



Hyphenated LC–MALDI–ToF/ToF and LC–ESI–QToF approach in proteomic characterization of honeybee venom

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ABSTRACT

To increase in the depth characterization of venom proteome of *Apis mellifera* the hyphenated LC–MALDI–ToF/ToF–MS (liquid chromatography–matrix-assisted laser desorption/ionization–time of flight/time of flight tandem mass spectrometry) and LC–ESI–QToF–MS (liquid chromatography–electrospray ionization–quadrupole time of flight tandem mass spectrometry) techniques combined with combinatorial peptide ligand library enrichment method is proposed in this study. The novel approach simplifies pretreatment protocol in venom investigation. By using the protein preparation kit with sequential multi-step elution, the honeybee venom was dispensed into four different fractions. In total 269 proteins were detected, among these 49 honeybee toxins, allergens and components involved in mechanism of envenoming belonging to venom enzyme classes of esterases, proteases/peptidases, protease inhibitors, hydrolases and major royal jelly proteins. Moreover 5 additional putative toxins were identified. Their role in envenoming process was discussed. We concluded that different mass spectrometry techniques increased the detection of the honeybee venom proteins, underscoring the complementary character of analytical methods. The combination of MALDI and ESI ionization has resulted in numerous proteins identifications, not possible to reach with single proteomic technique. The study will contribute to broadening the knowledge about the complexity of honeybee venom. The newly identified proteins may serve not only as toxins and allergens, but also as substances with potential pharmacological activity. Although, the most detected proteins belong to trace elements of honeybee venom without toxic activity or action on vital system of victims, they should be taken into account in characterization of living organism response on *Apis mellifera* sting.

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1. Introduction

Animal venoms and toxins consist of proteins and peptides with potential medical and biotechnological application [1]. The detailed characterization of different venoms allows to assign their components to specific groups of allergens and toxins [2]. Inconsistently, despite the fact that the bite of various animals can be deadly for humans, the venom may also contain components with therapeutic values. Depiction of relation between the structure of the molecule and its function provides further opportunities for the development of not only antidotes against specific toxins but also can provide many benefits in clinical diagnosis and the development of new drugs [3]. Honeybee (*Apis mellifera*) venom (HBV) due to a variety of biological and pharmacological properties is nowadays subjected to many clinical studies [4]. The

effectiveness of HBV in various pathological conditions such as: pain [5], rheumatism [6] or cancer [7] was documented. HBV is a very complex mixture of proteins, peptides and other compounds like: biogenic amines, sugars, phospholipids, amino acids and pheromones. All high abundance HBV components (melittin, phospholipase A₂, apamine, mast cell degranulating peptide) have been determined using liquid chromatography (LC) and capillary electrophoresis (CE) methods with UV–vis or diode array detection [8–13]. Mass spectrometers equipped with a MALDI (matrix assisted laser desorption/ionization) and ESI (electrospray ionization) ion source have now become essential tools for the analysis which is not only limited to the identification of compounds already known, but by using protein sequence databases it is possible to identify previously unknown proteins and peptides with potential biological activity. The *A. mellifera* genome was sequenced in 2006 and more than 10,000 protein-coding genes were confirmed [14]. Since the beginning of the 21st century new proteomic strategies based on different separation techniques followed by mass spectrometry (MS) allowed to boost the detection of new components

