo.com/). The bee venom has the following brief characteristics: loss of mass when dried, max. 12%; water-insoluble substances, max. 10%; total ash content, < 2%; authenticity (hemolysis period), max. 480 s; activity of phospholipase A2 (PLA2), no less than 100 IU/mg; activity of hyaluronidase, no less than 70 IU/mg; apamin concentration, ≥ 2%, PLA2 concentration, ≥ 12%; melittin concentration, ≥ 50%.

Detoxification of Bee Venom

Bee venom and ethanol (1:4 weight ratio) were mixed and filtered through filter paper 20 (Hyundai Micro, South Korea). The filtrate was passed through a cation exchange resin (Sigma-Aldrich, MO, USA) and freeze-dried. A total of 10 g of the freeze-dried powder was dissolved in 100 g of buffer solution, previously obtained by dissolving 60 g of anhydrous citric acid (Daejung, Korea) in 100 g of 90% ethanol (Daejung), and mixed at 40 °C for 6 h. Ethanol (90%) was added, as much as twice the weight of the buffer solution, precipitated at − 20 °C for 16 h, filtered with filter paper 20, and the residue on the paper was then dried at 70 °C for 1 h. Distilled water was added to the residue as much as nine times of the weight, sonicated for 1 h, and cooled to 25 °C room temperature. Then, in order to hydrolyze melittin, bromelain (Bision, South Korea), a natural hydrolase, was added by 1/2000 of the remaining amount and mixed at 45 °C. for 3 h with a roller mixer. And in order to further hydrolyze melittin, alcalase (Sigma-Aldrich), a natural hydrolase, was added by 1/500 of the remaining amount and mixed at 50 °C for 2 h with a roller mixer, followed by heating in a 95 °C. water bath for 30 m. Ethanol was added at three times the weight, precipitated at − 20 °C for 16 h, and then filtered with filter paper 20. The filtrate was filtered through a glass microfiber membrane filter (Whatman, UK), and 100% ethanol was added as much as the total weight and settled at − 20 °C for 16 h. After filtration through a 0.45-μm PVDF membrane filter (Hyundai Micro) and concentration at 60 °C with a vacuum evaporator, the concentrated solution was filtered through a 10 kDa cut-off ultrafiltration filter (Synopex, Korea) at a pressure of 20 psi. After adding 100% ethanol by the weight of the filtrate, it was precipitated at − 20 °C for 16 h. After filtration through a 0.20-μm polypropylene membrane filter (PALL) and concentration at 60 °C with a vacuum evaporator, the concentrate was subjected to gel filtration chromatography with Sephacryl S-300 high resolution (GE Healthcare, USA). The samples were injected at a flow rate of 1.3 mL/min, with water as the mobile phase, at a detection wavelength of 280 nm and fractionation time of 600 min using an FPLC equipment (Pharmacia Biotech, Sweden). Fractions were collected from 310 to 460 min and freeze-dried. The components of detoxified bee venom were analyzed through LC/MS. The samples were analyzed in a Triple TOF 5600 Q-TOF LC/MS/MS system (ABSciex, CA) using an Ultimate 3000 RSLC HPLC system (Thermo Fisher Scientific Inc., MA, USA). Mobile phase condition of LC/MS analysis is shown in Table 2 and the total ion chromatogram (TIC) obtained by LC/MS analysis is shown in Supplementary Fig. 1.

Table 2 The eluent gradient condition for LC/MS experiments

Time (min)	Eluent A 0.1% formic acid in DW (%)	Eluent B 0.1% formic acid in Acetonitrile (%)
0	95	5
1	95	5
17	55	45
24	0	100
26	0	100
26.5	95	5
27	95	5
30	95	5

Cell Culture

The human breast epithelial cell line MCF 10A, murine macrophage cell line RAW 264.7, and rat basophilic leukemia mast cell line RBL-2H3 cells were purchased from American Type Culture Collection (ATCC, VA, USA). The MCF 10A cells were cultured in Dulbecco's modified Eagle's medium (DMEM, WELGENE, Korea) supplemented with 5% heat-inactivated horse serum (WELGENE, Korea), 10 μg/mL insulin (Sigma-Aldrich), 100 ng/mL cholera toxin (Sigma-Aldrich), 0.5 μg/mL hydrocortisone (Sigma-Aldrich), 20 ng/mL recombinant human epithermal growth factor (Sigma-Aldrich), and 1% penicillin and streptomycin (WELGENE) at 37 °C in a humidified atmosphere of 5% CO_2. RAW 264.7 and RBL-2H3 cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS, WELGENE) and 1% penicillin and streptomycin at 37 °C in a humidified atmosphere of 5% CO_2.

Free Radical Scavenging Assay

The antioxidant activities of bee venom and detoxified bee venom were measured with the DPPH radical scavenging assay. DPPH powder (Sigma-Aldrich) was dissolved in methanol to a final concentration of 0.2 mM to prepare a radical DPPH working solution. DPPH working solution (100 μL) and sample (100 μL) were added to each well of a 96-well plate, followed by reaction in the dark for 4 min, and the absorbance was measured with a microplate reader (Molecular Devices EMax Plus, USA) at 515 nm. The formula used to calculate the antioxidant efficacy was as follows:

Radical scavenging activity (%) = {1 – (*A* [Sample] – *A* [Sample blank] / *A* [Blank])} × 100, where *A* is the absorbance for a given wavelength.

Cell Viability Assay

The effect of bee venom or detoxified bee venom on cell viability was evaluated using the MTS assay. MCF 10A or RAW 264.7 cells were seeded at 3000 cells/well in 96-well plates and incubated for 24 h; the medium with bee venom or detoxified bee venom was then

added, and the plates were incubated for 24 h. Thereafter, 20 μL of MTS reagent (Promega, WI, USA) was added, and absorbance was measured at 490 nm using a microplate reader (Molecular Devices EMax Plus). The formula used to calculate the cell viability was as follows:

$$\text{Cell viability}(\%) = \{(A\left[\text{Sample}\right] - A[\text{Blank}])/(A[\text{Control}]) - A[\text{Blank}])\} \times 100,$$

where A is the absorbance for a given wavelength.

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

RAW 264.7 cells were seeded at a density of 1×10^5 cells/well in 6-well plates and incubated for 24 h; the medium with bee venom or detoxified bee venom was then added, and the plates were incubated for 24 h. Thereafter, lipopolysaccharide (LPS) was added to the medium so that the concentration of LPS was 1 μg/mL, followed by incubation for 6 h. To obtain total RNA, the medium was completely removed, the cells were washed with PBS and then lysed with 1 mL of RiboEx (GeneAll, Korea), and RNA was extracted using a Hybrid-R RNA purification kit (GeneAll). To synthesize cDNA, RNA was quantified using a Nabi UV/Vis Nanospectrophotometer (MicroDigital, Korea), and 1 μL of random hexamer (100 pmol/μL) and 1 μL of dNTP mix (10 mM) were added to 1 μg of each RNA. The total volume was adjusted to 10 μL using DEPC-treated water. This mixture was subjected to heating at 65 °C for 5 min and immediate cooling on ice. Thereafter, 1 μL of M-MLV reverse transcriptase (Promega), 4 μL of 5X M-MLV RT reaction buffer (Promega), 1 μL of RNase inhibitor (Enzynomics, Korea), and 4 μL of DEPC-treated water were added to each RNA. The cDNA was synthesized by allowing the reaction at room temperature for 10 min and then at 50 °C for 1 h.

