



Shotgun proteome analysis of honeybee venom using targeted enrichment strategies



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ABSTRACT

The aim of this study was to explore the honeybee venom proteome applying a shotgun proteomics approach using different enrichment strategies (combinatorial peptide ligand libraries and solid phase extraction). The studies were conducted using nano-LC/MALDI-TOF/TOF-MS system. The MS analysis of peptide profiles (in the range of 900–4500 Da) and virtual gel-image of proteins from Lab-on-Chip assay (in the range of 10–250 kDa) confirm that use of targeted enrichment strategies increase detection of honeybee venom components. The gel-free shotgun strategy and sophisticated instrumentation led to a significant increase of the sensitivity and higher number of identified peptides in honeybee venom samples, comparing with the current literature. Moreover, 11 of 12 known honeybee venom allergens were acknowledged and 4 new, so far uncharacterized proteins were identified. In addition, similarity searches were performed in order to investigate biological relations and homology between newly identified proteins sequences from *Apis mellifera* and other *Hymenoptera*.

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1. Introduction

Stinging by *Hymenoptera* represents one of the main causes of anaphylaxis both in adults and in children (Matysiak et al., 2011a; Silva et al., 2012). In Central Europe most post-stinging anaphylactic reactions are caused by honey bees (*Apis mellifera*), and less by other *Hymenoptera*, such as wasps (*Vespula vulgaris*, *Vespula germanica*), hornets (*Vespa crabro*) and bumble bees (*Bombus* spp.). The frequency of post-stinging allergic reaction and its intensity may vary depending the intra-individual characteristics of both the person and of the stinging insect (Przybilla and Rueff, 2010). Consequently the qualitative and quantitative differences in composition of the venom may affect the severity of allergic reaction after the sting. At present 12

allergens are identified in honeybee venom and are listed by the Allergen Nomenclature Sub-Committee of the International Union of Immunology Societies (IUIS) (Cichocka-Jaroszc et al., 2011). According to the literature the most important bee venom allergens are: phospholipase A₂ (Api m1), hyaluronidase (Api m2), acid phosphatase (Api m3) and melittin (Api m4) (Bilo et al., 2005; Peiren et al., 2005). It is known that in 97% of patients with bee venom-specific IgE antibodies, IgE antibodies to Api m1 are detected (Dotimas and Hider, 1987; Muller et al., 2009). In addition, IgE antibodies to Api m2 (hyaluronidase), Api m3 (acid phosphatase) and Api m4 (melittin) are present in 51%, 60% and 31% of patients, respectively (Kemeny et al., 1983). Hence, whereas the most abundant allergens are the primary targets for venom immunotherapy, minor protein components may represent new opportunities of exploration into the organism response to this toxin. Due to the fact that the *A. mellifera* genome is fully sequenced, we

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can take advantage of searching the complete genomic DNA sequence and getting matches from unidentified open reading frames.

The current literature presents many strategies for proteomic characterization of complex biological matrices (Freour et al., 2013; Moore et al., 2013; Rees et al., 2013; Wang et al., 2013; Yu et al., 2013). Due to the complexity of this material, some laboratories employ bottom-up strategies, where two-dimensional SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) is used to separate protein mixture. Subsequently after in-gel digestion, the resulting peptide mixture is analyzed by tandem mass spectrometry techniques (Wilkins et al., 2007). Nonetheless, the results obtained from the bottom-up strategy often have consistency issues (low reproducibility, inter-sample agreement). SDS-PAGE is both sample and time-consuming, and becomes very tedious particularly when the runs are repeated several times. Advancement of mass spectrometry fragmentation methods allowed the development of a less complicated top-down strategy, which involves the analysis of purified proteins. This technique is preferably used for analysis of post-translational modifications of single proteins or isoforms of proteins. The limitations of bottom-up and top-down strategies include: complex process of sample preparation and relatively high costs of analysis. Accordingly, most of the recent proteomic studies are based on the shotgun strategy, which requires proteolytic in-solution digestion of the whole sample and skips the laborious and low reproducible 2-D PAGE steps. In view of the large number of the peptides obtained, optimization of the LC method (including selection of proper column and gradient program) in this strategy is required.

In complex biological matrices, such as bee venom, the dynamic range of the proteins is very high (Kokot et al., 2011). Thereby a major challenge is to concentrate the analyte in order to identify proteins present at lower levels. To overcome this problem and allow access to the “hidden proteome”, several multidimensional prefractionation methods and tools have been developed for removing the most abundant proteins. These methods include: organic solvent extraction (Chertov et al., 2004), ultrafiltration (Tirumalai et al., 2003; Zheng et al., 2006), solid phase extraction (Hu et al., 2009; Koomen et al., 2005), extraction by mesoporous silica particles (Tian et al., 2007), extraction using magnetic beads (Baumann et al., 2005), chromatography, electrophoresis (Marshall et al., 2004), immunoaffinity fractionation (Brand et al., 2006; Gong et al., 2006; Pieper et al., 2003) and fractionation using hydrogel nanoparticles (Rainczuk et al., 2010). Recently, especially methods using immunodepletion (only restricted to physiological fluids such as: serum, plasma, cerebrospinal fluid etc.) and the combinatorial peptide ligand library strategy (CPLL or ProteoMiner, Bio-Rad) have been employed (Krief et al., 2012; Whiteaker et al., 2007). The CPLL is helpful for revealing lower abundance proteins, undetectable by classical analytical methods (Boschetti and Righetti, 2008) and can be applied to the broad range of biological samples, including not only physiological fluids (Castagna et al., 2005; Sennels et al., 2007) but also extracts of plants (Boschetti et al., 2009; Boschetti and Righetti,

2014), food (D'Ambrosio et al., 2008; Farinazzo et al., 2009) or animal toxins (Calvete et al., 2009; Fasoli et al., 2010). This strategy is based on the interaction of complex protein matrices with a large, highly diverse library of hexapeptides bound to chromatographic supports. Due to the fact that bead capacity limits binding capacity of the proteins, the signal of high-abundance proteins is reduced while low-abundance proteins bring their signal increasing detection. Therefore, this technique is particularly useful for the removal of proteins with the highest concentrations, which can mask the other components.

In addition to the problem with compressing the dynamic range of protein concentration in complex biological samples, the second problem in the proteomic analysis is the contamination of the analyzed sample by salts, detergents and many other impurities (Gobom et al., 1999). The widely used solid phase extraction (SPE) with easy to handle pipette tips allows for desalting, concentration and purification of the sample, results in a significant improvement in the identification of the proteins and peptides analyzed by mass spectrometry techniques (Palmlad and Vogel, 2005). Commercially available pipette tips such as ZipTip (Millipore, Bedford, MS, USA) or NuTip (Glygen, Columbia, MD, USA) are packed with conventional particles, while MonoTip (GL Sciences, Tokyo, Japan) pipette tips are packed with functionalized monolithic silica. The OMIX tips (Agilent, Palo Alto, CA, USA) used in this study, are classified as a monolith-like silica gel product. However, there are divergent references in available literature about the effectiveness of extractive pretreatments of SPE techniques with use pipette tips in mass spectrometry analyses (Hennion, 1999; Huck and Bonn, 2000; Kumazawa et al., 2010; Rossi and Zhang, 2000).

The aim of this study was to examine the shotgun proteomics strategy for the characterization of honeybee venom samples, as well as the evaluation of suitable system for purification and concentration of the natural biological sample.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile (ACN), DL-dithiothreitol (DTT), ammonium phosphate monobasic, iodoacetamide, heptafluorobutyric acid, trifluoroacetic acid (TFA), ultrapure water and ammonium bicarbonate were supplied by Sigma–Aldrich (St. Louis, MO, USA). 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) and α -cyano-4-hydroxycinnamic acid (HCCA) were obtained from Bruker (Bremen, Germany). Ethanol and isopropanol was supplied by J. T. Baker (Center Valley, PA, USA). All reagents used were of analytical grade or better.

2.2. Honeybee venom samples

Samples of honeybee venom were collected from an apiary of the Department of Inorganic and Analytical Chemistry, Poznan University of Medical Sciences by stimulating the bees with electric current pulses. Venom collecting frames were placed in the upper body of the hive

in the middle space of the hive super. The duration of the venom-collecting event was 2 h during full activity of bees. Such schedule allowed obtaining the highest efficiency of bee venom production. The samples were stored until analysis at 5 °C in darkness.

2.3. Sample treatment with ProteoMiner and OMIX

Honeybee venom samples were treated with combinatorial protein ligand libraries (CPLL) which are commercially available under the name ProteoMiner, Bio-Rad (Hercules, CA, USA). The honeybee venom solutions were prepared by dissolving 50 mg of the product in 1 mL of ultrapure water. Then the samples were sonicated for 10 min and centrifuged for further 10 min. Next, the clear solution was transferred into a reaction tube. The whole procedure of depletion was performed according to the manufacturer's protocol. Honeybee venom solution used in CPLL studies was also used for concentration and purification with OMIX pipette tips (Agilent). Clean-up procedure was performed according to the manufacturer's instructions. Protein concentration of eluates after ProteoMiner and OMIX protocols was determined by Bradford protein assay (Bradford, 1976) and each sample was separately analyzed on MALDI-MS.

2.4. Nano-LC/MALDI-TOF/TOF-MS analysis

Prior to the shotgun analysis both the honeybee venom samples and eluates after ProteoMiner and OMIX protocols were digested according to the procedure modified from Pierce In-Solution Tryptic Digestion Kit. Studies were performed using nano-LC/MALDI-TOF/TOF-MS system which consisted of: nanoflow LC system EASY-nLC II (Bruker), fraction collector Proteineer-fc II and MALDI-TOF/TOF mass spectrometer UltrafleXtreme (Bruker). Peptides were concentrated on trap column Thermo Scientific EASY-Column C18 (5 µm, 100 µm × 20 mm) and separated on Thermo Scientific EASY-Column C18 (3 µm, 75 µm × 100 mm) by linear gradient of water (mobile phase A) and ACN (mobile phase B), both containing 0.1% TFA. The gradient elution method was: 0–100% B in 60 min. The flow rate was maintained at 300 nL min⁻¹ and the injection volume was 5 µL. 192 fractions of separated sample were automatically mixed with matrix solution and spotted on MALDI target. In order to optimize the method which allows obtaining the best identification results, two different target plates were tested: disposable prespotted AnchorChip (PAC target plates are prespotted with HCCA matrix and calibration spots) and standard AnchorChip targets. For AnchorChip plate nanoLC fraction matrix solution was used by mixing: 748 µL of 95:5 (v/v) acetonitrile: 0.1% TFA, 36 µL of saturated solution of HCCA in 90:10 (v/v) acetonitrile: 0.1% TFA, 8 µL of 10% TFA and 8 µL of 100 mM ammonium phosphate monobasic. By routine, a standard peptide calibration mixture in the mass range 700–3500 Da (Bruker) was analyzed for external calibration of the mass spectrometer. Typical instrument settings were as follows: ion source 1, 25.0 kV; ion source 2, 22.7 kV; lens voltage, 7.9 kV; pulsed ion extraction time, 120 ns; matrix suppression mass cut off, *m/z* 500. All spectra were

acquired by accumulating 2500 shots from 5 non-overlapping positions. The following settings for MALDI-MS/MS acquisition control were used: S/N threshold, 12; minimal mass distance to co-eluting compounds of higher intensity, 5 Da; maximum number of MS/MS per fraction, 10. The control of the instrument, data acquisition, and data evaluation were performed using the following software platforms: flexControl 3.4, FlexAnalysis 3.4 and WARP-LC 1.3 (Bruker).