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of this product and it was shown that the composition of HBV is more complex than it was supposed [15–21]. Independently conducted research in different groups confirmed the presence in HBV dozens of uncharacterized proteins which may potentially play role as toxins and/or allergens. The complexity of the HBV composition affects the difficulty of full proteomic analysis of this product. This problem can be partially dissolved by using different enrichment strategies and/or applying LC–MS techniques [22]. Despite the depletion and cleaning procedures HBV samples need to be separated before mass spectrometry analysis. There are many reports investigating the honeybee venom proteome applying the classical two-dimensional electrophoresis approach. However, as stated by Van Vaerenberg et al. [18], lack them sensitivity to explore low abundant venom components which may be masked by the presence of high abundance proteins such as phospholipase A₂ and melittin. Furthermore, low molecular weight fractions remain undetected due to poor resolution in classical two-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (2D-SDS–PAGE) separation. Applying complex methodologies based on different enrichment strategies followed by LC–MS analysis was found to be more effective than 2D gel separation. However, it is still needed to look for new approaches based on modern analytical techniques which will allow full proteomic characterization of HBV. Therefore the aim of this study was to characterize honeybee venom composition using hyphenated LC–MALDI–ToF/ToF and LC–ESI–QToF techniques combined with combinatorial peptide ligand library enrichment. The implemented ProteoMiner Sequential Elution Small-Capacity Kit, Bio-Rad (Hercules, CA, USA), allows us to obtain four different fractions from HBV, instead of one used in earlier venom studies [18]. The ProteoMiner protein enrichment technology enriches and concentrates low abundance proteins and peptides that cannot be detected without any sample pretreatment. The mechanism is based on reduction of protein concentration differences by utility of diverse library of combinatorial peptide ligands. The added proteome is equalized as the library beads have equivalent binding capacity to most of the proteins contained in the sample. Thereby, the high abundant analytes are reduced, when less abundant are concentrated. The sequential elution kit employ multiple elution reagents to sequentially elute proteins and peptides based on their different properties. This enrichment strategy highly compressed the dynamic range of proteins and peptides of HBV. The newly developed approach provides a broad proteomic characterization of HBV and allows for detection of new putative toxins and allergens. To our knowledge, this is the first report where the presented methodology has been applied in proteomic description of honeybee venom.

2. Material and methods

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 Daltonics (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 from *Apis mellifera carnica* 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 product was characterized by high purity and no contaminating substances which could affect the MS analysis were present. The purity of honeybee venom samples was confirmed using high performance liquid chromatography method (HPLC) with diode array detection. The method used for this purpose has been earlier developed and validated in our laboratory [8]. The collected samples were subjected to HPLC analysis in order to check venom quality and determine its major components. The dried samples were stored until analysis at 5 °C in darkness and dissolved directly before analysis.

2.3. ProteoMiner fractionation

Honeybee venom samples were treated with combinatorial proteoligand libraries (CPLL) which are commercially available under the name ProteoMiner Sequential Elution Small-Capacity Kit, Bio-Rad (Hercules, CA, USA). The honeybee venom solutions were prepared by dissolving 60 mg of dried venom (collected according to the procedure described in Section 2.2) in 1 mL of 3% ACN in 50 mM ABC solution. Then the samples were sonicated for 10 min and centrifuged for further 10 min. Next, the clear solution was transferred into a reaction tube. Fractionation was performed according to the manufacturer instructions.

2.4. Nano-LC separation

2.4.1. Nano-LC separation for MALDI–ToF/ToF–MS

Prior to the nanoLC analysis of crude honeybee venom samples and all honeybee venom fractions obtained from proteominer treatment were digested according to the procedure modified from pierce in-solution tryptic digestion kit and were subjected to nano-LC analysis. The system consisted of: nanoflow HPLC system EASY-nano LC II (Bruker Daltonics, Bremen, Germany) and fraction collector Proteineer-fc II (Bruker Daltonics, Bremen, Germany). Peptides were concentrated on a trap column, NS–MP-10 BioSphere C18 5 μ m particle size, 120-Å pore size, 100 μ m inner diameter, 20 mm length (NanoSeparations, Nieuwkoop, Netherlands) and separated on a Acclaim PepMap 100 column C18, 3 μ m, 100 Å, 75 μ m $\times$ 150 mm (Thermo Scientific, Sunnyvale, CA, USA) by linear gradient of water (mobile phase A) and 90% ACN (mobile phase B), both containing 0.05% TFA. The gradient elution method was: 2–50% B in 96 min. The flow rate was maintained at 300 nL min^{−1} and the injection volume was 15 μ L which correspond to 1.5 μ g. 384 fractions of separated sample were automatically mixed with matrix solution and spotted onto a AnchorChipTM MALDI target (Bruker Daltonics, Bremen, Germany). Per fraction 80 nL of eluent is mixed with 420 nL matrix solution. Matrix solution was generated by mixing: 748 μ L of 95:5 (v/v) acetonitrile: 0.1% TFA, 36 μ L of saturated solution of HCCA in 90:10 (v/v) acetonitrile:0.1% TFA, 8 μ L of 10% TFA and 8 μ L of 100 mM ammonium phosphate monobasic. The instruments were controlled using HyStar 3.2 software (Bruker Daltonics, Bremen, Germany).

