



Identification of a novel melittin isoform from Africanized *Apis mellifera* venom

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ABSTRACT

Apis mellifera, the European honey bee, is perhaps the most studied insect in the *Apidae* family. Its venom is comprised basically of melittin, phospholipase A₂, histamine, hyaluronidase, catecholamines and serotonin. Some of these components have been associated to allergic reactions, among several other symptoms. On the other hand, bee mass-stinging is increasingly becoming a serious public health issue; therefore, the development of efficient serum-therapies has become necessary, with a consequent better characterization of the venom. In this work, we report the isolation and biochemical characterization of melittin-S, an isoform of melittin comprising a Ser residue at the 10th position, from the venom of Africanized *A. mellifera*. This peptide demonstrated to be less hemolytic than melittin and to adopt a less organized secondary structure, as assessed by circular dichroism spectroscopy. Melittin-S venom contents varied seasonally, and the maximum secretion occurred during the (southern) winter months. Data on the variation of the honey bee venom composition are necessary to guide future immunological studies, aiming for the development of an efficient anti-serum against Africanized *A. mellifera* venom and, consequently, an effective treatment for the victims of mass-stinging.

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1. Introduction

The European honey bee, *Apis mellifera*, is one of the most studied insects [20,21,30,41]. As a consequence, its venom is probably the best characterized venom in the *Apidae* family, which includes both social and solitary species of bees and bumblebees. Among the primary and most important functions of the venom is the defense of the colony, mainly against insects, including other Hymenoptera, such as ants, wasps, bumblebees and bees from other colonies, to which the venom is generally fatal, but also against larger animals such as mammals.

The main component, ~50% by dry weight, in honey bee venom is melittin [15]. The active 26 amino acid peptide is released from its precursor, promelittin, during biosynthesis and presents a hemolytic activity, other than being the primary allergen in bee venom [9]. It is an amphiphilic peptide, with a predominantly hydrophobic N-terminal region and a hydrophilic C-terminal region, with the characteristic structure of membrane

bound cytolytic, channel-forming peptides and trans-membrane protein helices [8,32]. It acts synergistically [29,38] with phospholipase A₂ (PLA₂), being this enzyme correspondent to circa 11% of the venom dry weight [23].

It has been shown that the levels of different honey bee venom components, such as histamine [24], hyaluronidase [23], dopamine and noradrenaline [25], 5-hydroxytryptamine (5-HT) [28] and phospholipase A₂ [27] may change with the age of the individuals or the season of the year [6,24]. Specifically, Owen and Bridges [25] showed that melittin content changes as the summer progresses.

Toxic bee stinging effects have been mostly associated to allergic reactions; however, massive bee attacks can be toxic per se, e.g., large amounts of venom are inoculated into the victim that can even die as a consequence of the direct action of the toxins (melittin, PLA₂, etc). The initial symptoms from mass envenomation include edema, fatigue, dizziness, nausea, vomiting, fever, and unconsciousness. Response to the release of endogenous histamine includes diarrhea and incontinence. Systemic damage may develop from within 24 h to 6 days, including hemolysis, hemoglobinemia, levels of creatinine phosphatase elevated up to 500-fold and acute tubular necrosis. Other symptoms include muscular aches, cramps, hyperglycemia, and hypertension. Complete recovery require from 3 to 6 weeks, but death can occur within 6 h to 12

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days, generally as a result of renal failure due to breakdown products of hemolysis, myonecrosis, and the consequent renal damage; or from cardiac arrest due to complications of the venom toxicity (for further information see the review of Vetter et al. [37] and the stinging simulations of Francese et al. [7]).

The present work will focus on the biochemical characterization of the seasonal variation of the major components of the Africanized honey bee (AHB) venom. AHB was introduced in Brazil in 1956, in an attempt to find a subspecies of *A. mellifera* that was more adapted to a tropical climate. In 1957, 26 swarms escaped and started spreading through South and Central Americas [20] and have become a serious public health issue. Molecular homogeneity is a crucial requirement for the development of efficient serum-therapies, employed in massive-attack cases. In this work, we have analyzed the venom composition of one specific hive for 1 year and have been able to identify both a seasonal variation in the contents of the venom and the presence of a novel melittin isoform secreted only during the southern winter. These data will serve as guidance for subsequent immunology studies.