2.5. Database searching

The fragment ion spectra obtained from the MALDI-MS/MS measurements were processed with ProteinScape 3.0 platform. The tandem mass spectra were assigned to peptide sequences by searching the NCBI nr database with the Mascot search engine (version 2.4.0), Matrix Science, London, UK. The results were filtered to a false discovery rate at a peptide-spectrum match level of less than 1% based on decoy counts and only proteins with at least one unique protein were included. The searches were taxonomically restricted to the “Metazoa”. The search parameters were as follows: trypsin digestion with up to one missed cleavage; carbamidomethyl cysteine as fixed modification; peptide precursor mass tolerance: 25 ppm; fragment mass tolerance: 0.7 Da; peptide charge: +1; monoisotopic mass.

In order to find biological relationships and homologies between identified in this study proteins sequences from *A. mellifera* and other *Hymenoptera* the Basic Local Alignment Search Tool (BLAST) was used (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Two peptides were considered to be homologs when the bit score was higher than 40 and the E-value of their BLAST alignment was smaller than 0.01.

2.6. Peptide profiling experiments

The honeybee venom solutions were prepared by dissolving 5 mg of the product in 1 mL of ultrapure water. The samples were vortexed for 1 min followed by sonication for 10 min and then centrifuged. Each honeybee venom sample and eluates after ProteoMiner and OMIX protocols were separately analyzed on a ground steel target plate. Double layer preparation of spots was performed. Firstly 0.5 µL of saturated solution of matrix (sinapinic acid) in ethanol was deposited on MALDI target and dried. Next, 0.5 µL of matrix (saturated solution (30 mg mL⁻¹) of sinapinic acid in 30% ACN and 0.1% TFA)/analyte mixture was loaded onto the first layer of the matrix spot and dried at room temperature. Peptide profiling experiments were carried out with MALDI-TOF/TOF mass spectrometer UltrafleXtreme (Bruker) in the mass range between 900 Da and 4500 Da. Mass calibration was performed for each four samples in range of ~700 Da–3500 Da. The peptide calibration standard contained seven standard peptides. All analyses were performed in the positive-ion reflector mode by accumulating data from 2500 laser shots for each spectrum. Each sample analysis was carried out in triplicate. The instrument was controlled using Bruker's flexControl 3.4 software and mass spectra were analyzed in Bruker's FlexAnalysis 3.4 software.

2.7. On-Chip analysis of honeybee venom proteins

The chemicals used in this study were included in the Agilent High Sensitivity Protein 250 Kit. Ultrapure water was used for all preparations. On-Chip electrophoresis was performed on an Agilent 2100 Bioanalyzer system using the suitable kit for the sizing of proteins in the range from 10 to 250 kDa. The LabChip kit was purchased from Agilent Technologies. Before analysis, honeybee venom samples were adjusted to a protein concentration of $2 \mu\text{g } \mu\text{L}^{-1}$ by Bradford protein assay (Bradford, 1976). Afterward, all samples and standard were diluted 1:200 (v/v) in ultrapure water. 4 μL of analyte sample/standard was combined with 2 μL denaturing solution containing 1 M DTT as a reducing factor and sample buffer, then incubated at 95°C for 5 min. An appropriate aliquot of each protein/standard sample was loaded onto the chip, which beforehand was filled with the mixture of gel and dye and the destaining solution. The sample buffer used possessed a lower marker standard at known molecular weight. The chip was run for 30 min. Separated proteins were detected with laser-induced fluorescence. All the analyses were conducted in triplicate. Data evaluation was performed using Agilent 2100 Expert software (version B.02.08.S16).

3. Results & discussion

3.1. Honeybee venom analysis applying shotgun proteomic approach

The present study was focused on applying shotgun proteomics using different enrichment strategies to explore the honeybee venom proteome. The studies were conducted using nano-LC/MALDI. Overcoming the pitfalls of the classical two-dimensional electrophoresis approach conducted prior to mass spectrometric analysis allowed to develop a novel approach that is easier to handle. The gel-free shotgun proteomic strategy and sophisticated instrumentation led to a significant increase of the sensitivity and higher number of identified honeybee venom components, comparing with literature (Baracchi and Turillazzi, 2010; Francese et al., 2009; Matysiak et al., 2011b; Peiren et al., 2008). In the first part of this study Prespotted AnchorChip (PAC) targets were used (with HCCA matrix). According to manufacturer's information PAC targets are highly suitable for low to medium complex samples in LC-based proteomics applications. These MALDI plates enable highly sensitive measurements of protein digests in both MS and MS/MS mode with reasonable signal-to-noise ratios. In addition, they allow for very fast analysis without the time-consuming process of matrix preparation. The MS/MS results obtained were analyzed using Mascot against the comprehensive sequence database NCBI nr. The summary report of identified honeybee venom proteins is presented in Table 1. Detailed reports from sequence database searching are included in Supplementary materials (A.1, B.1, C.1, D.1), which can be found in the online version. The reproducibility of presented data was proved in the analyses performed in further studies. All identified proteins and peptides presented in this manuscript have been confirmed in additional LC/MALDI

experiments conducted in our laboratory (data available on request). The shotgun proteomics strategy (using PAC target) allowed the identification of 18 honeybee venom proteins. Noteworthy is the identification of 10 out of 12 proteins that are accepted by the International Union of Immunological Societies (IUIS) as bee venom allergens (phospholipase A_2 , hyaluronidase, venom allergen acid phosphatase, melittin, venom dipeptidyl peptidase 4, Api m6, venom protease, carboxylesterase, icarapin, major royal jelly protein). Moreover, the expression of two previously unidentified proteins (hypothetical protein: LOC725074 and LOC408666) was proved. Prior studies of honeybee venom employing both “bottom-up” and “top-down” strategies led to identification of only six known venom proteins and three unknown protein components, not identified earlier (Matysiak et al., 2011b; Peiren et al., 2005). Although, PAC targets are dedicated for the rapid screening analysis, many reports have shown that application of the classical AnchorChip MALDI plate facilitates better results (Du et al., 2010; Leung and Pitts, 2008; Zhang et al., 2008, 2007). This plate, according to the manufacturer is preferred for high-throughput MALDI measurements that are performed in unattended, fully automatic mode. Such applications include MALDI peptide mapping and subsequent MS/MS sequencing of LC/MALDI analyses of complex peptide mixtures. The plate is coated with hydrophilic patches surrounded by a hydrophobic ring which prevents sample spreading and concentrates the sample in a distinct location. The use of higher amounts of HCCA matrix (when compared with PAC targets) directly mixed and co-crystallized with the nanoLC fraction results in significant improvement of sensitivity, increases the number of identified compounds (Zenobi and Knochennuss, 1998). The MS/MS analysis using AnchorChip plates was conducted with the same parameters as with PAC plates. Applying this methodology 26 honeybee venom proteins were identified (Table 1). As in the case of using PAC plate 10 of honeybee venom allergens were identified (phospholipase A_2 , hyaluronidase, venom allergen acid phosphatase, melittin, venom dipeptidyl peptidase 4, Api m6, venom carboxylesterase, icarapin, major royal jelly protein, vitellogenin). Furthermore, three undescribed honeybee venom proteins were identified (hypothetical protein: LOC725074, LOC408666, LOC725163). Further MS/MS studies with additional concentration and purification steps (ProteoMiner and OMIX) of honeybee venom sample were carried out only with use the AnchorChip plate because of higher number of identified proteins.

In complex biological matrices such as bee venom, many proteins are present in small quantities, which do not allow direct detection of them (Li et al., 2013). It was shown that the content (relative to the dry venom mass) of the main honeybee venom constituents was as follows: apamine: $2.85 \pm 0.79\%$; mast cell degranulating peptide: $2.82 \pm 0.64\%$; phospholipase A_2 : $12.95 \pm 3.09\%$; melittin: $45.91 \pm 9.78\%$ (Kokot and Matysiak, 2009; Kokot et al., 2011). Thus, extensive annotation of honeybee venom proteome is challenged by the high concentration range of the protein components. To identify the low-abundance compounds more readily, it is crucial to concentrate the analyte. In order to explore the “hidden fraction” of

Table 1

Summary report of identified honeybee venom proteins applying shotgun proteomics methodology and different enrichment strategies. Detailed reports from sequence database searching are included in [Supplementary materials \(S1–S4\)](#), which can be found in the online version.