2.4.2. Nano-LC separation for ESI–q–ToF–MS/MS analysis

The analysis was performed using a split free nano LC system (Dionex nRSLC), Dionex, Sunnyvale, CA, USA. Before ESI–q–ToF–MS/MS analysis tryptic peptides were concentrated on a trap column, Acclaim PepMap C18, RSLC, 5 μ m, 100 Å, 100 μ m ID $\times$ 2 cm (Dionex, Sunnyvale, CA, USA) using 0.1% FA in water at a flow rate of 15 μ L min^{−1} and separated on a Acclaim PepMap 100

column C18, 2 μm , 100 $\text{\AA}$, 75 $\mu\text{m} \times 250 \text{ mm}$ (Thermo Scientific, Sunnyvale, CA, USA) by a linear gradient of water (mobile phase A) and ACN (mobile phase B), both containing 0.1% FA. The gradient elution method was: 4–40% B in 60 min. The flow rate was maintained at 300 nL min^{-1} and 1.5 μg was injected onto the column.

To enable automatic internal mass calibration of the data files in Data Analysis software (Bruker Daltonics, Bremen, Germany), the LC system was equipped with a 10-port valve on which a second nano trap column used as a loop to introduce a calibration solution, sodium formic acid (Na FA), into the mass spectrometer. In this setup valve position 1.2 is used for sample loading onto the trap column. At the same time Na FA cluster is eluting via the analytical column to the mass spectrometer. This produces a calibration segment at the beginning of the nano LC gradient. After the sample has been trapped (typically after 10 min at 15 $\mu\text{L/min}$), the valve is switched to position 10.1. This allows elution of peptides from trapping column to the analytical column and subsequent MS detection. During the elution of peptides (gradient) the second trap column is filled with Na FA via a syringe pump.

2.5. MALDI-ToF/ToF-MS analysis

Mass spectrometry analysis was performed using the MALDI-ToF/ToF instrument UltrafleXtreme equipped with a SmartBeam II laser [23]. (Bruker Daltonics, Bremen, Germany). Typical instrument setting for MS analysis was as follows: ion source 1, 25.09 kV; ion source 2, 22.59 kV; lens voltage, 7.89 kV; pulsed ion extraction time, 120 ns; matrix suppression mass cut off, m/z 700. All spectra were acquired by accumulating 4000 shots from 40 non-overlapping positions with a repetition rate of the pulsed laser of 2 kHz. By routine, a standard peptide calibration mixture in the mass range 700–3500 Da (Bruker Daltonics, Bremen, Germany) was analyzed for external calibration of the mass spectrometer. The MS/MS mode for protein identification was applied with the following setting: ion source 1, 7.50 kV; ion source 2, 6.75 kV; lens, 3.50 kV; reflectron 1, 29.50 kV; reflectron 2, 14.00; lift 1, 19.00 kV; lift 2, 3.00 kV, pulsed ion extraction time, 80 ns; fragments only. Precursor with a signal-to-noise ratio above 10 were automatically subjected to MS/MS analysis. The maximum number of MS/MS per fraction was limited defined by the signal-to-noise of 10 and reached up to 47. The control of the instrument, data acquisition, processing and evaluation was performed using the following software platforms: flexControl 3.4, FlexAnalysis 3.4 and WARP-LC 1.3 and ProteinScape 3.1 (Bruker Daltonics, Bremen, Germany).