2. Material and methods

2.1. Reagents

All the employed reagents were of analytical grade and were purchased from Sigma Co. (St. Louis, MO, USA), unless otherwise stated.

2.2. Bees

Africanized honey bee (*A. mellifera*) workers, aging 30–40 days, were obtained from a specific hive at the Apiary of Botucatu School of Veterinary Medicine and Animal Husbandry – UNESP (Brazil) once a month every 3rd week of the month, from October 2007 to September 2008.

2.2.1. Venom obtainment by means of electric stimulation

Venom was collected according to a slight modification of the Benton et al. protocol [3]. Briefly, a wired glass plated was placed in the entrance of the hive so that the homing bee had to land on the plate in order to gain access to the interior of the colony. The wires are electrified and apply mild shocks to the bees that respond by stinging the surface on which they are walking. Once it is unlikely that a bee will eject all the contents of its venom sac in one single attack, successive stinging occurs, typically 10 times, yielding approximately 1 μ L per attack. The venom dries rapidly on the glass plate and can be scraped off and processed as described above. Approximately 100 bees were allowed to land on the plate.

2.3. Chromatographic analyses

2.3.1. RP-HPLC profiling and purification

A reversed-phase binary HPLC system (20A Prominence, Shimadzu Co., Japan) was used for sample profiling and separation. The lyophilized crude venom powder was solubilized into 0.1% trifluoroacetic acid (TFA) and the 12-month collections were normalized to 1 mg mL⁻¹ solutions, based on sample mass. These solutions were centrifuged and the supernatant was separated for subsequent chromatographic analyses. Twenty-microliter aliquots were loaded in an ACE C8 column (ACE 3 μ m, C8, 300 Å, 100 mm $\times$ 2.1 mm) in a two-solvent system: (A) trifluoroacetic acid (TFA)/H₂O (1:1000) and (B) TFA/acetonitrile (ACN)/H₂O (1:900:100). The column was eluted at a constant flow rate of 0.2 mL min⁻¹ with a 10–100% gradient of solvent B over 31 min,

after a 5 min isocratic elution with 10% B. The HPLC column eluates were monitored by a Shimadzu SPD-M20A PDA detector scanning from 200 to 500 nm (1 nm steps). Background subtraction was performed for chromatogram superimposition and integration.

2.4. Mass spectrometry

The mass spectrometry analyses were performed in an ESI mass spectrometer (LCQ DuoTM, ThermoFinnigan, USA), equipped with a nanospray source and connected to nanoHPLC system (UltiMate HPLC System, LC Packings, Dionex, USA). The samples were diluted in a 5% ACN containing 0.2% formic acid, and were introduced in the spectrometer at a flow rate of 1 μ L min⁻¹. The spray voltage was kept at 1.8 kV, the capillary voltage at 46 V, the capillary temperature at 180 °C and the tube lens offset was kept at –5 V. MS spectra were collected in the 50–2000 *m/z* range. Instrument control, data acquisition and data processing were performed with the Xcalibur Suite.

2.5. Peptide sequencing

Complete peptide sequencing was performed by Edman degradation using a Shimadzu PPSQ-21 automated protein sequencer, following the manufacturer's standard instructions. Amino acid analyses were performed to quantify the different peptide batches used throughout this work.

2.6. Circular dichroism (CD)

CD measurements were carried out on a J-810 Circular Dichroism Spectropolarimeter (Jasco Inc.) coupled to a Peltier Jasco PFD-425S system for temperature control. Far-UV spectra were recorded in the spectral range 181–260 nm and averaged over 10 scans at 20 °C, using a 1-mm path-length quartz cell, 0.5 nm step resolution, 50 nm/min speed, 8 s response time, and 1 nm bandwidth. Spectral correction was performed by subtracting the blank. Measured ellipticity θ (mdegree) was converted to molar ellipticity $[\theta]$ (degree cm² dmol⁻¹) for structural calculations performed by CDSSTR algorithm [4,19,34] hosted at Dichroweb website (<http://dichroweb.cryst.bbk.ac.uk/html/home.shtml>) [17,39,40].