Applied sample enrichment methodology/MALDI target plate		Untreated/ PAC	Untreated/ AnchorChip	ProteoMiner/ AnchorChip	OMIX/ AnchorChip
Identified proteins	Icarapin (Api m10)	●	●	●	●
	Phospholipase A ₂ (Api m1)	●	●	●	●
	Venom carboxylesterase – 6 (Api m8)	●	●	●	●
	Mast cell degranulating peptide	●	●	●	●
	Melittin (Api m4)	●	●	●	●
	Venom dipeptyl peptidase 4 (Api m5)	●	●	●	●
	Apamine	●	●	●	●
	API m6 (Api m6)	●	●	●	●
	Secapin	●	●	●	●
	Chymotrypsin inhibitor	●	●	●	●
	Venom acid phosphatase	●	●	●	●
	Chain A, crystal structure of bee venom hyaluronidase	●	●	●	●
	Vitellogenin precursor (Api m12)	●	●	●	●
	Venom allergen acid phosphatase (Api m3)	●	●	●	●
	Hypothetical protein: LOC408666	●	●	●	●
	Major royal jelly protein 9 precursor (Api m11)	●	●	●	●
	Major royal jelly protein 8 precursor (Api m11)	●	●	●	●
	Hypothetical protein LOC725074	●	●	●	●
	Venom protease (Api m7)	●	●	●	●
	Major royal jelly protein MRJP5 (Api m11)	●	●	●	●
	Hypothetical protein: LOC725163	●	●	●	●
	Apolipophorins-like	●	●	●	●
	Apolipophorins isoform 1	●	●	●	●
	Sorbitol dehydrogenase-like isoform 2	●	●	●	●
	Chitinase-like protein Idgf4-like, partial	●	●	●	●
	Hexamerin	●	●	●	●
	Transferrin 1 precursor	●	●	●	●
	Nucleobindin – 2 like, partial	●	●	●	●
	Peptidyl-prolyl cis–trans isomerase B	●	●	●	●
	Lysosomal pro-x carboxypeptidase-like	●	●	●	●
	Renin receptor like	●	●	●	●
	Calreticulin isoform 1	●	●	●	●
	Hypothetical protein: LOC100577054	●	●	●	●
Total		18	26	14	17

honeybee venom proteome, affinity chromatography utilizing solid phase combinatorial peptide libraries (CPLL) was used. This technique allows eliminating the excess of ballast proteins and decreasing the dynamic range of concentration of different protein species in the sample. Upon the available literature it was decided to use ProteoMiner system, which was proved to be a good choice for the study of biological matrices of broad origin, including human physiological fluids (Castagna et al., 2005; Guerrier et al., 2007; Roux-Dalvai et al., 2008; Sennels et al., 2007), zoonotic samples (D'Ambrosio et al., 2008; Farinazzo et al., 2009), plant tissues (Boschetti et al., 2009) and venoms (Fasoli et al., 2010). Depletion of the most abundant proteins using ProteoMiner resulted in identification of 4 additional predicted proteins (lysosomal Pro-X carboxypeptidase-like, peptidyl-prolyl cis–trans isomerase B precursor, renin receptor-like, calreticulin isoform 1), which were masked in previous experiments by other components. Although the expression of these proteins is not surprising because they are encoded in *A. mellifera* DNA, none of them has been reported in the available literature as honeybee venom component.

The contamination of the samples with salts, surfactants and other impurities may interfere with mass spectrometry

techniques and impairs the proteomic analysis of complex biological matrices (39). Thus, in order to clean up the analytes before acquisition, OMIX micropipette tips were applied. MS/MS analysis after OMIX pretreatment led to the identification of a new and uncharacterized honeybee venom hypothetical protein: LOC100577054, in addition to previously detected proteins in this study.

Applying gel-free shotgun proteomics approach combined with different enrichment strategies proposed in this study as many as 33 proteins have been detected in honeybee venom collected by electrical stimulation of the bees (10 proteins identified based on one significant peptide match). Li et al. (2013) identified in the same material in total only 17 proteins using gel-based proteomics (14 identified proteins) and shotgun analysis (only 15 identified proteins). They compared the proteome of honeybee venom collected by electrical stimulation of the bees and manually extracted from the gland. It was suggested that venom collected directly from the gland contains more components in reason of the contamination by the tissue damage during the milking process. However, our findings prove that some of proteins identified by Li et al. (2013) recovered only in manually extracted venom were also present in venom collected by electro-stimulation. These

were the following proteins: venom serine protease 34 precursor, vitellogenin, transferrin 1 precursor, chitinase-like protein Idgf4-like, hexamerin, peptidyl-prolyl cis–trans isomerase B precursor, apolipoporphins isoform 1, apolipoporphins-like. Such discrepancies are probably due to difference of the LC fractionation methods. In our study the honeybee venom samples were separated into 192 different fractions (directly spotted on MALDI target plate) while in the aforementioned study only seven fractions were analyzed with a QqTOF instrument which clearly influenced the number of separated peptides.

In order to find out any similarities of the uncharacterized honeybee venom proteins from our study (hypothetical protein: LOC408666, LOC725074, LOC725163, LOC100577054) with the proteomes of other organisms, BLAST was used. The program's algorithm finds regions of local similarity between amino acid sequences from different databases (in this study NCBI database was used) and on the basis of calculated statistical significance of matches, the alignment may be estimated. The results of searches with BLAST are presented in Table 2. It was noted that the analyzed sequences which were the fragments of honeybee venom proteins demonstrate high homology with the proteins of other *Hymenoptera* insects. In the case of a protein encoded by an *A. mellifera* gene, LOC100869681, high homology with other hypothetical proteins from: *Apis florea*, *Camponotus floridanus*, *Megachile rotundata* and *Nasonia vitripennis* was found. For the protein encoded by *A. mellifera* gene: LOC725074, there are three database matches that align to most of the query sequence and they are derived from: *A. florea*, *Solenopsis invicta* and *Bombus terrestris*. The amino acid sequence of a protein encoded by *A. mellifera* gene, LOC100577054, shows the highest similarity only with a protein sequence from *B. terrestris*, while for the hypothetical protein LOC725163 (*A. mellifera*), no high-scoring alignment was found. However to explain the role of this molecules further studies need to be performed.

3.2. Honeybee venom analysis applying peptide profiling approach

Peptide profiling experiments were carried out with MALDI-TOF/TOF mass spectrometer UltrafleXtreme (Bruker) in mass range between 900 and 4500 Da. In order to achieve high mass accuracy and high resolution, all experiments were performed in the reflector positive-ion mode. As matrix sinapinic acid was used, which was demonstrated in our previous report to be a better matrix than HCCA in peptide profiling studies of honeybee venom (Matysiak et al., 2011b). Analyses were performed on MALDI ground steel target plate, which has a highly regular fine structure on the surface, enabling homogenous co-crystallized preparations. The experiments were repeated three times. Signal-to-noise ratios (S/N) lower threshold for peak picking was set to 6. The results obtained from peptide profiling experiments are presented in Table 3. It was clearly shown that the use of ProteoMiner depletion method allowed for the detection of new peptide candidates which have not been observed before performing any enrichment strategies (Fig. 1). By applying CPLL as a depletion method, as many as 26 unique peptides, which have been not observed before depletion process, were detected.

With respect to honeybee venom MS analysis, the efficiency of OMIX purification strategy was also examined with a peptide profiling approach. 100 distinct peptides were determined; whereof as 62 of them have not been reported before neither in samples without any cleaning procedure nor in samples depleted with CPLL. Lack of any pretreatment course resulted in low level of identification, only 17 unique peptides were found. At the same time, only 19 common peptides were identified after implementation of these various preparation strategies (no depletion/purifying, ProteoMiner, OMIX) (Table 2). In summary, MALDI-TOF mass spectra achieved by peptide profiling analysis

Table 2

BLAST report for sequences of *Apis mellifera* proteins encoded by the following genes: LOC408666, LOC725074, LOC725163, LOC100577054. Two peptides were considered to be homologs, when the bit score was higher than 40 and the E-value of their BLAST alignment was smaller than 0.01.

Peptide sequence	Protein sequence producing significant alignments	Score [bits]	E-value
IVPIEHTQCTCDCK	PREDICTED: uncharacterized protein LOC100869681 (<i>Apis florea</i>)	55.4	7e-08
	PREDICTED: hypothetical protein LOC408666 (<i>Apis mellifera</i>)	55.4	8e-08
	hypothetical protein EAG_09508 (<i>Camponotus floridanus</i>)	42.6	0.001
ETQECSTGFYFDQNSCR	PREDICTED: hypothetical protein LOC408666 (<i>Apis mellifera</i>)	60.9	1e-09
	PREDICTED: uncharacterized protein LOC100883244 (<i>Megachile rotundata</i>)	54.9	1e-07
	PREDICTED: uncharacterized protein LOC100869681 (<i>Apis florea</i>)	52.4	9e-07
	PREDICTED: vascular endothelial growth factor A–A-like (<i>Nasonia vitripennis</i>)	42.2	0.003
QSYVPEECTCACNNVDEQK	PREDICTED: hypothetical protein LOC408666 (<i>Apis mellifera</i>)	67.7	8e-12
	PREDICTED: uncharacterized protein LOC100869681 (<i>Apis florea</i>)	60.9	2e-09
	PREDICTED: uncharacterized protein LOC100883244 (<i>Megachile rotundata</i>)	43.5	0.001
CNFQIGIPVCTWPTTGGCR	PREDICTED: hypothetical protein LOC725163 (<i>Apis mellifera</i>)	67.7	3e-12
HGDSVCVSSDCCPGTWCHTYANR	PREDICTED: hypothetical protein LOC725074 (<i>Apis mellifera</i>)	81.2	3e-17
	PREDICTED: uncharacterized protein LOC100864630 (<i>Apis florea</i>)	79.1	2e-16
	ckin1 precursor (<i>Solenopsis invicta</i>)	52.4	9e-07
	hypothetical protein SINV_07967 (<i>Solenopsis invicta</i>)	52.4	1e-06
	PREDICTED: hypothetical protein LOC100644886 (<i>Bombus terrestris</i>)	48.1	3e-05
GEVCPVYLGCIK	PREDICTED: hypothetical protein LOC100577054 (<i>Apis mellifera</i>)	43.1	5e-04
	PREDICTED: hypothetical protein LOC100651794 (<i>Bombus terrestris</i>)	40.1	0.006

Table 3

Honeybee venom peptide candidates detected by peptide profiling analysis using different enrichment strategies. A – untreated sample; B – sample treated with ProteoMiner; C – sample treated with OMIX.