2.6. ESI-q-ToF-MS/MS analysis

ESI mass spectrometry analysis was performed using an impact II instrument (Bruker Daltonics, Bremen, Germany). Before nano LC MS/MS measurements an external mass calibration was done using “TuneMix low concentration” (Agilent Technologies, Palo Alto, CA, USA) covering a mass range from 322 m/z to 2,122 m/z . The MS was operated with a Captive Spray Source (Bruker Daltonics, Bremen, Germany) at a source voltage of 1,600 V. The drying gas flow was set to 3 L min^{-1} and the heater operated at 150 $^{\circ}\text{C}$. For data dependent auto MS/MS measurements one of the default application methods from oToFControl, version 4.0.59 (Bruker Daltonics, Bremen, Germany) was chosen. This method features the following settings:

A mass range from 150–2,200 m/z is being acquired. The MS acquisition speed was set to 2 Hz. Instead of a fixed number of precursors ions, a fixed cycle time of 3 s. was chosen in combination with IDAS (Intensity Dependent Acquisition Speed) ranging from 4–16 Hz for the MS/MS experiments. The lower m/z limit for selection of a precursor ion was set to 300. After the acquisition of one

MS/MS spectrum per precursor ion, this m/z is excluded from the precursor selection for 2 min. “Reconsider if” was activated and set to a value of 3, so this precursor will be included again in the precursor selection list in the case that its intensity is increasing by the set factor of 3. “Exclude singly” was used to avoid the selection of singly charged background ions.

2.7. Database searching

The fragment ion spectra obtained from the ESI- and MALDI-MS/MS measurements were processed with ProteinScape 3.1 platform (Bruker Daltonics, Bremen, Germany). Tandem mass spectra were analyzed by searching the NCBI nr database with Mascot 2.4.1 search engine (Matrix Science, London, UK). The LC-ESI 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 peptide were included. The searches were taxonomically restricted to the “*Apis mellifera*”. For the ESI data the percolator was used [24]. For the LC-MALDI data we used the significant threshold of $p > 0.05$ set by the search engine.

The general search parameters for the MALDI-ToF data were as follows: trypsin digestion, peptide precursor mass tolerance: 35 ppm; fragment mass tolerance: 0.7 Da; peptide charge: +1; monoisotopic mass. For the ESI-MS/MS spectra the following parameters were used: trypsin digestion, peptide precursor mass tolerance: 10 ppm; fragment mass tolerance: 0.05 Da; peptide charge: +2, +3 and +4; monoisotopic mass.

In Mascot the maximum number of variable modification is limited to 9, as a long list of variable modifications in a Mascot database search significantly slows down the search speed. In addition for a maximum number of protein identifications a minimum number of optional modifications combined with strict enzyme settings are beneficial. On the other hand for a maximum number of matching peptides a broader profile of optional modifications and relaxed (unspecific) enzyme settings is required. For these reasons in total four different sets of parameters were used as follows:

Set 1: two missed cleavages; carbamidomethylation of cysteine as fixed modification; variable modifications: oxidation of Methionine, Histidine and Tryptophan

Set 2: one missed cleavage; carbamidomethylation of cysteine as fixed modification; variable modifications: oxidation of Methionine, Histidine and Tryptophan; Methylation of Aspartic Acid and Glutamic Acid; Dioxidation of Methionine; Deamidation of Asparagine and Glutamine; Glutamine to Pyroglutamic Acid (N-term)

Set 3: semitrypsin; one missed cleavage; carbamidomethylation of cysteine as fixed modification; variable modifications: oxidation of Methionine, Histidine and Tryptophan

Set 4: semitrypsin; two missed cleavages; carbamidomethylation of cysteine as fixed modification; variable modifications: oxidation of Methionine

At the end for the ESI data the search results from Set 1, Set 2 and Set 4 were combined into one protein list using ProteinExtractor [25] algorithm in ProteinScape (Bruker Daltonics, Bremen, Germany). For the MALDI data all 4 sets were combined into one list of identified proteins.

All uncharacterized proteins were analyzed through Basic Local Alignment Search Tool (BLAST, <http://www.ncbi.nlm.nih.gov/>) similarity search using the National Center for Biotechnology Information (NCBI) gi identifiers as the query. The searches were performed against a nonredundant protein database using protein-protein BLAST algorithm. Hits were considered statistically confident with color code in accordance with the BLAST scoring scheme.