2.7. Biological assays

2.7.1. Hemolysis

The hemolytic assay was performed following the method described by Al-Badri et al. [1], with some modifications. Erythrocytes were obtained by centrifuging fresh whole blood (3000 rpm, 10 min), to remove plasma and white blood cells. One mL of red blood cells (RBC) was diluted with 9 mL of PBS buffer (10 mM phosphate buffer, pH 7 and 150 mM NaCl). The suspension was further diluted to give a RBC 4%. Melittin and melittin-S (3 μ M in 50 μ L, diluted in the assay buffer) were incubated with RBC 4% (20 μ L) and PBS buffer (50 μ L) at 37 °C for 1 h. The tube was centrifuged at 4000 rpm for 5 min and 60 μ L of supernatant were transferred to 96-well plate, and the hemoglobin released was measured at 414 nm. Data were compared with negative control (100 μ L PBS buffer and 20 μ L RBC 4%) and a positive control (50 μ L PBS buffer, 50 μ L Triton X-100 0.1% and 20 μ L RBC 4%). Experiments were performed in triplicate. Percent hemolysis was calculated as follows: (%) = $(A_{\text{sample}} - A_{\text{buffer}}) / (A_{\text{triton}} - A_{\text{buffer}}) \times 100$.

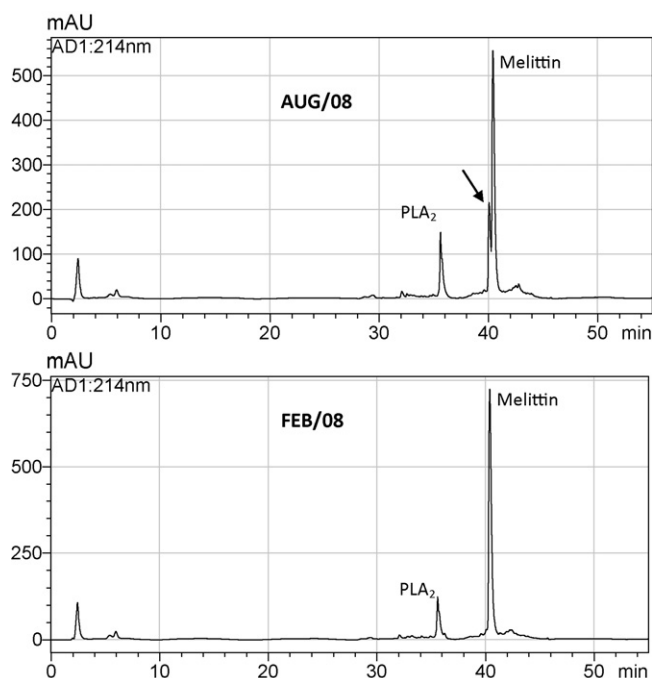


Fig. 1. Representative RP-HPLC profiles ($\lambda = 214$ nm) of Africanized *A. mellifera* venoms obtained by electrical stimulation. Melittin and phospholipase A₂ are annotated and the arrow denotes the peak varying according to the months.

2.8. Data fitting, statistical analyses and sequence alignment

When data fitting was performed, results are presented as the calculated value $\pm$ standard deviation (S.D.). Otherwise; data correspond to the mean of three individual experiments. Pep-

tide sequence alignment was performed using ClustalW software [16].

2.9. Molecular modeling and visualization

Molecular modeling was performed with SWISS-MODEL [2,10,33] and the figure was generated with Swiss-PdbViewer [10] (both features available at <http://www.expasy.org/>). Briefly and according to the SWISS-MODEL documentation, the homology modeling builds the three-dimensional protein structure models using experimentally determined structures of related family members as templates, on the basis of a sequence alignment between the target protein and the template structure. Besides, there are model quality assessment tools that estimate the reliability of the resulting models.