Samples	Unique monoisotopic masses [Da]				Common monoisotopic masses [Da]			
	A	B	C		A, B, C	A, B	B, C	A, C
Peptides masses	887.107	821.461	833.121	2895.391	917.256		3001.830	822.157
	891.160	1101.527	835.131	2917.384	919.272		3291.944	873.125
	915.110	1211.826	837.112	2935.381	933.234			889.125
	931.098	1215.027	861.124	2947.081	935.246			2808.077
	937.143	1229.850	877.124	2952.128	1141.333			2810.686
	973.082	1251.828	879.132	2965.027	2831.743			2828.537
	1113.184	1267.802	895.126	2976.385	2845.809			2858.597
	1139.164	1360.890	901.114	2983.370	2923.817			2867.454
	1157.177	1382.422	910.435	2990.488	2939.814			2883.422
	1159.203	1435.887	957.062	2993.453	2953.830			2887.030
	2026.720	1866.201	1423.194	2996.390	2967.814			2903.452
	2868.448	1991.218	1424.693	3004.420	2979.846			2909.358
	2872.438	3018.834	1477.693	3016.987	3007.872			2979.462
	2874.507	3031.871	1504.711	3036.402	3045.838			3019.446
	2992.437	3050.851	1525.717	3063.971	3085.913			3023.484
	2999.439	3057.858	1620.749	3066.397	3101.846			3027.498
	3002.445	3061.861	1676.766	3139.432	3115.894			3051.516
		3074.916	1908.904	3142.457	3129.886			3069.658
		3078.851	2150.016	3159.445	3169.978			3085.481
		3089.788	2306.110	3185.464				
		3141.915	2795.042	3213.433				
		3163.895	2801.328	3247.450				
		3193.923	2816.322	3258.456				
		3207.887	2820.439	3276.453				
		3223.924	2834.848	3457.522				
		3237.923	2842.648	3643.532				
			2844.039	3825.516				
			2857.436	3843.507				
			2863.915	4025.491				
			2873.421	4043.485				
			2877.323	4871.115				
Total	17	26	62	19	0	2	19	

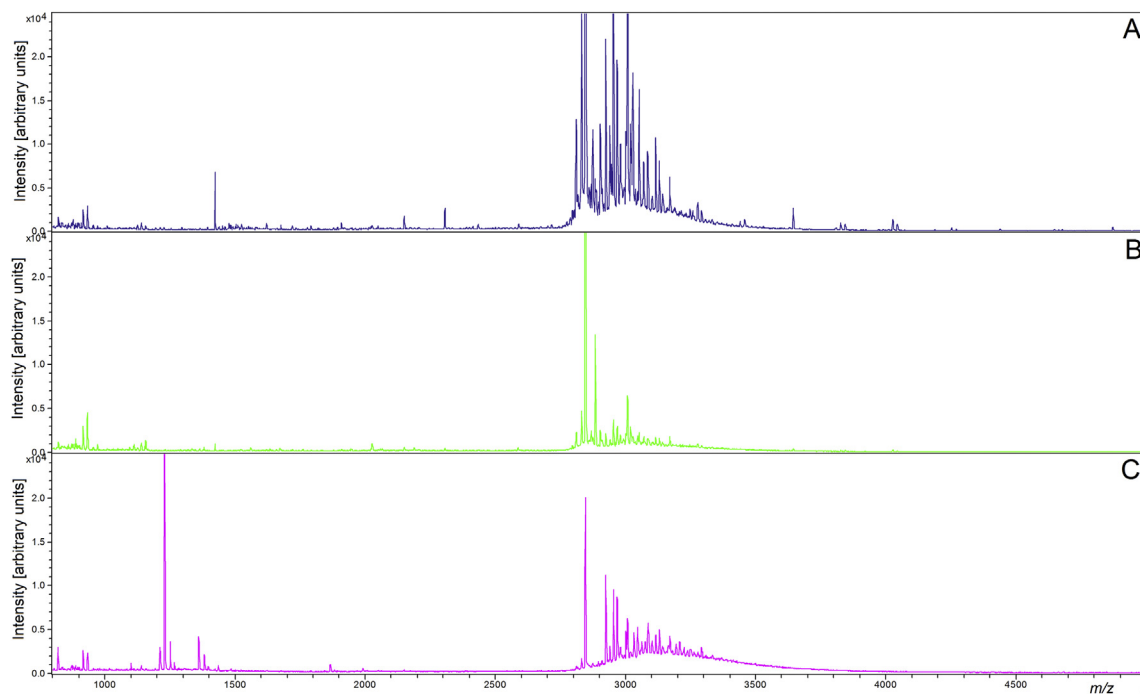


Fig. 1. MALDI TOF-MS spectra from peptide profiling analysis of honeybee venom using different methods of sample preparation. A – sample treated with OMIX; B – untreated sample; C – sample treated with ProteoMiner. All spectra are shown in the same scale.

of honeybee venom shows significant complementarity of data obtained by employing enrichment strategies (Fig. 1).

3.3. On-Chip analysis of honeybee venom proteins after different enrichment strategies

The honeybee venom samples treated with ProteoMiner and OMIX were additionally analyzed on Agilent 2100 bioanalyzer using LabChip 250 kit. This system provides ready-to-use and easy to handle on-chip electrophoresis. One of the main advantages over the traditional gel electrophoresis is the elimination of time-consuming procedures and minimization of the reagent and sample usage. Lab-on-Chip assay facilitates the process of data collecting and analysis down to a few steps: load sample, run analysis and view data. Effortless and quick analysis ensures valuable information about quality of the analyzed protein samples and allows estimating the effectiveness of the application of various purification and depletion approaches. Therefore, it fulfills a very good complement of the proteomic study for investigating differences in proteins profile, after performance of the various pretreatment strategies. On virtual gel-image (Fig. 2), the sample of honeybee venom without any depletion approach indicated high fluorescent intensity and a lack of separated bands above 100 kDa. However, the few prominent bands were obtained in range of 15–60 kDa. The sample of honeybee venom purified with the use of OMIX tips showed

bands especially above ~40 kDa, so detection and the appearance of new proteins were increased. The results for the use of ProteoMiner Protein Enrichment kit were different. The new bands in the range of 10–150 kDa were revealed, corresponding respectively to proteins with smaller molecular weight, which were not been seen before. A strong suppression of high molecular weight proteins was also observed.

4. Conclusions

The shotgun proteomic strategy and diverse sample enrichment procedures such as the use of combinatorial peptide ligand libraries or solid phase extraction allowed the identification of 33 different honeybee venom proteins. Thus, 11 out of 12 known honeybee venom allergens were confirmed and 4 new, so far uncharacterized honeybee venom proteins, were identified (hypothetical proteins: LOC408666, LOC725074, LOC725163, LOC100577054). It can be summarized that both MS analysis with peptide profiling approach in the range of 900–4500 Da and virtual gel-image of proteins profiles in the range of 10–250 kDa demonstrate that the use of targeted enrichment strategies are justified because of the increased detection of peptides/proteins and exposing the previously hidden fragment of proteome. The method proposed in this study yields comprehensive data in proteomic characterization of natural products like honeybee venom. Furthermore, the newly identified and yet uncharacterized proteins should be further investigated in order to elucidate their roles in the venom of *A. mellifera*. In order to fully validate the proposed model continuation of proteomic characterization of honeybee venom is necessary. The conducted research will be extended in larger scale proteomic experiments using different depletion strategies and various honeybee venom samples of different origin and times of collection.

Ethical statement

The manuscript “Shotgun proteome analysis of honeybee venom using targeted enrichment strategies” has not been previously published yet and no other submission or publication will be made with these data.

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Conflict of interest statement

There are no conflicts of interest.

Transparency document

Transparency document related to this article can be found online at <http://dx.doi.org/10.1016/j.toxicon.2014.08.069>.

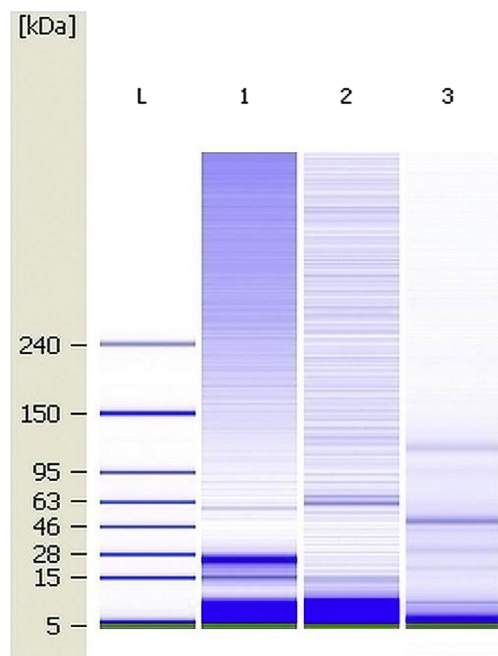


Fig. 2. Virtual gel-image of protein profiles of the honeybee venom samples run with LabChip High Sensitivity Protein 250 Kit. In the figure, lane L: protein ladder; lane 1: honeybee venom sample without depletion; lane 2: honeybee venom sample after use of OMIX tips; lane 3: honeybee venom sample after use of ProteoMiner Protein Enrichment kit. Green bands represent lower mass marker. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.toxicon.2014.08.069>.

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Table A.1. Honeybee venom proteins identified by nano-LC/MALDI-TOF/TOF-MS analysis conducted on Disposable Prespotted AnchorChip plate (based on NCBI nr database).

protein accession number	protein description	Mascot score	protein mass	protein cover [%]	protein isoelectric point	peptide expected mass	peptide calculated mass	peptide sequence
gi 5627 gi 58585172 gi 157833543 gi 385051077 gi 385051085 gi 24638082 gi 47117012 gi 229378	phospholipase A-2 [Apis mellifera] phospholipase A2 precursor [Apis mellifera] Chain A, Crystal Structure Of Bee-Venom Phospholipase A2 In A Complex With A Transition-State Analogue phospholipase A2 [Apis mellifera carnica] phospholipase A2 [Apis mellifera] RecName: Full=Phospholipase A2; AltName: Full=Phosphatidylcholine 2-acylhydrolase; AltName: Allergen=Api c 1 RecName: Full=Phospholipase A2; AltName: Full=Phosphatidylcholine 2-acylhydrolase; AltName: Allergen=Api d 1 phospholipase A	582	18475 19045 15239 19023 19015 15187 15236 14665	74.7 72.5 83.6 59.3 64.1 39.6 26.1 50.4	7.18 7.05 8.07 6.36 7.05 8.07 7.11 8.42	845.3857	844.3789	FYDCLK
						978.4521	977.4641	CLHYTVDK
						1035.4929	1034.4855	IGDNELEER
						1074.5078	1073.4989	VYQWFDLR
						1126.5708	1125.5607	MYFNLIDTK
						1144.5748	1143.5634	HGLTNTASHTR
						1160.5652	1159.5583	LEHPVTGCGGER
						1194.6056	1193.5901	NSADTISSYFVGK
						1197.5736	1196.5608	VYQWFDLRKY
						1254.5989	1253.5823	IIYPGTLWCGHGNK
						1388.6664	1387.6620	THDMCPDVMSAGESK
						1417.7330	1416.7190	LEHPVTGCGERTEGR
						1615.7992	1614.7977	CYKLEHPVTGCGGER
						1664.6677	1663.6640	LSCDCDDKFYDCLK
						1680.6564	1679.6590	
						1680.6584		
						1696.6526	1695.6539	
1697.8131	1696.7951							
1705.7589	1704.7712							
1781.6877	1780.7106							
1838.7100	1837.7321							