3. Results and discussion

The aim of this study was to propose a novel strategy based on hyphenated techniques to proteomic characterization of honeybee venom. To gain an insight to honeybee venom proteome, we combined combinatorial peptide ligand libraries (CPLL) and hyphenated mass spectrometric (nLC–MALDI–ToF/ToF–MS and nLC–ESI–Q–ToF–MS) characterization of in-solution digested proteins derived from *A. mellifera* venom. The CPLL methodology was broadly used in description of numerous biological samples, including human [26–29] and plant specimens [30–33]. In venomics investigations, this technique was first highlighted by Calvete et al. [34,35]. The analysis of *Cotinus atrox* venom proteome proved that each ligand library captured a different set of proteins, justifying the property of choice for this methodology in our study. Previously, the CPLL enrichment strategy was followed by gel electrophoresis separation (SDS–PAGE or 2-DE), where in-gel digested proteins were subjected to MS/MS analysis [18,34,35]. It is obvious that the concept of sample de-complexation and fractionation is an important issue before proteomic mass spectrometry investigation. However, the method is not limited only to gel electrophoresis, which is more labor intensive considering slice-by-slice analysis and is lacking in lower molecular weight proteins in the gel [36]. Therefore, the approach of direct in-solution analysis merged with fractionation based on “democratic proteome” may raise a novel work flow in exhaustive mass spectrometry analysis in the field of venomics. The main novelty of this study compared to previous published works by our group [19,20], is the implementation of the combinatorial heksapeptide ligand library with multistep elution approach, which allow us to obtain four different fractions from honeybee venom, instead of one. The gradient elution of various proteins and peptides with diverse characteristics has been never applied to honeybee venom investigation before. Moreover, the employment of both MALDI and ESI ionization techniques extended the sensitivity to detection of analyzed compounds, providing complementary data impossible to obtain by single run. Unambiguously, the proposed methodology revealed a detailed composition of honeybee venom proteome, as the highest so far identification of 269 proteins and peptides was observed [19,37,38]. Additionally, the presented strategy is characterized by less complicated and faster procedure in regard to sample fractionation, allowing the same to overcome the high dynamic range of proteins comprised in analyzed venom.

In order to gain the highest resolution in conducted studies, new nanoLC–MALDI ionization method has been developed, comparing to previous study conducted by our group [20]. The 150 mm column dedicated to peptides separation and 96 min gradient allowed to obtain 384 fractions in 15 s intervals.

The newly developed nanoLC method yielded sufficient peak separation (sharp and regular shape of peaks with typical width of <2 min), what fulfilled optimal requirements for further MS/MS analysis. Using 1 Da as a minimal mass distance to co-eluting compounds the mean number of MS/MS per fraction are about 20, resulting in high identification score.

The analysis of the crude, none prefractionated, venom gives in LC–MALDI and LC–ESI, 662 and 2688 unique tryptic peptides respectively which provides biological evidence for 60 (LC–MALDI) and 163 (LC–ESI) venom proteins and peptides respectively. The detailed summary reports of identified honeybee venom proteins are presented in Tables S1–S6. To decrease the dynamic range of HBV components, in next step the venom samples were subjected to enrichment procedure applying ProteoMiner. High abundant peptides, particularly melittin and phospholipase A₂, block binding sites at the C18-ligands of the solid phase of the column. Therefore using the ProteoMiner approach was justified for enrichment of low-abundance proteins and increasing its capability of binding to column source. The prefractionation strategy reduced the