3. Results

The monthly venom solutions were normalized to 1 mg mL^{-1} and $20 \mu\text{L}$ aliquots were analyzed by reversed-phase HPLC, according to Section 2. Background subtracted chromatograms were individually integrated and analyzed, as the representative chromatograms pictured in Fig. 1 show. On average, 100 different peaks could be detected in each month solution, of which less than 10 (the major peaks) would serve for quantitative-based comparison. Moreover, from our previous studies [6], we are aware that the largest peak in the chromatogram corresponds to melittin and the second largest, to PLA₂. However, depending on the month, there was a different second largest peak, as indicated by the arrow in Fig. 1, AUG/08. This peak, together with melittin, were purified and analyzed by MS (Fig. 2) and Edman degradation, and the chemically determined sequence is presented on Table 1. Since it differs from melittin only by a Ser residue at the 10th position, it was termed *melittin-S*.

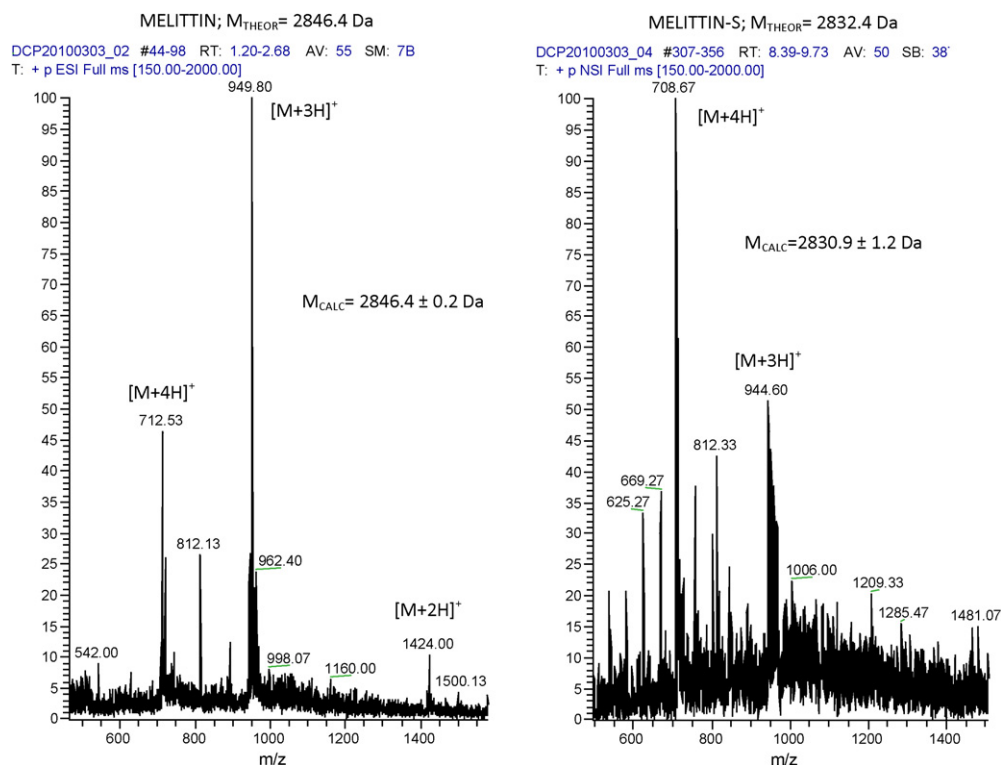


Fig. 2. Mass spectrometry profiles of melittin and melittin-S. The parent ion envelopes for both peptides are annotated. Other evident ions for melittin: $m/z = 812.13$ corresponds to $[y_{13}]^{2+}$; $m/z = 893.20$ corresponds to $[\text{PALISWIK-NH}_3]^+$ and $m/z = 542.00$ corresponds to $[y_4\text{-NH}_3]^+$. For melittin-S: $m/z = 812.33$ corresponds to $[\text{STGLPALIS-28}]^+$; $m/z = 625.27$ is $[\text{LKVLSST-NH}_3]^+$; $m/z = 669.27$ is $[\text{KRKRQ-28}]^+$; $m/z = 757.4$ corresponds to $[\text{ISWIKR-28}]^+$; $m/z = 537.33$ to $[\text{GLPALI-28}]^+$ and $m/z = 581.27$ to $[\text{IGAVLK}]^+$.