						2514.2166	2513.2148	NSADTISSYFVGKMYFNLDITK
gi 110758297	PREDICTED: phospholipase A2-like [Apis mellifera]	152	20520	21.3	8.81	1263.5542 1370.7332 1969.8180	1262.5237 1369.7255 1968.8207	EHDYCPDSIK HNLWNASLFLR NYNDLGFNHITDACCR
gi 328789531	PREDICTED: hypothetical protein LOC408666 [Apis mellifera]	245	35883	16.0	7.30	1889.8121 2128.8328 2330.9194	1888.8118 2127.8262 2329.9249	IVPIEHTQCTCDCK ETQECSTGFYFDQNSCR QSYVPEECTCACNNVDEQK
gi 66821891 gi 74835477 gi 301601654 gi 208342441	venom allergen acid phosphatase [Apis mellifera] RecName: Full=Venom acid phosphatase Acph-1; AltName: Allergen=Api m 3; Flags: Precursor venom acid phosphatase Acph-1 precursor [Apis mellifera] venom acid phosphatase [Apis mellifera]	228 208	43877 45360 46842 45251	40.8 39.2 37.9 27.6	5.63 5.74 5.53	888.5365 889.5229 1153.6249 1214.6074 1380.7518 1753.8099 1818.9102 1983.9994 2069.0098 2446.2651 2634.1858	887.5229 888.5181 1152.5927 1213.6125 1379.7449 1752.8035 1817.9056 1982.9850 2068.0000 2445.2573 2633.1809	LYGGPLLR QINVIFR EYQLGQFLR HMLDVVSGTQK IVYYLGIPSEAR DPYLYYDFYPLER ELQLPGCEVLCPLYK LQQWNEDLNWQPIATK FVDESANNLSIEELDFVK YLQLIENVIPSNEELICDKR YGDFLGDIYTEESVSALSSFYDR
gi 15988107 gi 58585182 gi 385051079	Chain A, Crystal Structure (Monoclinic) Of Bee Venom Hyaluronidase hyaluronidase precursor [Apis mellifera] hyaluronidase [Apis mellifera carnica]	184	40823 44232 44102	24.0 22.0 19.2	8.67 8.82	888.5182 1131.5928 1192.6322 1229.4987 1234.6187 1346.6794 1358.6691 1385.6309 1971.8743	887.4712 1130.5800 1191.6248 1228.5261 1233.6142 1345.6877 1357.6588 1384.6272 1970.8808	ADLEATLR VLPYYWYK DHLINQIPDK EHPFWDDQR QNWASLQPYK EYLNNELGPAVK YGQLFMEETLK REHPFWDDQR EFNVYWNVPFMCHK
gi 187281543	venom dipeptidyl peptidase 4 precursor [Apis mellifera]	167	87882	10.7	5.72	1316.5867 1355.6257 1599.8955 1744.8127	1315.5680 1354.6266 1598.8879 1743.8104	YEPLEEDHR NFNVNANGYTNK LGTVEIEDQIIITR VPFNLEETYDQSFR

						1910.8654	1909.8483	NDIYYQVFEGGSDTR
						2057.9272	2056.9126	GNANNIYEEETEGWLR
gi 229444 gi 223015 gi 14495012 gi 28201825 gi 58585154 gi 253722741 gi 354459555	melittin,pro melittin,prepro melittin [Polistes sp. HQL-2001] RecName: Full=Melittin; Flags: Precursor melittin precursor [Apis mellifera] Chain A, Structural Studies Of D-Pro Melittin, Nmr, 20 Structures Chain B, Chlamydomonas Reinhardtii Centrin Bound To Melittin	120 166	3700 7601 7497 8510 7580 2957 2846	41.2 21.4 22.4 19.5 55.6 57.7	4.87 4.69 10.03 6.03 4.69 12.03	1527.9016 1527.9028 1543.9009 1555.8752 1668.0099	1526.9072 1542.8844 1554.9021 1667.0134	VLTTGLPSLISWIK VLTTGLPCLISWIK VLTTGLPDLISWIK VLTTGLPALISWIKR
gi 110756431	PREDICTED: hypothetical protein LOC725074 [Apis mellifera]	157	8292	31.1	8.76	2714.0474	2713.0163	HGDSCVSSDCCPGTWCHTYANR
gi 60115688 gi 74835476	icarapin-like precursor [Apis mellifera] RecName: Full=Icarapin-like; AltName: Full=Venom carbohydrate-rich protein; Flags: Precursor	150	24773 24803	6.3	4.51	1454.8171 1582.9199	1453.8140 1581.9090	NVDTVLVLPSIER KNVDTVLVLPSIER
gi 34921475 gi 328794325 gi 94400907	RecName: Full=Allergen Api m 6; AltName: Allergen=Api m 6 PREDICTED: allergen Api m 6-like [Apis mellifera] allergen Api m 6 precursor [Apis mellifera]	141 90	7593 10008 9812	43.7 19.1 19.6	9.70 9.83	1052.4819 1092.4724 1109.5040 1185.5831	1051.4757 1091.4675 1108.4971 1184.5727	CPSNEIFSR ICAPGCVCRR CPSNEIFSR FGGFGGFGGLGGR
gi 187281550	venom carboxylesterase-6 precursor [Apis mellifera]	104	63596	4.7	9.33	994.5211 1884.9391	993.5244 1883.9305	VSTFTGNIR LYEAYEGIPYAQSPVGK
gi 229289 gi 1708948 gi 74787607 gi 296785039	mast cell degranulating peptide RecName: Full=Mast cell degranulating peptide; Short=MCD peptide; Short=MCDP; AltName: Full=Peptide 401; Flags: Precursor RecName: Full=Mast cell degranulating peptide; Flags: Precursor mast cell degranulating peptide preproprotein [Apis mellifera]	79	2590 5777 5816 5763	45.5 20.0	9.96 9.87 10.33 9.87	1102.5824 1159.6583 1315.7650	1101.6229 1158.6444 1314.7455	HVIKPHICR RHVIKPHICR
gi 229231 gi 58585166 gi 74813756	apamine apamin preproprotein [Apis mellifera] RecName: Full=Apamin; Flags: Precursor	70	2031 5220 5104	50.0 19.6	8.55 8.77 8.78	931.4595 988.4847	930.4593 987.4807	APETALCAR
gi 328781948	PREDICTED: venom acid phosphatase Acph-1-like [Apis mellifera]	63	48366	2.2	6.61	1153.6249	1152.6325	EYKIGKMLR
gi 58585070 gi 189212375 gi 58585188	major royal jelly protein 8 precursor [Apis mellifera] major royal jelly protein 8 [Apis mellifera] major royal jelly protein 6 precursor [Apis mellifera]	62 45	46927 46940 49754	6.0 2.7	6.00 6.54 5.89	1507.6487	1506.6627	YIDYDFGSDEKR

						1605.7725	1604.7624	VIYGFDFNDVNFR
gi 67010041 gi 189212377	major royal jelly protein 9 precursor [Apis mellifera] major royal jelly protein 9 [Apis mellifera]	56	48658 48487	9.0	8.70	849.5212 1041.5388 1250.7623 1384.5667	848.5120 1040.5251 1249.7506 1383.5619	TFVTILR NNVPIDVDR ILNAPVNQLIR YFDYNFGSDEK
gi 223850 gi 58585180 gi 74811304 gi 74811305 gi 74811306 gi 74811307 gi 110282998	secapin secapin preproprotein [Apis mellifera] RecName: Full=Secapin; Flags: Precursor	54	2867 8674 8675 8673 8758 8728 8659	32.0 10.4	9.84 9.51 9.21 9.26 9.45 9.26	972.5483	971.5440	YIIDVPPR
gi 88985004	RecName: Full=Vitellogenin-1; AltName: Full=Vitellogenin I; Short=VTG I; Contains: RecName: Full=Lipovitellin-1; Short=LV1; Contains: RecName: Full=Phosvitin; Short=PV; Contains: RecName: Full=Lipovitellin-2; Short=LV2; Flags: Precursor	53	187805	0.8	8.93	1340.6978	1339.7347	ISDAPAQVAEVLK
gi 66507455	PREDICTED: venom protease [Apis mellifera]	49	39458	3.4	6.39	1542.6365	1541.6279	YENNDPFVCCPK
gi 5902765 gi 328783352	RecName: Full=Chymotrypsin inhibitor; AltName: Full=AMCI PREDICTED: chymotrypsin inhibitor [Apis mellifera]		5969 7039	19.6 16.7	4.77 4.76	1367.6113 1367.6113	1366.6122	IGCQCQEGFLR

Table B.1. Honeybee venom proteins identified by nano-LC/MALDI-TOF/TOF-MS analysis conducted on AnchorChip Standard plate (based on NCBI database).

protein accession number	protein description	Mascot score	protein mass	protein cover [%]	protein isoelectric point	peptide expected mass	peptide calculated mass	peptide sequence
gi 5627 gi 58585172 gi 157833543 gi 385051077 gi 385051085 gi 24638082	phospholipase A-2 [Apis mellifera] phospholipase A2 precursor [Apis mellifera] Chain A, Crystal Structure Of Bee-Venom Phospholipase A2 In A Complex With A Transition-State Analogue phospholipase A2 [Apis mellifera carnica] phospholipase A2 [Apis mellifera] RecName: Full=Phospholipase A2; AltName: Full=Phosphatidylcholine 2-acylhydrolase; AltName: Allergen=Api c 1	615	19045	71.0	7.18	845.3829	844.3789	FYDCLK
						845.4027		
						1035.5002	1034.4855	CLHYTVDK
						1035.5055		
						1126.5740	1125.5607	VYQWFDLR
						1144.5735	1143.5634	MYFNLDTK
						1144.5808		
						1194.5238	1193.5070	FKHTDACC
						1194.5875	1193.5901	HGLTNTASHT
						1254.5948	1253.5823	LEHPVTGCGER
						1254.5994		
						1388.6719	1387.6620	NSADTISSYFVGK
						1615.8079	1614.7977	IYPGTLWCGHGNK
						1664.6667	1663.6640	THDMCPDVMSAGESK
						1664.6836		
gi 58585104 <gi 351637527; gi 351637643> <gi 351636801; gi 351636949> gi 351636957 <gi 351637149; gi 351637325> <gi 351637571; gi 351637623> <gi 351637329; gi 351637487> <gi 351637517; gi 351637689>	vitellogenin precursor [Apis mellifera] vitellogenin, partial [Apis mellifera]	527	202117	13.5	6.29	982.4993	981.4953	SFMELSLR
		71	<41709; 45132>	<10.9; 11.3>	<5.25; 5.31>	1051.5292	1050.5247	QNTWTVFR
		247	<33050; 34941>	<17.5; 18.9>	<8.70; 8.89>			
		56	25937	30.1	5.24	1188.6083	1187.6088	VGFVTPFEHR
		48	<24220; 25208>	7.8, 7.6	<5.61; 6.22>			
		55	<41735; 45158>	<8.1; 8.8>	<5.29; 5.49>	1197.5955	1196.5868	FMILCNLMR
		63	<15037; 15493>	<19.3; 19.8>	7.71, 7.74			
		188, 173,	<44057; 45408>	<10.9; 11.3>	<5.25; 5.50>			