The mRNA expression of inflammatory cytokines was analyzed by qRT-PCR. A total of 10 μL of 2X Prime Q-master Mix (GENET BIO, Korea), 1.5 μL of 10 pmol/μL forward primer, 1.5 μL of 10 pmol/μL reverse primer, and 5 μL of nuclease-free water were added to 2 μL of cDNA diluted to 1/10, and qRT-PCR was performed using AriaMx (Agilent, USA). qRT-PCR was performed with 40 cycles of denaturation at 95 °C for 20 s, annealing at 58 °C for 20 s, and elongation at 72 °C for 20 s. The primers used to perform qRT-PCR are indicated in Table 1.

Immunoblotting Assay

RAW 264.7 cells were seeded at a density of 1×10^5 cells/well in a 6-well plate and incubated for 24 h; the medium with bee venom or detoxified bee venom was then added, and the plates were incubated for 24 h. Thereafter, LPS was added to the medium so that the concentration of LPS was 1 μg/mL, followed by incubation for 1 h. To obtain protein samples, the medium was completely removed, and cells were washed with PBS and then lysed with lysis buffer (10 mM Tris–HCl [pH 7.5], 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1%, Triton X-100, 10% glycerol, 0.1% SDS) with a protease inhibitor cocktail. Protein samples were subjected to sonication and centrifuged at $16{,}000 \times g$ for 10 min. The supernatant was separated and used for immunoblotting. Protein samples were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were transferred onto a nitrocellulose membrane (0.45 μm, Merck Millipore, USA). PBST with 5% nonfat skim milk was used as a blocking buffer for 1 h, and the

nitrocellulose membrane was treated with anti-phospho-IκBα (9246S, 1:500, Cell Signaling Technology), anti-IκBα (L35A5, 1:500, Cell Signaling Technology,), and anti-GAPDH (H86504M, 1:2000, Meridian Life Science) antibodies diluted in blocking buffer overnight at 4 °C. The unbound antibody was washed with PBST, and the antibodies bound to the nitrocellulose membrane were detected with the Super Signal system using horseradish peroxidase-conjugated secondary antibodies.

β-Hexosaminidase Release Assay

The β-hexosaminidase release assay was performed as previously described [31–33]. RBL-2H3 cells were seeded at 5×10^4 cells/well in 96-well plates and incubated for 24 h. The medium was aspirated, and cells were washed twice with siraganian buffer (119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM $MgCl_2$, 25 mM PIPES, 40 mM NaOH, 1 mM $CaCl_2$, and 0.1% BSA, pH 7.2) and then incubated in 100 μL siraganian buffer supplemented with compound 48/80 (Sigma-Aldrich), bee venom or detoxified bee venom for 1 h. Compound 48/80 was used as an alternative to histamine as a positive control. The reaction was terminated at 4 °C for 10 min. The fluorescence of β-hexosaminidase in the supernatant was measured using a β-hexosaminidase activity assay kit (Cell Biolabs, USA), and the fluorescence of β-hexosaminidase was measured using 2104 EnVision Multilabel Plate Readers (PerkinElmer, USA) at emission wavelength of 340 nm and excitation wavelength of 450 nm. β-Hexosaminidase release (RFU) was calculated by subtracting the fluorescence intensity of the control group from that of the experimental group.

Statistical Analysis

All experiments have at least three independent biological repeats. The data are presented as the mean ± standard error, and statistical analysis was performed using Student's *t*-test for the significance. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Results

Detoxification of Bee Venom

To efficiently use bee venom, it is very important to remove its toxicity. Melittin, a peptide composed of 26 amino acids, is the main component responsible for the pharmacological effect of bee venom and exhibits cytotoxicity at high concentrations [34]. In addition, the other major constituents of bee venom that show toxicity include PLA2, hyaluronidase, histamine, apamin, and MCD. Therefore, in this study, to remove the toxicity of bee venom, toxic substances were filtered out, and melittin was hydrolyzed to perform a detoxification process. The detoxification process is shown in Fig. 1. As a result of analyzing the components of detoxified bee venom through LC/MS, components other than melittin were not detected and fragmented melittin was detected (Table 3 and Supplementary Table 1). The detoxified bee venom was dissolved in tertiary distilled water and used for the subsequent experiments.

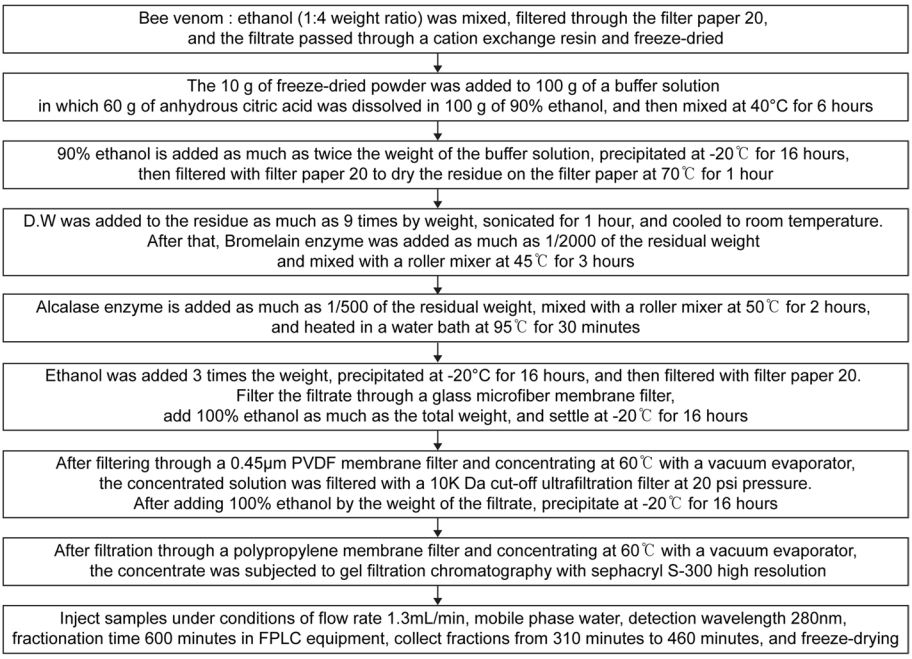

Fig. 1 Process of detoxification of bee venom

Table 3 Peptides identified in detoxified bee venom

No	Sequence	Molecular weight (Da)	Detection count	Detection rate (%)
1	TTGLPALISWIKRKRQQ	1994	27	14.3
2	TGLPALISWIKRKRQQ	1893	35	18.5
3	LISWIKRKRQQ	1453	102	54.0
4	GIGAVLKVL	868	21	11.1
5	WIKRKRQQ	1140	4	2.1