Table 1
Bee and wasp melittins alignment.

		↓
Melittin-S	GIGAVLKVL	S TGLPALISWIKRKRQQ
P01501	GIGAVLKVL	T TGLPALISWIKRKRQQ
P01502	GIGAILKVL	S TGLPALISWIKRKRQE
P01504	GIGAILKVL	A TGLPTLISWIKNRKQ
P59261	GIGAVLKVL	A TGLPALISWIKRKRQQ
P59262	GIGAVLKVL	T TGLPALISWIKRKRQQ
P68407	GIGAVLKVL	T TGLPALIGWIKRKRQQ
P68408	GIGAVLKVL	T TGLPALISWIKRKRQQ
P68409	GIGAVLKVL	T TGLPALISWIKRKRQQ
Q8LW54	GIGAVLKVL	T TGLPALISWIKRKRQQ
Q95Z19	GIGAVLKVL	T TGLPALISWIKRKRQQ
Consensus	****:****:****:*.***.***:	
	10	20

Uniprot accession numbers: P01501: *A. mellifera*; P01502: *A. dorsata*; P01504: *A. florea*; P059261: *Polistes hebraeus*; P59262: *Vespula maculifrons*; P68407: *A. cerana cerana*; P68408: *Vespa magnifica*; P68409: *Vespa velutina nigrithorax*; Q8LW54: *A. cerana*; Q95Z19: *Polistes* sp. Consensus sequence as calculated by ClustalW. (*) identity; (:) conserved substitution and (.) semi-conserved substitution, according to [16] and <http://www.ebi.ac.uk/Tools/clustalw2>.

In light of the apparent monthly variation of the novel peptide content, its normalized percent area was plotted as a function of the month of the year, as presented in Fig. 3. As one can see, melittin-S secretion remains constant during the year and suddenly rises during the (southern) winter, and then gets back to its normal values. Data are presented as normalized peak area percent ± S.D.

Next, in order to assess whether this conservative amino acid substitution would arouse any conformation change, CD analyses were performed with both melittin and melittin-S, as depicted in Fig. 4 that contains the spectra obtained in 25% TFE. One can observe that the global shape of the spectra is the same, but there are changes that indicate possible structural variation (the valley at 222 nm, for instance). These variations were confirmed by the calculation of the secondary structure content and its fractions, as presented in Table 2 that shows that there is a major change from helix to unordered structures in melittin-S, rather than a change from helices to strands.

Once melittin has had its 3D structure determined and it is available at the PDB (DOI:10.2210/pdb2mlt/pdb), the effect of such

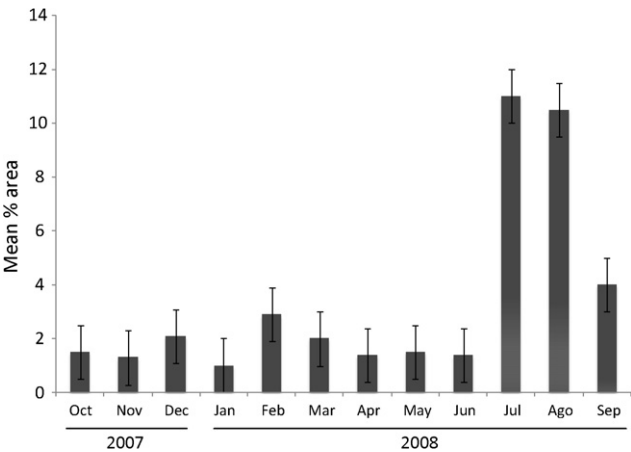


Fig. 3. Normalized monthly percent peak area variation of melittin-S.