<gij351636997; gij351637131> <gij351636969; gij351637141>		160 232	<200878; 25042> <22758; 25695>	<24.1; 29.2> <30.4;34.2>	<510; 5.22> <5.23; 5.12>	1221.6140	1220.6037	FDVSLALDGER
						1222.6471	1221.6394	SFFELLQNPK
						1313.7109	1312.7027	DVPTWEVNILK
						1370.7740	1369.7718	DLPISGKPFQIR
						1421.6476	1420.6445	LPYPWTMDNER
						1454.6552	1453.6408	INYHFGMTDNSR
						1525.7684	1524.7711	VEENEIIFEIYK
						1541.7649	1540.7535	SIHNNYPVHTFGR
						1688.7985	1687.7963	LGPCWHAVMTTYPR
						1776.8226	1775.8149	VMTRPDTDSENWTPK
						1785.9515	1784.9268	SIVGQLQVDTQGENAVK
						1825.9635	1824.9404	GTRPMIPDDNTSLALPK
						1910.8768	1909.8846	HDNSLYDEYIPFLER
						2047.0427	2046.0244	CEVLYDIAPLSDFVIHR
						2112.9919	2112.0024	SVNHLLTHHEYDYELSR
gij15988107gij58585182gij385051079	Chain A, Crystal Structure (Monoclinic) Of Bee Venom Hyaluronidasehyaluronidase precursor [Apis mellifera]hyaluronidase [Apis mellifera carnica]	467441	410514446044331	38.935.632.8	8.678.82	888.4956	887.4712	ADLEATLR
						1114.5582	1113.5526	IALNNNANDR
						1131.5864	1130.5800	VLPHYWYK
						1192.6400	1191.6248	DHLINQIPDK
						1229.5374	1228.5261	EHPFWDDQR
						1234.6252	1233.6142	QNWASLQPYK
						1346.7045	1345.6877	EYLNNELGPAVK
						1358.6589	1357.6588	YQQLFMEETLK
						1385.6436	1384.6272	REHPFWDDQR
						1977.0603	1976.0329	GEEIAILYDPGMFPALLK
gij328780886 gij328780884	PREDICTED: apolipophorins-like [Apis mellifera] PREDICTED: apolipophorins isoform 1 [Apis mellifera]	324 244	85160 202119	13.0 4.7	8.80 8.65	944.5002	943.4875	IDYLHQR
						994.5706	993.5647	LFLDALFR
						1000.5822	999.5865	LSVWNLLR
						1034.5748	1033.5457	YPERPGAAR
						1072.6323	1071.6189	NQFGLIKPR
						1122.5918	1121.5717	LTDAYEAAIR

						1231.7214	1230.7085	LGYLGVGQVIGR
						1267.6547	1266.6357	NFNFLELNTR
						1623.6543	1622.6527	DCSCLYEYGMIGR
						1698.7889	1697.7798	SGVVTDGGHFFTFDGR
						1742.7521	1741.7399	YCQQNSCENVAEIK
gi 66821891 gi 74835477 gi 301601654 gi 208342441	venom allergen acid phosphatase [Apis mellifera] RecName: Full=Venom acid phosphatase Acph-1; AltName: Allergen=Api m 3; Flags: Precursor venom acid phosphatase Acph-1 precursor [Apis mellifera] venom acid phosphatase [Apis mellifera]	291	44106	22.8	5.63	888.5192	887.5229	LYGGPLLR
			45588	21.9		889.5204	888.5181	QINVIFR
			47070	21.2	5.74	1214.6127	1213.6125	HMLDVVSGTQK
		168	45479	14.2	5.53	1380.7478	1379.7449	IVYYLGIPSEAR
						1753.8026	1752.8035	DPYLYDYFYPLER
						1983.9764	1982.9850	LQQWNEDLNWQPIATK
						2068.9998	2068.0000	FVDESANNLSIEELDFVK
gi 58585070 gi 189212375	major royal jelly protein 8 precursor [Apis mellifera] major royal jelly protein 8 [Apis mellifera]	212	47326 47339	12.3	6.00 6.54	1498.8203	1497.8304	LWVLDSGLINNVR
						1507.6754	1506.6627	YIDYDFGSDEKR
						1536.7272	1535.7117	SQYQANNVHYQGK
						1605.7750	1604.7624	VIYGFDFNDVNFR
gi 58585188	major royal jelly protein 6 precursor [Apis mellifera]	81	50154	2.7	5.89	1507.6754	1506.6627	YLDYDFGSDEKR
gi 187281543	venom dipeptidyl peptidase 4 precursor [Apis mellifera]	190	88338	15.7	5.72	1138.5940	1137.5931	IQPLYHDR
						1174.5837	1173.5778	QSIWEENR
						1261.6165	1260.6099	DGEIVATWTNR
						1599.8890	1598.8879	LGTVEIEDQIITR
						1744.8225	1743.8104	VPFNLEETYDQSFR
						2057.9160	2056.9126	GNANNIYEEETEGWLR
						2342.9739	2342.0121	FMGLPTPEDNQSGYNDTDVSR
						3018.4731	3017.4223	NLYSVQLSGNEPPVCLSCDVLTPEGNR
gi 60115688 gi 74835476 gi 94471624	icarapin-like precursor [Apis mellifera] RecName: Full=Icarapin-like; AltName: Full=Venom carbohydrate-rich protein; Flags: Precursor icarapin variant 2 precursor [Apis mellifera]	187	24830	15.2	4.51	1004.5175	1003.5120	EQMAGILSR
		89	24860	11.4	4.37	1283.6824	1282.6670	IPEQGVVWNK
						1454.8223	1453.8140	NVDTVLVLPsIER
						1582.9065	1581.9090	KNVDTVLVLPsIER
gi 328789531	PREDICTED: hypothetical protein LOC408666 [Apis mellifera]	186	37195	13.8	7.30	1668.6425	1667.6466	MWHPDLCSCFCR
						1889.8301	1888.8118	IVPIEHTQCTCDCK

						2128.8350	2127.8262	ETQECSTGFYFDQNSCR
gi 110756311 gi 328790048 gi 328780728 gi 328792775 gi 66531797	PREDICTED: ubiquitin-60S ribosomal protein L40 isoform 1 [Apis mellifera] PREDICTED: ubiquitin-60S ribosomal protein L40 isoform 3 [Apis mellifera] PREDICTED: polyubiquitin-A-like [Apis mellifera] PREDICTED: polyubiquitin-A-like isoform 1 [Apis mellifera] PREDICTED: ubiquitin-40S ribosomal protein S27a [Apis mellifera]	174 116	14979 15475 76874 89421 18083	24.2 23.5 4.5 3.9 11.5	9.87 9.84 7.71 6.86 9.83	1523.7902 2130.1809	1522.7740 2129.1480	IQDKEGIPPDQQR TLSDYNIQESTLHLVLR
gi 34921475 gi 328794325 gi 94400907	RecName: Full=Allergen Api m 6; AltName: Allergen=Api m 6 PREDICTED: allergen Api m 6-like [Apis mellifera] allergen Api m 6 precursor [Apis mellifera]	171 122	8163 10578 10382	46.5 21.3 21.7	9.70 9.83	1092.4893 1092.4948 1109.5028 1109.5170 1185.5825 1294.6241	1091.4675 1108.4971 1184.5727 1293.6136	ICAPGCVCVR CPSNEIFSR FGGFGGFGGLGGR GKCPSNEIFSR
gi 110758297	PREDICTED: phospholipase A2-like [Apis mellifera]	154	21148	15.5	8.81	1370.7369 1969.8258	1369.7255 1968.8207	HNLWNASLFLR NYNDLGFNHITDACC
gi 229444 gi 14495012 gi 223015 gi 28201825 gi 58585154 gi 253722741	melittin,pro melittin [Polistes sp. HQL-2001] melittin,prepro RecName: Full=Melittin; Flags: Precursor melittin precursor [Apis mellifera] Chain A, Structural Studies Of D-Pro Melittin, Nmr, 20 Structures	144 138	3700 7668 7601 8567 7580 2957	41.2 22.4 21.4 21.4 55.6	4.87 10.03 4.69 4.69 12.03	1511.9304 1527.9216 1568.9413	1510.9123 1526.9072 1567.9701	VLTTGLPALISWIK VLTTGLPSLISWIK VLTTGLPKLISWIK
gi 110756431	PREDICTED: hypothetical protein LOC725074 [Apis mellifera]	142	8691	31.1	8.76	2714.0645	2713.0163	HGDSCVSSDCCPGTWCHTYANR
gi 149939403gi 160425209	hexamerin [Apis mellifera]hexamerin 70a precursor [Apis mellifera]	119112	8154981522	2.36.3	6.436.46	1913.9211	1912.9166	IETSLNSDKPFTYNER
gi 48096138	PREDICTED: sorbitol dehydrogenase-like isoform 2 [Apis mellifera]	112	38575	6.3	6.71	805.4422 1670.7849	804.4494 1669.7947	IGDFVVR YANDYADALLASR
gi 66514614 gi 110773851	PREDICTED: chitinase-like protein Idgf4-like [Apis mellifera] PREDICTED: chitinase-like protein Idgf4-like, partial [Apis mellifera]	92	48996 42136	5.7 6.6	8.06 6.52	1296.6851	1295.6721	YLVLTETSQSR