Melittin amino acids sequence: GIGAVLKVLTTGLPALISWIKRKRQQ

Estimation of Antioxidant Activity of Detoxified Bee Venom

Reactive oxygen species (ROS) are high-energy, unstable, and highly reactive molecules containing an oxygen atom and unpaired electrons [35]. When ROS exceed a particular amount in the body, they attack lipids, proteins, DNA, and polymers, thereby hindering cell function [36]. In particular, active oxygen has an important effect on inflammation control. It has been reported that ROS are regulated by the inflammatory regulatory protein TXNIP and the antioxidant enzyme TRX [37, 38]. Therefore, it is very important to effectively control ROS during the anti-inflammatory reaction, for which it is necessary to study antioxidant substances. In this study, a DPPH assay was performed to estimate the antioxidant activity of the detoxified bee venom. To prove the reliability of the experiment, resveratrol, which is known to have excellent antioxidant efficacy, was used as a positive control.

In the DPPH assay, resveratrol showed radical scavenging activity of 23.67% ± 1.68%, 32.89% ± 1.04%, and 37.47% ± 1.16% at concentrations of 25, 50, and 100 μM, respectively. Bee venom showed radical scavenging activity of 5.59% ± 0.39%, 39.56% ± 1.16%, 91.82% ± 2.36%, and 98.03% ± 0.38%, whereas detoxified bee venom showed radical scavenging activity of 7.48% ± 0.53%, 21.3% ± 0.63%, 58.02% ± 1.49%, and 89.52% ± 0.34%, at concentrations of 0.1, 1, 10, and 100 μg/mL, respectively (Fig. 2). These results showed that the detoxified bee venom had excellent antioxidant activity.

Comparison of Cytotoxicity Between Detoxified Bee Venom and Bee Venom

It is known that bee venom has a strong anti-inflammatory activity and can cause a fatal allergic reaction and hepatotoxicity [39]. In order to confirm whether the cytotoxicity of detoxified bee venom was effectively reduced, the effects of treatment with detoxified bee venom on the viability of human breast epithelial MCF 10A and murine macrophage RAW 264.7 cells were measured using the MTS assay. The MTS assay was used to assess the mitochondrial activity of cells by evaluating the concentration of formazan produced by the reduction of tetrazolium salt by NADH and NADPH, by measuring absorbance at 490 nm [40]. The viability of bee venom– and detoxified bee venom–treated cells was measured as 100% of the viability of cells untreated as a control. In the case of bee venom–treated cells, the cell viability significantly decreased when the venom concentration was more than 15 μg/mL, but detoxified bee venom–treated cells did not show any cytotoxicity (Fig. 3). These results suggested that the cytotoxicity of the detoxified bee venom was efficiently removed from the bee venom after the detoxification process.

Effect of Detoxified Bee Venom on the mRNA Expression of Inflammatory Cytokines

When an inflammatory reaction occurs, immune cells secrete pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, which signal danger around them. Subsequently, macrophages promote the synthesis of nitric oxide by expressing *iNOS* via cytokines that

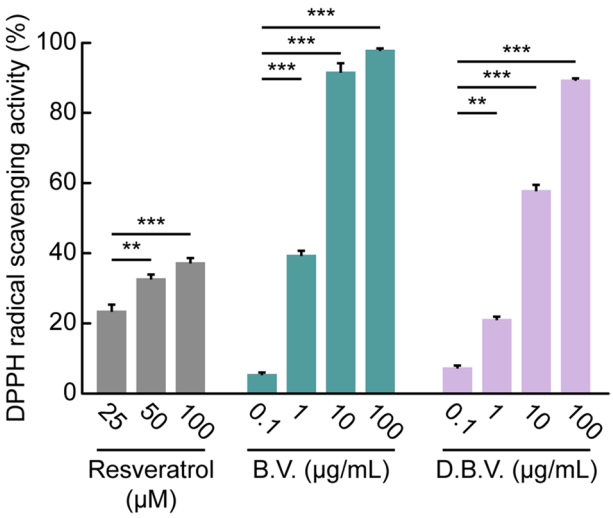

Fig. 2 The in vitro antioxidant activities of the bee venom and detoxified bee venom. DPPH radical scavenging activity of resveratrol, the bee venom (B.V.), and the detoxified bee venom (D.B.V.). Results are expressed as means ± SD ($n = 3$). $^{**}p < 0.01$, $^{***}p < 0.001$

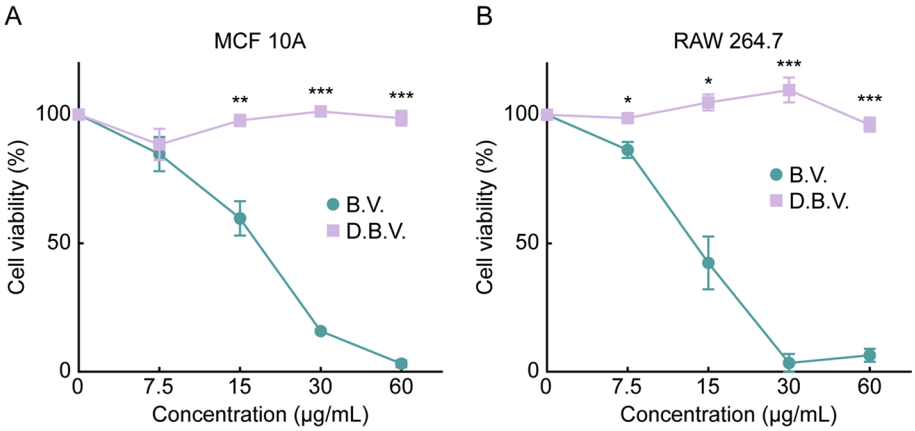

Fig. 3 Comparison of cytotoxicity between bee venom and detoxified bee venom. MCF 10A (**A**) and RAW 264.7 (**B**) cells were treated with indicated concentrations of bee venom (B.V.) or detoxified bee venom (D.B.V) for 24 h. Cell viability was determined using the MTS assay. Results are expressed as means ± SD (**n** = 3). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

promote the inflammatory reaction [41, 42]. To confirm the anti-inflammatory activity of detoxified bee venom, expression of two representative inflammatory cytokine mRNAs, *TNF-α* and *IL-6*, and a representative nitric oxide-generating enzyme, *iNOS*, during the inflammatory reaction was confirmed in RAW 264.7 cells. When LPS induced an inflammatory reaction in RAW 264.7 cells grown in culture medium without treatment, the mRNA expression levels of *TNF-α*, *IL-6*, and *iNOS* increased. When the inflammatory reaction was induced by LPS treatment in RAW 264.7 cells grown in 7.5 μg/mL of bee venom–treated culture medium, the mRNA expression levels of *TNF-α*, *IL-6*, and *iNOS* were similarly increased. In contrast, when LPS treatment induced inflammatory reactions in RAW 264.7 cells grown in a culture medium treated with 7.5 μg/mL of detoxified bee venom, the mRNA expression level of *TNF-α*, *IL-6*, and *iNOS* was decreased compared to RAW 264.7 cells grown in a culture medium without treatment. Accordingly, it was confirmed that the mRNA expression levels of *TNF-α*, *IL-6*, and *iNOS* were remarkably suppressed in RAW 264.7 cells treated with detoxified bee venom (Fig. 4). Therefore, it can be inferred that detoxified bee venom could effectively inhibit the inflammatory reaction in RAW 264.7 cells.