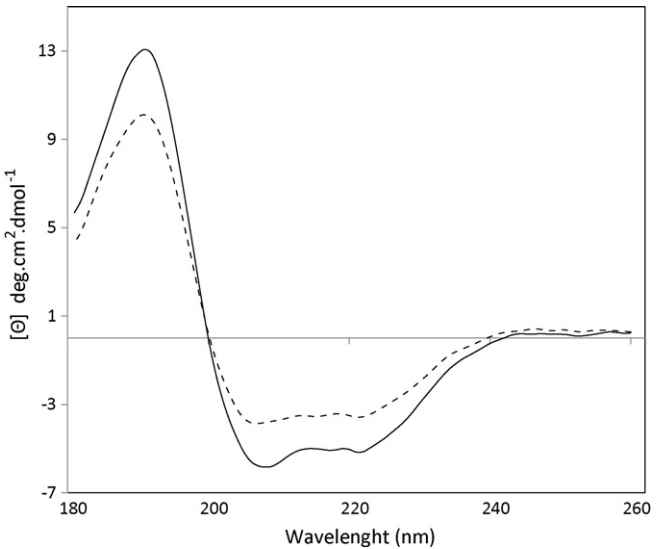


Fig. 4. Superimposed circular dichroism (CD) spectra of 7 μM melittin (solid line) and 7 μM melittin-S (dashed line), in the presence of 25% trifluoroethanol.

amino acid substitution could be probed by means of molecular modeling, as presented in Fig. 5. This figure shows the melittin model, with the Ser substitution as in melittin-S, calculated by the SWISS-MODEL. Peptide structure is presented as sticks, and surface potential is represented by the wireframe. In yellow, the substituted amino acid showing the high degree of exposure to the solvent and the particular ‘hinge’ region that it occupies.

Once the major toxic effect known to melittin is hemolysis, basically due to membrane penetration and pore formation, this assay was performed comparing both the natural and the novel melittin variant. These results are presented in Table 3 that shows a much greater reduction in the hemolysis level (95–35%) than in alpha-helix content (82–64%, Table 2), suggesting a complementary mechanism.

4. Discussion

The development of an anti-serum against *A. mellifera* venom would provide a faster and more efficient treatment for victims of mass stinging. But an effective anti-serum must take into account that it has to be the outcome of the immunization of animals with a representative venom pool of honey bee venoms from the very different ambient conditions, developmental stages, season of milking and diet that the insect may be subjected to.

Venom components variation has been described by Neumann et al. [22], Habermann [11,12] (in the 60s) and more recently, by

Table 2
Calculated secondary structure fractions.

	TFE 25%				Total
	Total ^a helix	Total ^a strand	Turns	Unordered	
Melittin	0.82	0.05	0.02	0.10	0.99
Melittin-S	0.64	0.09	0.05	0.21	0.99
	TFE 100%				Total
	Total helix	Total strand	Turns	Unordered	
Melittin	0.85	0.06	0.03	0.06	1
Melittin-S	0.81	0.07	0.02	0.11	1.01

^a Total corresponds to the sum of both helices or strands contents (regular and distorted) as calculated by the software.