limitation of the dynamic range with respect to the nanoLC separation. Although the CPLL technology has been used for exploring honeybee venom proteome, we have utilized fractionation gradient elution, which allows to elute low abundant HBV constituents of different hydrophobicity. All the obtained fractions (F1–F4) were analyzed with hyphenated LC–MS techniques (Table 1 and Tables S1–S6). According to the literature data, the 1-D SDS–PAGE separation followed by the LC–MS/MS analysis yields the maximum protein coverage [18,36]. However, in our presented shotgun based approach it was demonstrated that time consuming and laborious electrophoretic prefractionation can be omitted, giving complementary data of investigated proteome. Fig. 1 presents the work flow of our approach and number of proteins identified using LC–MALDI and LC–ESI in four fractions. LC–ESI technique allowed to identify more proteins than LC–MALDI in case of fraction F1, F3 and F4. Finally, combining the results from crude venom and CPLL fractions allows to detect 135 unique proteins using LC–ESI and 27 unique proteins using LC–MALDI. From both LC–MS analysis we obtained in total as many as 269 proteins. The extra protein identifications by the LC–ESI are partially accounted due to the analytical set up on one hand and due to different search parameters in the Mascot DB searches on the other. As mentioned above for the LC–ESI–MS we used a longer analytical column with smaller particle size which increases the density of the chromatographic peaks as well as the number of overall MS/MS spectra recorded. In the database search parameter we used for each instrument the corresponding mass tolerances for precursor and fragment masses which are for LC–ESI–MS precursor 10 ppm versus 35 ppm for the LC–MALDI–MS and for the LC–ESI–MS/MS fragments 0.05 Da versus 0.7 Da for the LC–MALDI–MS/MS data. Also due to the usage of Mascot's percolator [39] in the database search of the LC–ESI–MS/MS data we increased protein id's up to 20%. This was not feasible for the LC–MALDI–MS/MS data due to lack of the total number of MS/MS spectra which we limited to a signal-to-noise of 10. A basic difference effecting sensitivity is due to differences in the ionization process of ESI versus MALDI. The fact that there are extra proteins found in the MALDI measurements and vice versa emphasizes the complementary character of these two mass spectrometry techniques. However, the aim of this study was not primarily a comparison of LC–ESI–MS versus LC–MALDI–MS but the increase in the depth of analysis of honeybee venom by the application of CPLL in combination with hyphenated mass spectrometric techniques.

Although a long list of 269 proteins was found in the investigated venom, several compounds that were already found in other studies are missing in this experimental approach. For instance, some extra venom compounds were found using LC–ESI–FT–MS analysis [18]. Comparison of the experimental set ups reveal differences in methodology as well as in the mass spectrometric analysis which both result in an increased resolution in the detection of peptides. The increased resolution of the FT–ICR–MS instrument (if compare with ESI–qTOF and MALDI–TOF/TOF, the resolution is 2–3 and 5–10 higher, respectively) makes it less co-eluting peptides which still can be detected, isolated and fragmented in a FT–ICR–MS instrument where as in our set-up they might not be separated leading to a faulty mass assignment and overlaying fragment spectra which both reduces the chance of identification in a subsequent data base search. The differences between obtained results may be also due to the venom sample variation caused by different methods of sample collection [21] or seasonal variation [40]. Moreover, these discrepancies may also derive from variant pretreatment protocols used in individual laboratories [41].

The biological function of all identified proteins in honeybee venom samples was determined using different proteomic platforms UniProtKB (<http://www.uniprot.org/>), EMBL–EBI (<http://www.ebi.ac.uk/>). The HBV proteins (Table 1) were classified into different groups according to biological function, while ca. 13%