Effect of Detoxified Bee Venom on the Phosphorylation of IκBα

The inflammatory reaction induced by LPS is regulated by NF-κB through the Toll-like receptor 4 (TLR 4) signaling pathway [43]. NF-κB forms a complex with IκBα in the cytoplasm. The IκB kinase (IKK) activated by LPS stimulation phosphorylates IκBα, and the NF-κB and IκBα complexes are separated. Then, IκBα is decomposed and NF-κB is activated by moving to the nucleus, thereby regulating the production and synthesis of inflammatory cytokines within the nucleus [44]. These processes are related to the signaling system of inflammatory reactions. Our study confirmed the excellent anti-inflammatory activity of detoxified bee venom in RAW 264.7 cells. The anti-inflammatory activity of detoxified bee venom was additionally confirmed through the phosphorylation of IκBα during the inflammatory reaction in RAW 264.7 cells.

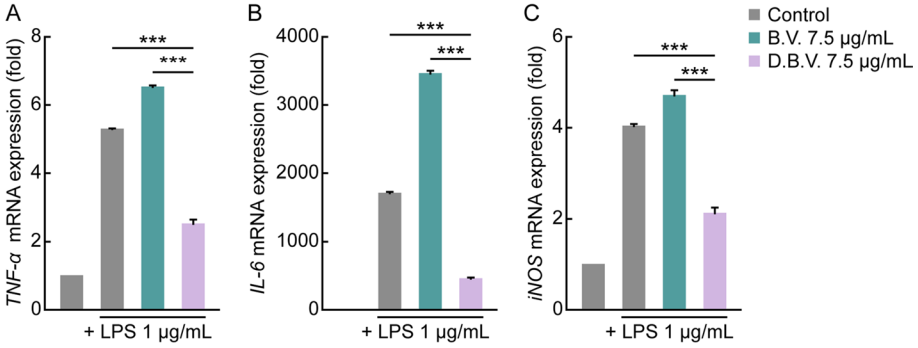

Fig. 4 Comparison of anti-inflammatory activity between bee venom and detoxified bee venom. RAW 264.7 cells were pretreated with 7.5 μg/mL bee venom (B.V.) or detoxified bee venom (D.B.V) for 24 h and then stimulated with LPS (1 μg/mL) for 6 h. qRT-PCR was performed to analyze expression of the pro-inflammatory cytokine mRNAs *TNF-α* (**A**), *IL-6* (**B**), and *iNOS* (**C**). Results are expressed as means ± SD ($n = 3$). ***$p < 0.001$

When the inflammatory reaction was induced by LPS in RAW 264.7 cells grown in the culture medium without treatment, the level of IκBα phosphorylation increased. In turn, the expression of phospho-IκBα was similar when the inflammatory reaction was induced through LPS in RAW 264.7 cells grown in the 7.5 μg/mL of bee venom–treated culture medium and in the culture medium without treatment. Conversely, when the inflammatory reaction was induced through LPS in RAW 264.7 cells grown in 7.5 μg/mL of detoxified bee venom–treated culture medium, the expression level of phospho-IκBα was significantly reduced. Therefore, it was confirmed that detoxification of bee venom greatly inhibited the phosphorylation of IκBα induced by LPS in RAW 264.7 cells (Fig. 5). Hence, it was shown that the detoxified bee venom inhibited IκBα phosphorylation and thus exhibited great anti-inflammatory activity in RAW 264.7 cells.

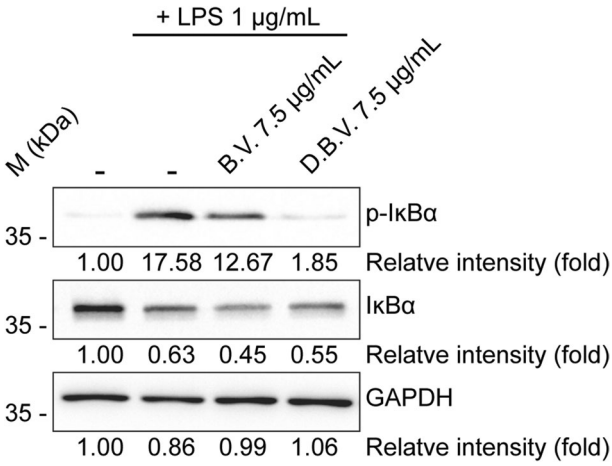

Fig. 5 Comparison of the activity of IκBα phosphorylation inhibition between bee venom and detoxified bee venom in RAW 264.7 cells. RAW 264.7 cells were pretreated with 7.5 μg/mL bee venom (B.V.) or detoxified bee venom (D.B.V.) for 24 h and then stimulated with LPS (1 μg/mL) for 1 h. The cell extracts were subjected to immunoblotting with anti-IκBα and anti-phospho-IκBα antibodies. GAPDH was used as a loading control

Comparison of the Effects of Detoxified Bee Venom and Bee Venom on β-Hexosaminidase Release

Because bee venom is a well-known allergen, it is not safe to use despite its excellent pharmacological effects [45]. When the immune system is exposed to an allergen, activated mast cells induce degranulation and secrete inflammatory mediators such as histamine, cytokines, chemokines, and β-hexosaminidase, which are present in the granules, to the outside of the cells [46]. This secretion induces symptoms such as hives, angioedema, and anaphylaxis at the secretion site, leading to allergic reactions. Therefore, proper control of mast cell degranulation can be an effective method for treating allergic reactions [47]. To confirm the allergic reaction against detoxified bee venom, the release of β-hexosaminidase was examined after treatment of RBL-2H3 cells with detoxified bee venom. In order to confirm whether the degranulation reaction occurred properly, the cells were treated with 10 μg/mL of compound 48/80, which is an oligomeric mixture of condensation products of N-methyl-p-methoxyphenethylamine and formaldehyde and it is known to promote mast cell degranulation, as a positive control, and the amount of β-hexosaminidase released was measured. It was found that a higher amount of β-hexosaminidase was released in cells treated with bee venom compared to that in cells treated with detoxified bee venom at both concentrations of 3.75 μg/mL and 7.5 μg/mL (Fig. 6). From this result, it was confirmed that detoxified bee venom in RBL-2H3 cells did not induce allergies compared to bee venom, and thus, toxicity was eliminated through the detoxification process.