						1616.7141	1615.7154	EGDYPAPIYESYGR
gi 67010041gi 189212377gi 66544205	major royal jelly protein 9 precursor [Apis mellifera]major royal jelly protein 9 [Apis mellifera]PREDICTED: major royal jelly protein 1-like, partial [Apis mellifera]	8752	489434877322989	9.57.6	8.708.87	849.5225 1017.5070 1250.7533 1622.7959	848.5120 1016.4927 1249.7506 1621.7849	TFVTILR IAIDEWDR ILNAPVNQLIR IANGDLNFNEVNFR
gi 229231 gi 58585166 gi 74813756	apamine apamin preproprotein [Apis mellifera] RecName: Full=Apamin; Flags: Precursor	79	2259 5505 5389	50.0 19.6	8.55 8.77 8.78	988.4928 988.5021	987.4807	APETALCAR
gi 5902765 gi 328783352	RecName: Full=Chymotrypsin inhibitor; AltName: Full=AMCI PREDICTED: chymotrypsin inhibitor [Apis mellifera]	70	6539 7723	16.7	4.77 4.76	1367.6232	1366.6122	IGCQCQEGFLR
gi 66535209 gi 328787562	PREDICTED: tubulin alpha-1 chain-like [Apis mellifera]	69	50619 53467	3.3 3.2	5.01 5.12	1701.9092	1700.8985	AVFIDLEPTVVDEV
gi 187281550	venom carboxylesterase-6 precursor [Apis mellifera]	64	63938	9.3	9.33	962.5041 982.5887 1208.6852 1484.6956 1797.8826	961.4869 981.5760 1207.6713 1483.6772 1796.8767	TIPFNENK FQPPRIK NIVFVAINYR SPVWYTYYSYR GSEDCLYINIYVPVR
gi 229289 gi 1708948 gi 74787607 gi 296785039	mast cell degranulating peptide RecName: Full=Mast cell degranulating peptide; Short=MCD peptide; Short=MCDP; AltName: Full=Peptide 401; Flags: Precursor RecName: Full=Mast cell degranulating peptide; Flags: Precursor mast cell degranulating peptide preproprotein [Apis mellifera]	61	2818 6062 6044 6048	40.9 18.0	9.96 9.87 10.33 9.87	1159.6583 1159.6658	1158.6444	HVIKPHICR
gi 58585086	transferrin 1 precursor [Apis mellifera]	54	80033	2.9	6.77	907.4807 1533.8007	906.4811 1532.7947	YLDVIER LASNAGYNVIEQVR
gi 328789222	PREDICTED: nucleobindin-2-like, partial [Apis mellifera]		61892	2.1	5.20	1332.6774	1331.6582	IPELHDHTNTR
gi 328782499	PREDICTED: proactivator polypeptide isoform 1 [Apis mellifera]	44	103057	1.1	5.32	1196.6511	1195.6383	LCEQLVGHLK

gi 223850 gi 58585180 gi 74811304 gi 74811305 gi 74811306 gi 74811307 gi 110282998	secapin secapin preproprotein [Apis mellifera] RecName: Full=Secapin; Flags: Precursor	43	2981 8845 8846 8844 8929 8899 9930	32.0 10.4	9.84 9.51 9.21 9.26 9.45 9.26	972.5572	971.5440	YIIDVPPR
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Table C.1. Honeybee venom proteins identified by nano-LC/MALDI-TOF/TOF-MS analysis with ProteoMiner strategy applied, conducted on AnchorChip Standard plate (based on NCBI database).

protein accession number	protein description	Mascot score	protein mass	protein cover [%]	protein isoelectric point	peptide expected mass	peptide calculated mass	peptide sequence
gi 229444 gi 223015 gi 14495012 gi 28201825 gi 58585154 gi 253722741	melittin.pro melittin.prepro melittin [Polistes sp. HQL-2001] RecName: Full=Melittin; Flags: Precursor melittin precursor [Apis mellifera] Chain A, Structural Studies Of D-Pro Melittin, Nmr, 20 Structures	265 316	3700 7601 7668 8567 7580 2957	41.2 21.4 22.4 19.5 21.4 55.6	4.87 4.69 10.03 6.03 4.69 12.03	1511.9221 1511.9230 1511.9334 1527.9153 1527.9248 1554.9316 1569.9257 1668.0217	1510.9123 1526.9072 1553.9181 1568.9178 1667.0134	VLTTGLPALISWIK VLTTGLPSLISWIK VLTTGLPNLISWIK VLTTGLPELISWIK VLTTGLPALISWIKR
gi 15988107 gi 58585182 gi 385051079	Chain A, Crystal Structure (Monoclinic) Of Bee Venom Hyaluronidase hyaluronidase precursor [Apis mellifera] hyaluronidase [Apis mellifera carnica]	211	41051 44460 44331	9.4 8.6 8.7	8.67 8.67 8.82	1971.8969 2184.1204	1970.8808 2183.0973	EFNVYWNVPTFMCHK MSWLFESDVLPSVYLR
gi 5627 gi 58585172 gi 157833543 gi 229378 gi 385051077 gi 385051085 gi 47117012 gi 24638082	phospholipase A-2 [Apis mellifera] phospholipase A2 precursor [Apis mellifera] Chain A, Crystal Structure Of Bee-Venom Phospholipase A2 In A Complex With A Transition-State Analogue phospholipase A phospholipase A2 [Apis mellifera carnica] phospholipase A2 [Apis mellifera] RecName: Full=Phospholipase A2; AltName: Full=Phosphatidylcholine 2-acylhydrolase; AltName: Allergen=Api d 1 RecName: Full=Phospholipase A2; AltName: Full=Phosphatidylcholine 2-acylhydrolase; AltName: Allergen=Api c 1	132 117 112 75 58 53	19045 19615 15809 15121 19536 19585 15806 15757	32.7 31.7 39.6 25.6 26.9 23.4 14.2 23.1	7.18 7.05 8.07 8.42 6.36 7.05 7.11 8.07	1126.5785 1144.5720 1194.6143 1254.6019	1125.5607 1143.5634 1193.5901 1253.5823	VYQWFDLR MYFNLIDTK HGLTNTASHTR LEHPVTGCGER

						1615.8099	1614.7977	IYPGTLWCGHGK
gi 60115688 gi 74835476 gi 94471624	icarapin-like precursor [Apis mellifera] RecName: Full=Icarapin-like; AltName: Full=Venom carbohydrate-rich protein; Flags: Precursor icarapin variant 2 precursor [Apis mellifera]	129 72	24830 24860 19609	11.2 6.3	4.51 4.37	1283.6743 1454.8257 1582.9230	1282.6670 1453.8140 1581.9090	IPEQGVVNWNK NVDTVLVLPsIER KNVDTVLVLPsIER
gi 328789222	PREDICTED: nucleobindin-2-like, partial [Apis mellifera]	99	61892	3.4	5.20	2097.0210	2096.0136	YLMEVVQALESDPEFQAK
gi 58585104 gi 351636773 gi 351636781 gi 351636785 gi 351636791 gi 351636795 gi 351636801 gi 351636809 gi 351636811 gi 351636821 gi 351636829 gi 351636833 gi 351636851 gi 351636873 gi 351636875 gi 351636877 gi 351636885 gi 351636889 gi 351636895 gi 351636897 gi 351636901 gi 351636909 gi 351636911 gi 351636913 gi 351636917 gi 351636921 gi 351636925 gi 351636929 gi 351636931 gi 351636935 gi 351636937 gi 351636943 gi 351636949	vitellogenin precursor [Apis mellifera] vitellogenin, partial [Apis mellifera]	97	202117 32115 32254 32155 32253 32281 33419 33331 33330 32722 33815 33619 32240 33718 33688 32835 33177 34228 33176 33078 32355 32799 32780 34941 33064 32908 33050 33336 33260 33320 33050 33517 32822	0.8 5.4 5.2 5.3 5.1 5.2 5.4 5.1 5.3 5.2 5.1 5.2 5.4 5.1 5.3 5.2 5.1 5.2 5.3 5.3 5.0 5.2 5.3 5.2 5.3	6.29 8.89 9.03 9.15 9.03 9.02 8.42 8.70 9.02 9.03 8.70 5.2 8.89 8.88 9.13 8.89 9.03 8.70 8.89 8.89 9.01 8.89 8.70 8.88 8.89 9.02 8.89 9.02 9.03	1910.8966 1909.8846	HDNSLYDEYIPFLER	

gi 66821891 gi 74835477 gi 208342441 gi 301601654	venom allergen acid phosphatase [Apis mellifera] RecName: Full=Venom acid phosphatase Acph-1; AltName: Allergen=Api m 3; Flags: Precursor venom acid phosphatase [Apis mellifera] venom acid phosphatase Acph-1 precursor [Apis mellifera]	96	44106 45588 45479 47070	5.9 5.7 5.5	5.63 5.53 5.74	1153.6023 1753.8079	1152.5927 1752.8035	EYQLGQFLR DPYLYYDFYPLER
gi 328778095	PREDICTED: lysosomal Pro-X carboxypeptidase-like [Apis mellifera]	87	56966	5.1	8.17	2637.3279	2636.2905	DLSTATNIVFSNGLMDPWSSGGVLR
gi 66507455	PREDICTED: venom protease [Apis mellifera]	86	40313	5.4	6.39	2175.1565	2174.1372	LIHPDYSTTTFVNDAVLR
gi 187281550	venom carboxylesterase-6 precursor [Apis mellifera]	78	63938	4.5	9.33	1208.6808 1797.8970	1207.6713 1796.8767	NIVFVAINYR GSEDCLYINIYVPVR
gi 335892796	peptidyl-prolyl cis-trans isomerase B precursor [Apis mellifera]	61	22744	4.9	8.42	1206.6209	1205.6081	VWFDINIDGK
gi 328776891	PREDICTED: renin receptor-like [Apis mellifera]	59	44295	4.3	5.03	2046.0525	2045.0218	FPLNVNEVEETTWQALR
gi 66545506	PREDICTED: calreticulin isoform 1 [Apis mellifera]	37	47487	3.5	4.45	1721.8427	1720.8243	DEICAIGFDLWQVR
gi 229289 gi 1708948 gi 74787607 gi 296785039	mast cell degranulating peptide RecName: Full=Mast cell degranulating peptide; Short=MCD peptide; Short=MCDP; AltName: Full=Peptide 401; Flags: Precursor RecName: Full=Mast cell degranulating peptide; Flags: Precursor mast cell degranulating peptide preproprotein [Apis mellifera]	32	2818 6062 6044 6048	40.9 18.0	9.96 9.87 10.33 9.87	1159.6567	1158.6444	HVIKPHICR