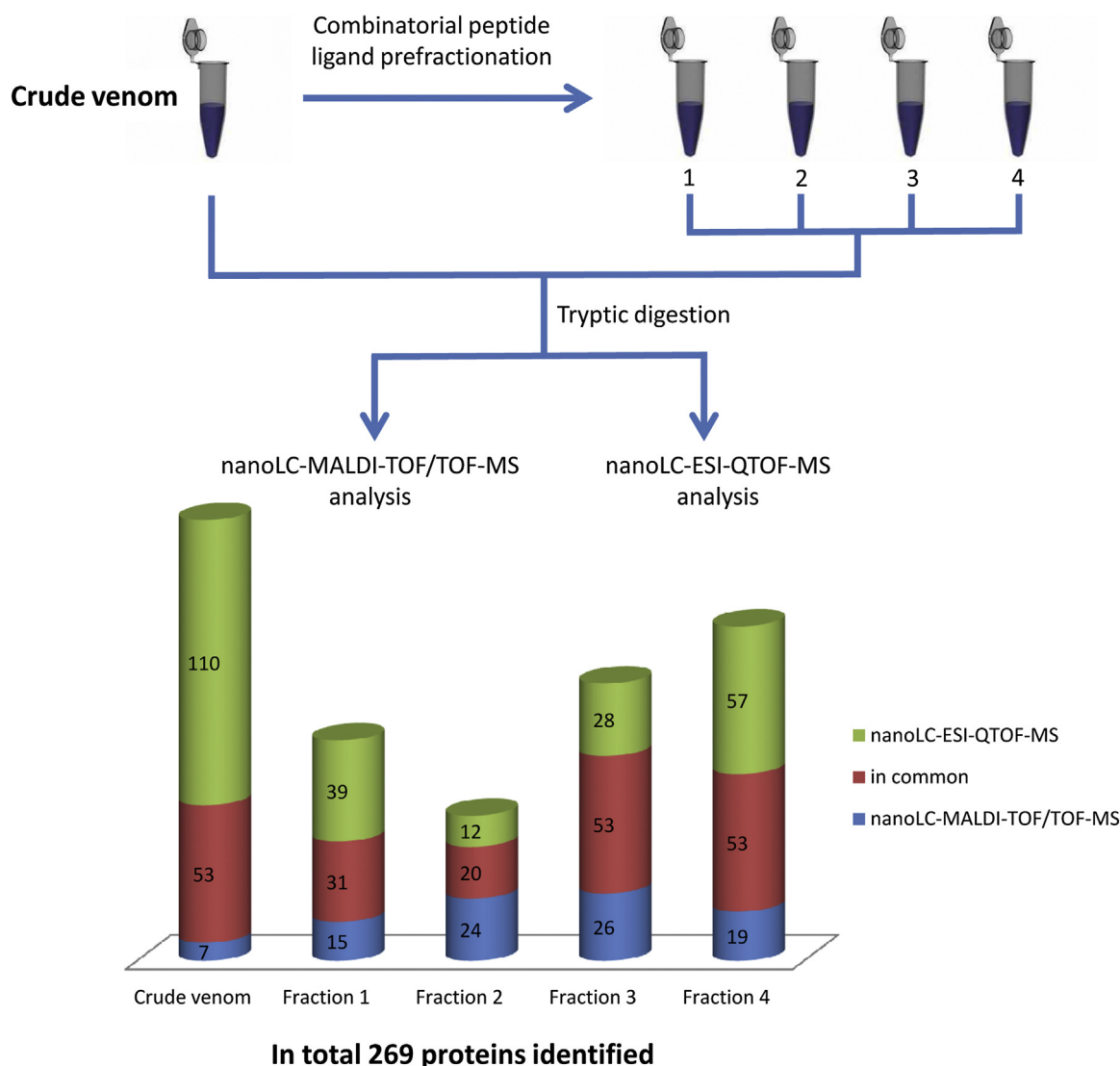


Fig. 1. The work flow of characterization of *A. mellifera* venom proteome by using hyphenated LC–MALDI–ToF/ToF and LC–ESI–QToF techniques combined with combinatorial peptide ligand library (CPLL) enrichment method. The number of proteins identified in four CPLL fractions is shown.

were uncharacterized with having unknown function (Table S1). In total 49 toxins, allergens and components involved in mechanism of envenoming were recognized, including all 12 honeybee venom components approved as allergens by International Union of Immunological Societies (Table 1). It should be highlighted that 5 toxins (antitrypsin-like, venom serine protease 34 precursor, A disintegrin and metalloproteinase with thrombospondin motifs 3-like isoform X1, putative serine protease K12H4.7-like isoform X2, serine protease 53) were only observed in LC–ESI analysis. 10 toxins were detected in crude venom as well as in all CPLL fractions using both methods. The main toxins belong to different enzyme classes, among this: esterases, proteases/peptidases, protease inhibitors, hydrolases and major royal jelly proteins. Six major royal jelly proteins (MRJPs) were recognized in our study. Besides two well reported MRJPs 8 and 9 [42], we identified MRJPs 1, 2, 3 and 5 [43], which may play an important role in honeybee nutrition [44]. The combined use of CPLL technology and high performance mass spectrometric methods allow to identify a broad spectrum of venom enzymes, in this rare described glucosidase 2 and β -galactosidase [18], involved in carbohydrate metabolism. Moreover, the proteomic characterization revealed two antioxidant enzymes, superoxide dismutase 1 and glutathione

S-transferase D1, known to protect against oxidative stress and tissue damage [45]. Although we were lacking in tertiapin identification, other small peptides with neurotoxic and antimicrobial function like mast cell degranulating peptide (MCDP) [19] and apidaecin [46] were detected. Finally, two high molecular weights proteins, hexamerin [47] and vitellogenin [48] were identified either in crude venom or in CPLL fractions, not observed in nanoLC FT–ICR MS/MS analysis conducted by Van Vaerenbergh et al. [18].