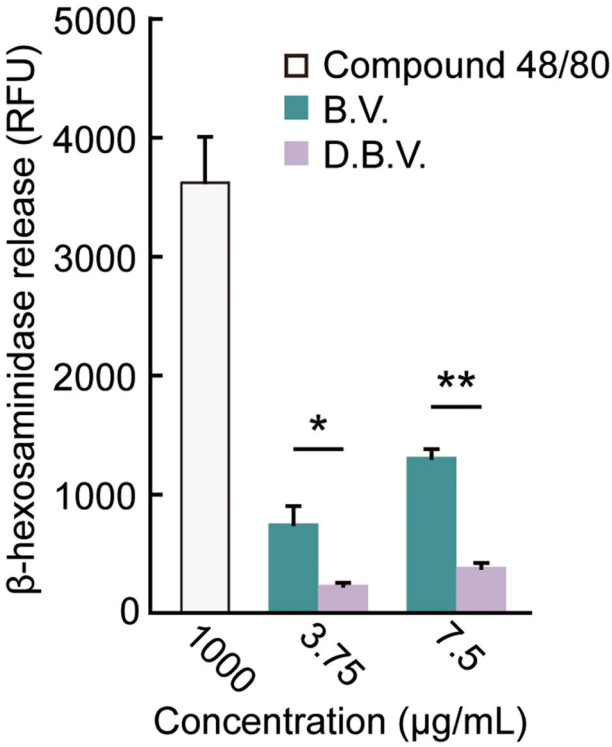

Fig. 6 Comparison of degranulation activity between bee venom and detoxified bee venom in RBL-2H3 cells. RBL-2H3 cells were treated with indicated concentrations of compound 48/80, bee venom (B.V.), or detoxified bee venom (D.B.V.) for 1 h. Compound 48/80 was used as a positive control for degranulation. Release of β-hexosaminidase was measured by fluorometric analysis. Results are expressed as means ± SD ($n = 3$). *$p < 0.05$, **$p < 0.01$

Discussion

Bee venom has a strong pharmacological effect, but at the same time, it has strong toxicity and may cause serious side effects depending on the individual [48, 49]. Typical side effects include hyperventilation, fatigue, loss of appetite, strong pain, vomiting, and anaphylaxis [14, 15]. In severe cases, death may occur. According to a study, an average of 29% of patients experienced side effects after treatment [16]. Therefore, it is important to reduce the side effects of bee venom. In this study, detoxified bee venom was manufactured to reduce side effects and maintain pharmacological effects such as anti-inflammatory activity by hydrolyzing melittin and adjusting the ratio of constituents in bee venom. During the initiation of the inflammatory reaction, blood vessels are expanded by various inflammatory mediators, blood flow is increased, capillaries are expanded, and the permeability of blood vessels is increased, so that plasma and immune cells can gather at the infected site. The inflammatory mediators include kinin, complement proteins, blood coagulation substances, histamine, pro-inflammatory cytokines, NO, and ROS. ROS are known to mediate many inflammatory diseases [50]. In this study, the antioxidant activity of detoxified bee venom was evaluated using the DPPH assay, and it was confirmed that detoxified bee venom has strong antioxidant activity compared to the positive control resveratrol, which has excellent antioxidant effects. And, to confirm that the cytotoxicity of detoxified bee venom was reduced, the cytotoxicity of detoxified bee venom and bee venom was compared using the MTS assay. In both MCF 10A and RAW 264.7 cells, bee venom showed cytotoxicity at a concentration of 15 μg/mL, whereas detoxified bee venom showed no cytotoxicity even at a concentration of 60 μg/mL or more. It was found that cytotoxicity was greatly reduced. In addition, after treatment with bee venom and detoxified bee venom at the same concentration in RAW 264.7 cells for 24 h, the expression levels of the representative pro-inflammatory cytokine mRNAs *TNF-α*, *IL-6*, and *iNOS* were compared. The mRNA expression levels of *TNF-α*, *IL-6*, and *iNOS* did not change in RAW 264.7 cells treated with bee venom after LPS-induced inflammatory reaction compared to the control group. In RAW 264.7 cells treated with detoxified bee venom, the mRNA expression levels of *TNF-α*, *IL-6*, and *iNOS* were significantly decreased compared to the control group, confirming the strong anti-inflammatory activity of detoxified bee venom. To further verify its anti-inflammatory activity, phosphorylation of IκBα, a component of the NF-κB pathway involved in the regulation of inflammatory reactions, was confirmed by immunoblotting analysis. After treating RAW 264.7 cells with bee venom and detoxified bee venom at the same concentration for 24 h, the phosphorylation of IκBα was confirmed after inducing an inflammatory reaction with LPS. As a result, the level of phosphorylation of IκBα in RAW 264.7 cells treated with bee venom was similar to that in the control group, but the phosphorylation of IκBα was significantly reduced in RAW 264.7 cells treated with detoxified bee venom. And degranulation was confirmed in RBL-2H3 cells to verify that the detoxified bee venom, which has strong anti-inflammatory activity, had reduced allergenicity. The degranulation reaction was confirmed by measuring the release of β-hexosaminidase, which is formed when mast cells are degranulated. The amount of β-hexosaminidase in RBL 2H3 cells treated with detoxified bee venom was found to be very low compared to the amount of β-hexosaminidase in RBL- 2H3 cells treated with bee venom. In this study, it was confirmed that detoxification of bee venom reduced the cytotoxicity and allergenicity of bee venom while maintaining its inherent anti-inflammatory activity. Thus, the pharmacological efficacy of bee venom was maintained, while the risk of side

effects was reduced, which is expected to further increase the possibility of using bee venom for medicinal applications.

Conclusion

Taken together, the results of this study suggest that detoxified bee venom has a strong antioxidant effect and significantly reduced cytotoxicity. In addition, compared to bee venom, non-toxic bee venom strongly inhibits mRNA expression of *TNF-α*, *IL-6*, and *iNOS* and inhibits phosphorylation of IκBα in RAW 264.7 cells, as well as degranulation in RBL-2H3 cells. The results of this study show that through our detoxification process, bee venom's toxicity can be significantly reduced and its efficacy is maintained. Accordingly, the side effects of bee venom are greatly reduced, which is expected to be of great help in medical use of detoxified bee venom.

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Author Contribution HL and KL performed research and wrote the paper. YK, CL, and KK designed the study and wrote the paper. YK, CL, and KK designed the study, provided funding, and edited the paper. HL and KK analyzed the data and edited the paper.

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Data Availability The data is included in the article. If you need further information, please contact the corresponding author.

Declarations

Ethics Approval and Consent to Participate No conflicts, informed consent, and human or animal rights applicable.