Table D.1. Honeybee venom peptides identified by nano-LC/MALDI-TOF/TOF-MS analysis with OMIX strategy applied, conducted on AnchorChip Standard plate (based on NCBI database).

protein accession number	protein description	Mascot score	protein mass	protein cover [%]	protein isoelectric point	peptide expected mass	peptide calculated mass	peptide sequence
gi 15988107 gi 58585182 gi 385051079	Chain A, Crystal Structure (Monoclinic) Of Bee Venom Hyaluronidase hyaluronidase precursor [Apis mellifera] hyaluronidase [Apis mellifera carnica]	627	41051 44460 44331	43.4 39.8 37.0	8.67 8.82	888.4721	887.4712	ADLEATLR
						1131.5850	1130.5800	VLPYYWYK
						1192.6323	1191.6248	DHLINQIPDK
						1229.5310	1228.5261	EHPFWDQQR
						1234.6449	1233.6142	QNWASLQPYK
						1267.6244	1266.6067	YGILQNWMDK
						1346.7029	1345.6877	EYLNNELGPAVK
						1358.6660	1357.6588	YGQLFMEETLK
						1385.6339	1384.6272	REHPFWDQQR
						1971.8699	1970.8808	EFNVYWNVPTFMCHK
						2109.1099	2108.0844	SFPGVGVIDFESWRPIFR
						2184.1052	2183.0973	MSWLFESDVLPSVYLR
						2238.0730	2237.0852	ITDLGADGFHWGSSDDINTK
gi 5627 gi 58585172 gi 157833543 gi 385051077 gi 385051085 gi 24638082 gi 229378 gi 47117012	Chain A, Crystal Structure Of Bee-Venom Phospholipase A2 In A Complex With A Transition-State Analogue phospholipase A2 [Apis mellifera] phospholipase A2 [Apis mellifera carnica] phospholipase A2 [Apis mellifera] RecName: Full=Phospholipase A2; AltName: Full=Phosphatidylcholine 2-acylhydrolase; AltName: Allergen=Api c 1 phospholipase A RecName: Full=Phospholipase A2; AltName: Full=Phosphatidylcholine 2-acylhydrolase; AltName: Allergen=Api d 1	451	19045 19615 15809	66.7 64.7 73.9	7.18 7.05 8.07	845.3870	844.3789	FYDCLK
						1035.4877	1034.4855	CLHYTVDK
						1074.5040	1073.4989	IGDNELEER
		433	19536 19585	52.7 56.3	6.36 7.05	1126.5690	1125.5607	VYQWFDLR
						1126.5769		
						1144.5702		
		124	15757 15121 15806	28.4 37.2 19.4	8.07 8.42 7.11	1144.5728	1143.5634	MYFNIDTK
						1254.5956		
						1253.5823		
		124	15757 15121 15806	28.4 37.2 19.4	8.07 8.42 7.11	1144.5728	1143.5634	MYFNIDTK
						1254.5956		
						1253.5823		
		124	15757 15121 15806	28.4 37.2 19.4	8.07 8.42 7.11	1144.5728	1143.5634	MYFNIDTK
						1254.5956		
						1253.5823		

						1388.6704 1388.6785 1566.8602 1615.8096 1664.6947 1705.7775 1838.7389	1387.6620 1565.8354 1614.7977 1663.6640 1704.7712 1837.7321	NSADTISSYFVGK SKPKVYQWFDLR IYPGTLWCGHGNK THDMCPDVM SAGESK CYKLEHPVTGCCGER LSCDCDDKFYDCLK
gi 110758297	PREDICTED: phospholipase A2-like [Apis mellifera]	142	21148	15.5	8.81	1370.7344 1969.8059	1369.7255 1968.8207	HNLWNASLFLR NYNDLGFNHITDACCR
gi 229444 gi 14495012 gi 223015 gi 28201825 gi 55977270 gi 58585154 gi 253722741	melittin,pro melittin [Polistes sp. HQL-2001] melittin,prepro RecName: Full=Melittin; Flags: Precursor melittin precursor [Apis mellifera] Chain A, Structural Studies Of D-Pro Melittin, Nmr, 20 Structures	330 310	3700 7668 7601 8567 7538 7580 2957	41.2 22.4 21.4 19.5 21.4 55.6	4.87 10.03 4.69 6.03 4.69 12.03	1511.9222 1511.9291 1511.9373 1511.9569 1527.9181 1527.9236 1555.8943 1668.0238	1510.9123 1526.9072 1554.9021 1667.0134	VLTTGLPALISWIK VLTTGLPSLISWIK VLTTGLPDLISWIK VLTTGLPALISWIKR
gi 66821891 gi 74835477 gi 208342441 gi 301601654	venom allergen acid phosphatase [Apis mellifera] RecName: Full=Venom acid phosphatase Acph-1; AltName: Allergen=Api m 3; Flags: Precursor venom acid phosphatase [Apis mellifera] venom acid phosphatase Acph-1 precursor [Apis mellifera]	255	44106 45588 45479 47070	17.2 16.5 16.0	5.63 5.53 5.74	888.5470 889.5298 1753.7998 1983.9982 2446.2954	887.5229 888.5181 1752.8035 1982.9850 2445.2573	LYGGPLL R QINVIFR DPYLYYDFYPLER LQQWNEDLNWQPIATK YLQLIENVIPSNEELICDKR
gi 187281543	venom dipeptidyl peptidase 4 precursor [Apis mellifera]	176	88338	9.8	5.72	1138.6116 1174.5863 1261.6133 1744.8365 1910.8602 2057.9290	1137.5931 1173.5778 1260.6099 1743.8104 1909.8483 2056.9126	IQPPLYHDR QISIWEENR DGEIVATWTNR VPFNLEETYDQSFR NDIYYQVFEFGSDTR GNANNIYEEETEGWLR

gi 58585070 gi 189212375 gi 58585188	major royal jelly protein 8 precursor [Apis mellifera] major royal jelly protein 8 [Apis mellifera] major royal jelly protein 6 precursor [Apis mellifera]	162	47326	9.1	6.00	1498.8304	1497.8304	LWVLDSGLINNVR
		49	47339 50154	9.2 2.7	6.54 5.89	1507.6744	1506.6627	YIDYDFGSDEKR
						1605.7710	1604.7624	VIYGFDNDVNFR
gi 328789531	PREDICTED: hypothetical protein LOC408666 [Apis mellifera]	156	37195	5.3	7.30	2128.8291	2127.8262	ETQECSTGFYFDQNSCR
gi 110756431	PREDICTED: hypothetical protein LOC725074 [Apis mellifera]	152	8691	31.1	8.76	2714.0493	2713.0163	HGDSCVSSSDCCPGTWCHTYANR
gi 60115688 gi 74835476 gi 94471624	icarapin-like precursor [Apis mellifera] RecName: Full=Icarapin-like; AltName: Full=Venom carbohydrate-rich protein; Flags: Precursor icarapin variant 2 precursor [Apis mellifera]	151	24830	14.8	4.51	1004.5128	1003.5120	EQMAGILSR
		92	24860 19609	11.4	4.37	1283.6771	1282.6670	IPEQGVVNWNK
						1454.8240	1453.8140	NVDTVLVLPSIER
gi 34921475 gi 328794325 gi 94400907	RecName: Full=Allergen Api m 6; AltName: Allergen=Api m 6 PREDICTED: allergen Api m 6-like [Apis mellifera] allergen Api m 6 precursor [Apis mellifera]	123	8163	46.5	9.70	1092.4751	1091.4675	ICAPGCVCRC
		74	10578 10382	21.3 21.7	9.83	1109.5063	1108.4971	CPSNEIFSR
						1185.5837	1184.5727	FGGFGGFGGLGGR
						1294.6318	1293.6136	GKCPSEIFSR
gi 229289 gi 1708948 gi 74787607 gi 296785039	mast cell degranulating peptide RecName: Full=Mast cell degranulating peptide; Short=MCD peptide; Short=MCDP; AltName: Full=Peptide 401; Flags: Precursor RecName: Full=Mast cell degranulating peptide; Flags: Precursor mast cell degranulating peptide preproprotein [Apis mellifera]	81	2818	45.5	9.96	1159.6526	1158.6444	HVIKPHICR
			6062 6044	20.0	9.87 10.33			
			6048		9.87	1315.7518	1314.7455	RHVIKPHICR
gi 229231 gi 58585166 gi 74813756	apamine apamin preproprotein [Apis mellifera] RecName: Full=Apamin; Flags: Precursor	61	2259	50.0	8.55			
			5505 5389	19.6	8.77 8.78	988.4910	987.4807	APETALCAR
gi 328782499	PREDICTED: proactivator polypeptide isoform 1 [Apis mellifera]	58	103057	1.1	5.32	1196.6500	1195.6383	LCEQLVGHLK
gi 223850 gi 58585180 gi 74811304 gi 74811305 gi 74811306 gi 74811307	secapin secapin preproprotein [Apis mellifera] RecName: Full=Secapin; Flags: Precursor	56	2981	32.0	9.84	972.5546	971.5440	YIIDVPPR
			8845	10.4	9.51			
			8846		9.21			
			8844		9.26			
			8929		9.45	972.5547		
			8899					

gi 110282998			8830		9.26	972.5604		
gi 187281550	venom carboxylesterase-6 precursor [Apis mellifera]	51	63938	7.5	9.33	920.4940 982.5919 994.5357 1025.5305 1208.6849	919.4916 981.5760 993.5244 1024.5229 1207.6713	WVFNNIK FQPPRPIK VSTFTGNIR LFNIDFEK NIVFVAINYR
gi 5902765 gi 328783352	RecName: Full=Chymotrypsin inhibitor; AltName: Full=AMCI PREDICTED: chymotrypsin inhibitor [Apis mellifera]	49	6539 7723	19.6 16.7	4.77 4.76	1367.6163	1366.6122	IGCQCQEGFLR
gi 67010041 gi 189212377	major royal jelly protein 9 precursor [Apis mellifera] major royal jelly protein 9 [Apis mellifera]	43	48943 48773	4.5	8.70	1017.5177 1250.7612	1016.4927 1249.7506	IAIDEWDR ILNAPVNQLIR
gi 328787755	PREDICTED: hypothetical protein LOC100577054 [Apis mellifera]		11512	12.1	6.81	1394.6783	1393.6734	GEVCVPYLGCIK