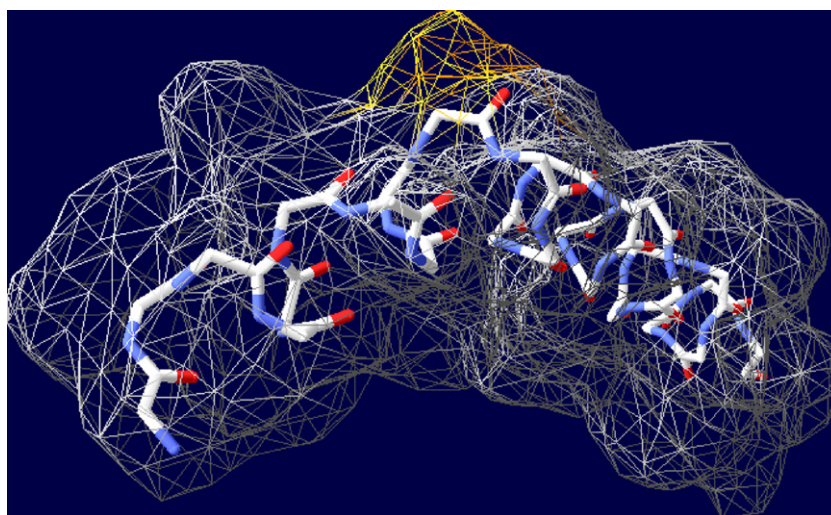


Fig. 5. 3D representation, as calculated by SWISS-MODEL, of the spatial structure of melittin-S. In yellow the substituted residue, indicating its high degree of exposition to the solvent and particular hinge region that it occupies. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 3
Hemolysis parameters.

A_{414}	Controls ^a		Melittin ^b	
	+	–	W.T. ^c	S ^d
AVG ^e ± S.D. (%) ^f	0.079 ± 0.007 (100)	0.044 ± 0.007 (0)	0.077 ± 0.002 (95)	0.056 ± 0.002 (35)

^a Positive control was triton X-100, negative control was sterile PBS solution.

^b Melittin concentration (both) was 3 μ M.

^c Wild-type melittin (Uniprot P01501).

^d Melittin-S.

^e Results correspond to data from independent triplicates.

^f Percent hemolytic activity compared to control.

Owen (in the 90s) [26,27]; however, no current study has been performed employing contemporary analytical tools such as those depicted here and in another recent work by our group [6].

In this way, knowledge of the presence and possible variation of the lesser components become important. Mainly, when one considers the massive attacks that AHB is likely to originate. When a bee stings the mammalian skin it will probably inject the whole contents of its venom sac (0.15–0.3 mg), due to the loss of the sting apparatus, including the venom sac, and its accessory muscles and nerve tissues that will keep contracting until the venom sac becomes empty [5]. In a true massive attack, 500–1000 stings are likely to occur. As a consequence, the victim would receive approximately 0.5 g of venom. At this level, minor components or isoforms start playing significant roles.

This may be the case for melittin-S, which remains in the low 1–2% level throughout the year, but abruptly rises to 10% during the southern winter months, thus becoming a ‘new’ major component, in the same level of PLA₂ (Fig. 1). Due to the close proximity of this new peak to melittin, and the similar molecular mass (Fig. 2), we have performed Edman degradation in order to assess whether this could be a melittin variation or isoform. Table 1 shows the chemically determined amino acid sequence that confirms the 16 Da mass difference observed in the MS spectra (Fig. 2).

Interestingly, the 10th position of melittin has already been probed by Nature (Table 1): *Apis dorsata* has the same Ser residue at this position, but has a Gln/Glu substitution. On the other hand *A. florea* and *Polistes hebraeus* (the common paper nest wasp, belonging to another superfamily of the suborder Apocrita of the Hymenoptera family) have opted for a neutral (in all senses) substitution by putting Ala at this position. All other known insect

melittins (up to this date and excluding the *Rana*’s melittin-like peptides) have Thr at the 10th position.

Kreil [14] has commented on the presence of the Ser residue in *A. dorsata* melittin when discussing the close relationship, molecularly and phylogenetically, between *A. mellifera* and *A. cerana* when compared to the other bees, and the possibility of the cross breeding between these *Apis*. However, that would not be the case for this work, once the hives were extremely homogeneous and the variation was clearly seasonal. Moreover, *wild-type* melittin content has not varied (data not shown), indicating that the bee is responding to some environmental change by synthesizing melittin-S.