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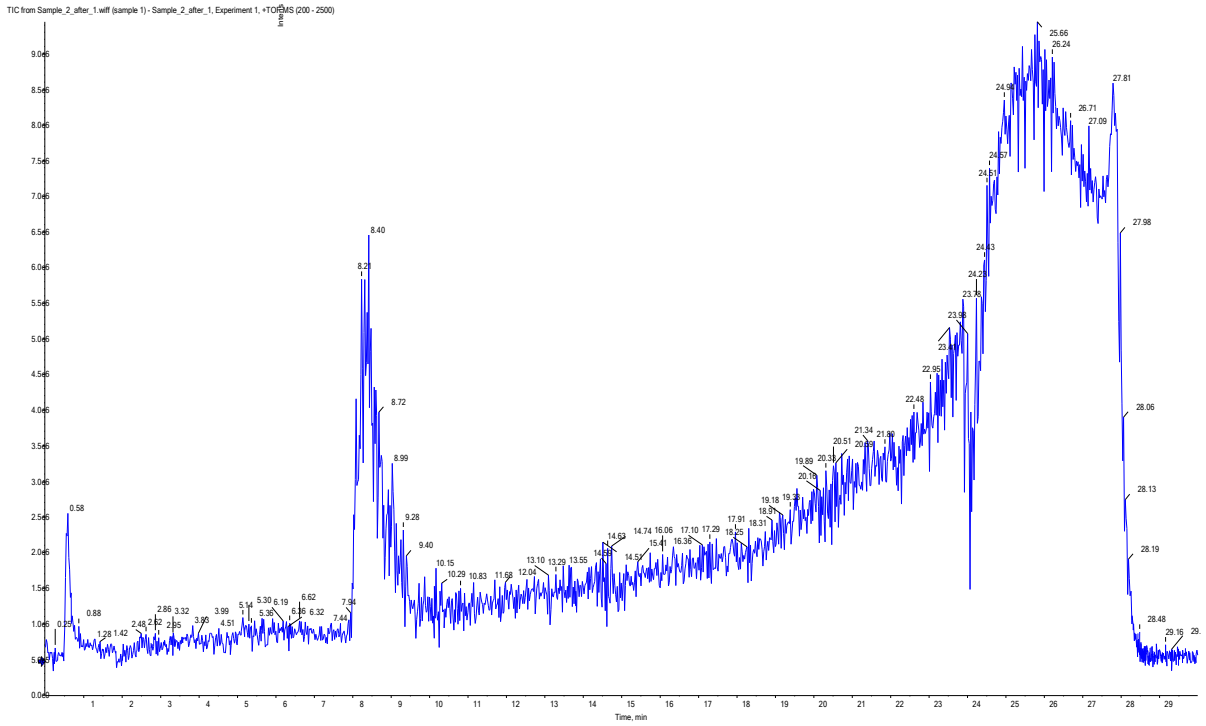

Supplementary Figure 1. Total ion chromatogram of detoxified peptide mixture of bee venom by LC/MS. The X-axis represents retention time and the Y-axis represents intensity of detected ions.

Supplementary Table 1. Peptides identified in detoxified bee venom.

% Cov	Names	Conf	Sequence	Modification	Prec MW	Prec m/z	Theor MW	Theor m/z	Theor z	Time	amino
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	97.28	TTGLPALISWIKRKRQQ	Amidated@C-term	1994.183	665.7349	1994.19	665.7373	3	10.1733	17
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	83.17	TTGLPALISWIKRKRQQ	Amidated@C-term	1994.18	665.734	1994.19	665.7373	3	10.1823	17
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TTGLPALISWIKRKRQQ	Amidated@C-term	1994.194	499.5558	1994.19	499.5548	4	10.2068	17
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	97.99	TTGLPALISWIKRKRQQ	Amidated@C-term	1994.183	665.7349	1994.19	665.7373	3	10.2153	17
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TTGLPALISWIKRKRQQ	Amidated@C-term	1994.187	499.554	1994.19	499.5548	4	10.242	17
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TTGLPALISWIKRKRQQ	Amidated@C-term	1994.187	499.5541	1994.19	499.5548	4	10.2573	17
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TTGLPALISWIKRKRQQ	Amidated@C-term	1994.184	665.7354	1994.19	665.7373	3	10.2853	17
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TTGLPALISWIKRKRQQ	Amidated@C-term	1994.179	665.7337	1994.19	665.7373	3	10.2871	17
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TTGLPALISWIKRKRQQ	Amidated@C-term	1994.188	499.5542	1994.19	499.5548	4	10.2997	17
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TTGLPALISWIKRKRQQ	Amidated@C-term	1994.179	665.7337	1994.19	665.7373	3	10.324	17
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TTGLPALISWIKRKRQQ	Amidated@C-term	1994.184	665.7354	1994.19	665.7373	3	10.3323	17
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TTGLPALISWIKRKRQQ	Amidated@C-term	1994.183	665.7349	1994.19	665.7373	3	10.342	17
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TTGLPALISWIKRKRQQ	Amidated@C-term	1994.187	499.554	1994.19	499.5548	4	10.366	17
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TTGLPALISWIKRKRQQ	Amidated@C-term	1994.184	665.7354	1994.19	665.7373	3	10.3743	17
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TTGLPALISWIKRKRQQ	Amidated@C-term	1994.18	665.7339	1994.19	665.7373	3	10.402	17
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TTGLPALISWIKRKRQQ	Amidated@C-term	1994.18	665.734	1994.19	665.7373	3	10.419	17
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TTGLPALISWIKRKRQQ	Amidated@C-term	1994.179	665.7337	1994.19	665.7373	3	10.4343	17
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	97.7	TTGLPALISWIKRKRQQ	Amidated@C-term	1994.18	665.7339	1994.19	665.7373	3	10.4458	17
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TTGLPALISWIKRKRQQ	Amidated@C-term	1994.18	665.734	1994.19	665.7373	3	10.461	17
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TTGLPALISWIKRKRQQ	Amidated@C-term	1994.18	665.7339	1994.19	665.7373	3	10.4895	17
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TTGLPALISWIKRKRQQ	Amidated@C-term	1994.18	665.734	1994.19	665.7373	3	10.5245	17
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	97.38	TTGLPALISWIKRKRQQ	Amidated@C-term	1994.183	499.553	1994.19	499.5548	4	10.5569	17
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TTGLPALISWIKRKRQQ	Amidated@C-term	1994.176	998.0954	1994.19	998.1024	2	10.6061	17
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TTGLPALISWIKRKRQQ	Amidated@C-term	1994.185	998.1	1994.19	998.1024	2	10.6088	17
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TTGLPALISWIKRKRQQ	Amidated@C-term	1994.18	665.7338	1994.19	665.7373	3	10.616	17
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TTGLPALISWIKRKRQQ	Amidated@C-term	1994.187	499.554	1994.19	499.5548	4	10.6671	17
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	83.47	TTGLPALISWIKRKRQQ	Amidated@C-term	1994.183	665.7349	1994.19	665.7373	3	10.7171	17
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	82.96	TGLPALISWIKRKRQQ	Amidated@C-term	1893.139	632.0535	1893.142	632.0547	3	9.839	16
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TGLPALISWIKRKRQQ	Amidated@C-term	1893.139	632.0535	1893.142	632.0547	3	9.8807	16
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TGLPALISWIKRKRQQ	Amidated@C-term	1893.139	632.0536	1893.142	632.0547	3	9.8883	16
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TGLPALISWIKRKRQQ	Amidated@C-term	1893.135	947.575	1893.142	947.5785	2	9.8902	16
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TGLPALISWIKRKRQQ	Amidated@C-term	1893.144	474.2932	1893.142	474.2929	4	9.9058	16
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TGLPALISWIKRKRQQ	Amidated@C-term	1893.135	474.2909	1893.142	474.2929	4	9.911	16
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TGLPALISWIKRKRQQ	Amidated@C-term	1893.139	632.0535	1893.142	632.0547	3	9.9224	16
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TGLPALISWIKRKRQQ	Amidated@C-term	1893.133	632.0515	1893.142	632.0547	3	9.9318	16
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TGLPALISWIKRKRQQ	Amidated@C-term	1893.139	632.0536	1893.142	632.0547	3	9.9505	16
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	49.2	TGLPALISWIKRKRQQ	Amidated@C-term	1893.13	947.5723	1893.142	947.5785	2	9.9638	16
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TGLPALISWIKRKRQQ	Amidated@C-term	1893.133	632.0515	1893.142	632.0547	3	9.967	16