Despite of well described honeybee venom toxins [15–21], other uncharacterized proteins, which have been never observed before, were recognized in our study. Sequence homologies between these proteins and known toxins from different venomous organisms were determined using the BLAST server (Table S1). Among 34 predicted unknown bee venom constituents, 5 of them shown high-scoring homologies with toxic or allergic proteins. Protein encoded by *A. mellifera* gene, LOC100576326, was found to produce significant alignments with several forms of serine protease from: *Apis dorsata*, *Apis florea*, *Bombus terrestris*, *Megachile rotundata*, *Cerapachys biroi* and many other stinging insects. High degree of homology between Hymenoptera venom proteomes was clearly proven by Van Vaerenbergh et al. [41]. Applying the high resolution mass spectrometry preceded by CPLL pretreatment protocol to

analysis of venom derived from *Bombus terrestris* (bumblebee), they demonstrated 72% homology of identified compounds to honeybee venom composition. Thereby, Van Vaerenbergh et al. hypothesized that these constituents may share similar functions and subcellular locations. The newly identified protein LOC100576326 with potential toxic activity of serine protease can contribute in envenoming process. In bee venom these enzymes indicate dual function, acting either as an arthropod prophenoloxidase activating factor in phenoloxidase cascade causing lethal melanization or likewise to snake venom serine protease, showing fibrinolytic activity [49]. Further, sequence encoded by gene LOC100577725 shows very high similarities with venom chitinases with hydrolytic activity directed towards chitin (linear polysaccharide of (1,4)-linked-2-acetamido-2-deoxy-D-glucose) expressed i.a. in: *Cerapachys biroi* and *Nasonia vitripennis* [50]. For uncharacterized protein LOC100578816 significant match was found with probable weak neurotoxin NNAM3 present in *Pogonomyrmex barbatus*. The secreted neurotoxin binds with low affinity to muscular and neuronal nicotinic acetylcholine receptor. BLAST search with amino acid sequence encoded by LOC725074 allowed to find similarities with omega-conotoxin-like protein 1 (OCPL1) from different species of Hymenoptera order, which interact as an ion channel inhibitor toxin [51]. It was also shown that uncharacterized protein LOC100578392 shared the same conserved domain as insect allergen related repeat superfamily. These results pave a way for further detailed structure–function correlation analysis based on MS data of the unknown toxins and allergens present in the *A. mellifera* venom.

4. Conclusions

Applying the shotgun analysis, which include in-solution digestion of fractions and hyphenated mass spectrometric (LC–MS/MS) analysis, we were able to broadly characterize the HBV components. Additionally, the combination of two different mass spectrometry techniques, which are LC–MALDI–ToF/ToF–MS and LC–ESI–QToF–MS, increased the detection of the proteins, underscoring the complementary character of mass spectrometry performance. For the estimation of effectiveness of the CPLL strategy, the positive control–crude venom–was included. The results revealed that CPLL strategy allowed to obtain additional 75 proteins in LC–MALDI–MS and 79 proteins in LC–ESI–q–ToF–MS, if compared with crude venom analyzed with these two LC–MS techniques, respectively. We concluded that the highest number of identification was reached in LC–ESI–q–ToF–MS analysis of single crude venom. However, the most presented proteins belong to trace elements of honeybee venom, without toxic activity or action on vital system of victims. Regarding to dynamic range of proteins in complex samples like venom, detection of every component of a proteome is not possible with use of single proteomic technique. In our study, we have proved that the combination of MALDI and ESI ionization with different types of mass analyzer has resulted in numerous protein identifications, gaining the visualization of honeybee venom proteome.

Conflict of interest statement

There are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jpba.2016.01.009>.

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