The chosen biological assay performed in this work for melittin-S was hemolysis and this peptide showed very different lytic levels as compared to melittin. Moreover, according to the interpretation of the 3D structure of melittin performed by Terwilliger and Eisenberg [36], there is a water soluble tetramer that is stabilized by the interactions of the hydrophobic residues that face each other and that are coated by polar residues. All interactions seem to be maintained by helix–helix contacts. Once the –OH groups present either in naturally occurring Thr or the novel Ser residues of melittins are known to be key players in the stabilization of helical structures due to their ability to participate in hydrogen bonds, and that Ser and Thr share a different χ_1 rotamer probabilistic distributions, it would be expected the secondary structure of melittin-S would be compromised. Stapley and Doig [35] data corroborate ours as those authors demonstrate that, based on compiled PDB data, Thr is more thermodynamically favored to assume a g^- rotamer in an α -helical structure than Ser, which rotates equally both ways, not constraining any preferential structure. Furthermore, this particular substitution occurs in a naturally present hinge region of melittin (Fig. 5), making any subtle change even more pronounced.

Data we have obtained corroborate this idea: the CD spectra of melittin-S obtained in 25% TFE (Fig. 4) clearly presents that there is a difference, in terms of secondary structure contents, between these two melittins. Interestingly, this change seems to be highly dependent on the environment (buffer) in which the experiment was performed. The secondary structure fraction distribution for melittin-S, determined at 25% TFE, is the same as that obtained in 100% aqueous buffer (data not shown), but is significantly different from those measured at 100% TFE (Table 2). TFE is commonly used in CD experiments not because it creates any secondary structure, but because it stabilizes pre-conditioned structures that, for some reason, would not form at those specific conditions [18].

The reason for the secretion of a melittin isoform is, yet, unknown. However, two factors must be taken into account: (i) melittin-S does exist, although in low levels, throughout the year. It is not suddenly synthesized as a completely new product. Therefore, the genetic repertoire and rationale behind its synthesis is already present; and (ii) this variation is not age-based. The worker bees (the ones harvested in this work) only live for a few weeks in mild climates (such it is in Botucatu) due to constant harsh work (no hibernation during winter).

The presence of isoforms of venom components in *A. mellifera* has been previously reported by Kettner et al. [13], when describing the allergen Api m 6. This molecule can be found in four different (iso)forms in the venom, which share a common 67 amino acid kernel and the three variants possess either a N-terminal tetra-peptide extend or a C-terminal dipeptide or both. These authors postulate that unidentified proteases would process Api m 6, resulting in its shedding into the venom sac lumen and subsequent formation of the isoforms. Later, in a subsequent study by Peiren et al. [31], authors showed that Api m 6 is derived from a single locus and that the protein-level variation has a simple genome-level cause, without the need to invoke multiple loci or alternatively spliced exons, nor different proteolytic processing pathways.

Although noteworthy, Api m 6 isoforms secretion into the venom follow a completely different rationale than melittin and melittin-S. The bee venom contains 1–2% Api m 6 at approximately equimolar amounts [13] and its secretion represents a ‘choice’ taken at the nucleic acid level. Melittin and melittin-S, on the other hand, ought to be constitutively secreted as parallel independent gene products and their contents vary significantly and seasonally (this work and Ferreira Junior et al., respectively [6]) throughout the year. Moreover, despite Api m 6 three-dimensional structure is not available, the kind of amino acid substitution among its isoforms is not likely to cause as significant structural changes (Api m 6 is highly constrained by S–S bridges) as those described in this work for melittin-S.

All together, these results indicate that the full comprehension of any given venom mechanism of action will rely on the thorough identification of the venom components. This becomes more important when the envenomation accident is caused by the injection of fairly large amounts of venom, such as biting of an adult *Bothrops jararaca*, in which 3–4 g of venom will be injected, or can also be the case for *A. mellifera* massive attacks, when 1000 bees will inject up to half a gram of venom. In either case, efficient neutralization of the toxic components must be quickly achieved in order to save lives. Such effectiveness will only be possible once every toxic component of the venom is characterized, including isoforms and variants.

Acknowledgments

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