63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TGLPALISWIKRKRQQ	Amidated@C-term	1893.132	947.5731	1893.142	947.5785	2	9.9943	16
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TGLPALISWIKRKRQQ	Amidated@C-term	1893.139	632.0535	1893.142	632.0547	3	10.0055	16
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TGLPALISWIKRKRQQ	Amidated@C-term	1893.136	947.5754	1893.142	947.5785	2	10.0153	16
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TGLPALISWIKRKRQQ	Amidated@C-term	1893.133	632.0516	1893.142	632.0547	3	10.0361	16
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	97.56	TGLPALISWIKRKRQQ	Amidated@C-term	1893.133	632.0517	1893.142	632.0547	3	10.0457	16
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TGLPALISWIKRKRQQ	Amidated@C-term	1893.133	632.0515	1893.142	632.0547	3	10.0573	16
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TGLPALISWIKRKRQQ	Amidated@C-term	1893.132	947.5731	1893.142	947.5785	2	10.0778	16
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	84.43	TGLPALISWIKRKRQQ	Amidated@C-term	1893.133	632.0517	1893.142	632.0547	3	10.0877	16
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TGLPALISWIKRKRQQ	Amidated@C-term	1893.136	947.5754	1893.142	947.5785	2	10.0993	16
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TGLPALISWIKRKRQQ	Amidated@C-term	1893.132	947.5731	1893.142	947.5785	2	10.1196	16
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TGLPALISWIKRKRQQ	Amidated@C-term	1893.135	632.0523	1893.142	632.0547	3	10.1315	16
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	97.86	TGLPALISWIKRKRQQ	Amidated@C-term	1893.135	632.0523	1893.142	632.0547	3	10.1438	16
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	84.32	TGLPALISWIKRKRQQ	Amidated@C-term	1893.144	474.2932	1893.142	474.2929	4	10.1633	16
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	98.15	TGLPALISWIKRKRQQ	Amidated@C-term	1893.135	632.0523	1893.142	632.0547	3	10.1858	16
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TGLPALISWIKRKRQQ	Amidated@C-term	1893.134	632.052	1893.142	632.0547	3	10.2033	16
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	97.87	TGLPALISWIKRKRQQ	Amidated@C-term	1893.135	632.0523	1893.142	632.0547	3	10.2487	16
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TGLPALISWIKRKRQQ	Amidated@C-term	1893.143	474.2931	1893.142	474.2929	4	10.2661	16
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TGLPALISWIKRKRQQ	Amidated@C-term	1893.135	947.575	1893.142	947.5785	2	10.2785	16
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TGLPALISWIKRKRQQ	Amidated@C-term	1893.136	947.5752	1893.142	947.5785	2	10.2903	16
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TGLPALISWIKRKRQQ	Amidated@C-term	1893.132	947.5731	1893.142	947.5785	2	10.329	16
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TGLPALISWIKRKRQQ	Amidated@C-term	1893.135	632.0522	1893.142	632.0547	3	10.329	16
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TGLPALISWIKRKRQQ	Amidated@C-term	1893.135	632.0523	1893.142	632.0547	3	10.3582	16
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	TGLPALISWIKRKRQQ	Amidated@C-term	1893.13	947.5725	1893.142	947.5785	2	10.4053	16
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	83.02	TGLPALISWIKRKRQQ	Amidated@C-term	1893.134	632.052	1893.142	632.0547	3	10.4123	16
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	95.14	LISWIKRKRQQ	Amidated@C-term	1453.893	727.9537	1453.899	727.957	2	4.9914	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9547	1453.899	727.957	2	5.0209	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.893	727.9537	1453.899	727.957	2	5.0371	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	64.97	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9531	1453.899	727.957	2	5.0506	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	66.83	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9547	1453.899	727.957	2	5.0634	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.893	727.9537	1453.899	727.957	2	5.0799	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9534	1453.899	727.957	2	5.0931	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	93.8	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9547	1453.899	727.957	2	5.1056	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9546	1453.899	727.957	2	5.1388	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	72.34	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9547	1453.899	727.957	2	5.1479	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.893	727.9537	1453.899	727.957	2	5.1644	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9547	1453.899	727.957	2	5.1806	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	93	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9546	1453.899	727.957	2	5.1813	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.893	727.9537	1453.899	727.957	2	5.2069	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	63.75	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9546	1453.899	727.957	2	5.2231	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	96.25	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9548	1453.899	727.957	2	5.2361	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	93.61	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9546	1453.899	727.957	2	5.2721	11

63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9547	1453.899	727.957	2	5.291	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9533	1453.899	727.957	2	5.3144	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9548	1453.899	727.957	2	5.3236	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	95.66	LISWIKRKRQQ	Amidated@C-term	1453.893	727.9538	1453.899	727.957	2	5.3364	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9533	1453.899	727.957	2	5.3566	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9535	1453.899	727.957	2	5.3659	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9549	1453.899	727.957	2	5.3787	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	93.64	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9535	1453.899	727.957	2	5.4086	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	92.92	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9549	1453.899	727.957	2	5.4207	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	64.43	LISWIKRKRQQ	Amidated@C-term	1453.893	727.9536	1453.899	727.957	2	5.4439	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9535	1453.899	727.957	2	5.4509	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9549	1453.899	727.957	2	5.4592	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	98.99	LISWIKRKRQQ	Amidated@C-term	1453.893	727.9536	1453.899	727.957	2	5.4859	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9546	1453.899	727.957	2	5.4932	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	97.11	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9549	1453.899	727.957	2	5.5077	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9546	1453.899	727.957	2	5.5252	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9548	1453.899	727.957	2	5.55	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9547	1453.899	727.957	2	5.5706	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9548	1453.899	727.957	2	5.5922	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	94.56	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9531	1453.899	727.957	2	5.6127	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	23.4	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9547	1453.899	727.957	2	5.6131	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9535	1453.899	727.957	2	5.6404	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9547	1453.899	727.957	2	5.6582	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	70.08	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9535	1453.899	727.957	2	5.6825	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9547	1453.899	727.957	2	5.7006	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9535	1453.899	727.957	2	5.7247	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9547	1453.899	727.957	2	5.7422	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9547	1453.899	727.957	2	5.7669	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	93.68	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9547	1453.899	727.957	2	5.7842	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9547	1453.899	727.957	2	5.8095	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	20.31	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9534	1453.899	727.957	2	5.8266	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.893	727.9537	1453.899	727.957	2	5.8401	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9547	1453.899	727.957	2	5.8522	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	94.97	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9534	1453.899	727.957	2	5.869	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.893	727.9537	1453.899	727.957	2	5.8824	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9547	1453.899	727.957	2	5.8882	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9534	1453.899	727.957	2	5.9115	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	26.78	LISWIKRKRQQ	Amidated@C-term	1453.893	727.9537	1453.899	727.957	2	5.9182	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9547	1453.899	727.957	2	5.9375	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9534	1453.899	727.957	2	5.9539	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.893	727.9537	1453.899	727.957	2	5.9667	11

63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9547	1453.899	727.957	2	5.9802	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9534	1453.899	727.957	2	5.996	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.893	727.9537	1453.899	727.957	2	6.0089	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9547	1453.899	727.957	2	6.0228	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.893	727.9537	1453.899	727.957	2	6.051	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	93.45	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9535	1453.899	727.957	2	6.0807	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9535	1453.899	727.957	2	6.1234	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	92.98	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9534	1453.899	727.957	2	6.1502	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	95.63	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9535	1453.899	727.957	2	6.1652	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	93.04	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9532	1453.899	727.957	2	6.1774	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	23.28	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9534	1453.899	727.957	2	6.1928	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	95.82	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9535	1453.899	727.957	2	6.2075	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	22.8	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9532	1453.899	727.957	2	6.2177	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9534	1453.899	727.957	2	6.2353	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	21.98	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9532	1453.899	727.957	2	6.25	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9532	1453.899	727.957	2	6.2924	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9532	1453.899	727.957	2	6.304	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	97.11	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9532	1453.899	727.957	2	6.3342	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	68.05	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9549	1453.899	727.957	2	6.3622	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	17.67	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9535	1453.899	727.957	2	6.367	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	94.47	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9532	1453.899	727.957	2	6.3765	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9549	1453.899	727.957	2	6.4047	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	92.71	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9535	1453.899	727.957	2	6.4092	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	64.47	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9532	1453.899	727.957	2	6.4224	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9549	1453.899	727.957	2	6.447	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	95	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9532	1453.899	727.957	2	6.4647	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9535	1453.899	727.957	2	6.473	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	94.18	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9549	1453.899	727.957	2	6.4893	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	93.03	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9535	1453.899	727.957	2	6.5157	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	95.2	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9535	1453.899	727.957	2	6.535	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	73.5	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9534	1453.899	727.957	2	6.5495	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9535	1453.899	727.957	2	6.5577	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	19.64	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9547	1453.899	727.957	2	6.5986	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	93.67	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9533	1453.899	727.957	2	6.6125	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	59.39	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9533	1453.899	727.957	2	6.6553	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9534	1453.899	727.957	2	6.6879	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	67.3	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9533	1453.899	727.957	2	6.6978	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.895	727.9547	1453.899	727.957	2	6.7081	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	71.86	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9533	1453.899	727.957	2	6.7304	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	21.43	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9533	1453.899	727.957	2	6.7403	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	93.82	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9533	1453.899	727.957	2	6.7727	11

63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9533	1453.899	727.957	2	6.7828	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	70.25	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9532	1453.899	727.957	2	6.8358	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	93.7	LISWIKRKRQQ	Amidated@C-term	1453.892	727.9534	1453.899	727.957	2	6.8462	11
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	45	GIGAVLKVL		868.5721	869.5794	868.5746	869.5818	1	9.659	9
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	GIGAVLKVL		868.5749	435.2947	868.5746	435.2946	2	9.6941	9
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	GIGAVLKVL		868.5755	435.295	868.5746	435.2946	2	9.7029	9
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	76.88	GIGAVLKVL		868.5653	869.5726	868.5746	869.5818	1	9.703	9
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	GIGAVLKVL		868.5755	435.295	868.5746	435.2946	2	9.738	9
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	82.56	GIGAVLKVL		868.5676	869.5749	868.5746	869.5818	1	9.7435	9
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	71.02	GIGAVLKVL		868.5736	869.5809	868.5746	869.5818	1	9.7557	9
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	GIGAVLKVL		868.5755	435.295	868.5746	435.2946	2	9.7812	9
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	44.58	GIGAVLKVL		868.5714	869.5786	868.5746	869.5818	1	9.7972	9
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	87.33	GIGAVLKVL		868.5714	869.5786	868.5746	869.5818	1	9.8245	9
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	92.46	GIGAVLKVL		868.5755	435.295	868.5746	435.2946	2	9.8277	9
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	GIGAVLKVL		868.5759	435.2952	868.5746	435.2946	2	9.8357	9
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	41.04	GIGAVLKVL		868.5678	869.5751	868.5746	869.5818	1	9.8485	9
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	GIGAVLKVL		868.5759	435.2952	868.5746	435.2946	2	9.874	9
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	GIGAVLKVL		868.5782	435.2964	868.5746	435.2946	2	9.88	9
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	54.06	GIGAVLKVL		868.5643	869.5716	868.5746	869.5818	1	9.8868	9
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	GIGAVLKVL		868.5746	435.2946	868.5746	435.2946	2	9.9173	9
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	72	GIGAVLKVL		868.5755	435.295	868.5746	435.2946	2	9.9202	9
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	99	GIGAVLKVL		868.5782	435.2964	868.5746	435.2946	2	9.9248	9
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	89.63	GIGAVLKVL		868.564	869.5712	868.5746	869.5818	1	9.9711	9
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	84.61	GIGAVLKVL		868.5703	869.5776	868.5746	869.5818	1	9.9737	9
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	33.96	WIKRKRQQ	Amidated@C-term	1140.694	571.3541	1140.699	571.3569	2	0.8415	8
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	33.22	WIKRKRQQ	Amidated@C-term	1140.694	571.3544	1140.699	571.3569	2	0.8598	8
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	33.39	WIKRKRQQ	Amidated@C-term	1140.694	571.3543	1140.699	571.3569	2	1.078	8
63.64	RecName: Full=Melittin; Short=MEL; Short=MLT; Flags: Precursor	30.19	WIKRKRQQ	Amidated@C-term	1140.694	571.3544	1140.699	571.3569	2	1.2258	8