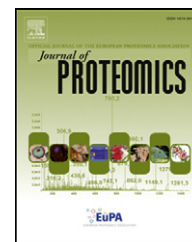


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Exploring the hidden honeybee (*Apis mellifera*) venom proteome by integrating a combinatorial peptide ligand library approach with FTMS

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ABSTRACT

At present, 30 compounds have been described in the venom of the honeybee, and 16 of them were confirmed by mass spectrometry. Previous studies typically combined 2-D PAGE with MALDI-TOF/TOF MS, a technology which now appears to lack sensitivity to detect additional venom compounds. Here, we report an in-depth study of the honeybee venom proteome using a combinatorial peptide ligand library sample pretreatment to enrich for minor components followed by shotgun LC-FT-ICR MS analysis. This strategy revealed an unexpectedly rich venom composition: in total 102 proteins and peptides were found, with 83 of them never described in bee venom samples before. Based on their predicted function and subcellular location, the proteins could be divided into two groups. A group of 33 putative toxins is proposed to contribute to venom activity by exerting toxic functions or by playing a role in social immunity. The other group, considered as venom trace molecules, appears to be secreted for their functions in the extracellular space, or is unintentionally secreted by the venom gland cells due to insufficient protein recycling or co-secretion with other compounds. In conclusion, our approach allowed to explore the hidden honeybee venom proteome and extended the list of potential venom allergens.

Biological significance

This study dug deeper into the complex honeybee venom proteome than ever before by applying a highly performing sample pretreatment and mass spectrometric technology. We present putative biological functions for all identified compounds, largely extending our knowledge of the venom toxicity. In addition, this study offers a long list of potential new venom allergens.

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1. Introduction

Honeybee venom is composed of a mixture of biogenic amines, peptides and proteins. The venom causes local tissue damage, which induces death in other insects and pain and inflammation in higher organisms [1]. Worker bees use their sting apparatus in order to defend the colony and their food stock.

Moreover, as it was recently demonstrated that in the bee hive venom peptides are smeared on the body surface of females and on wax combs, an additional function of bee venom in social immunity has been hypothesized [2].

Early efforts to unravel the bee venom composition date back to the work of Langer in 1897 [3]. Today, up to 30 venom proteins and peptides are described [4–22]. However, some of

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those lack a proper characterization. Several venom constituents isolated in the 1970s and 80s by chromatographic means are described by their enzymatic activity or amino acid composition, but amino acid sequences are often lacking, e.g. for minimine [21], cardioprep [4] and adolapin [5]. The development of proteomic methods, and later of the *Apis mellifera* genome [23], has boosted the detection of new bee venom compounds significantly [6,8,10,18,19].

Preceding studies investigating the honeybee venom proteome often combined 2-DE with MALDI-TOF/TOF MS [18,20]. However, this method lacks dynamic range and sensitivity to allow the detection of low abundant compounds. In addition, low molecular weight fractions remain largely unexplored due to poor resolution in classical SDS-PAGE separation. Therefore, previous studies suggested the existence of yet unknown venom compounds [18,20]. For example, a large part of a typical honeybee 2DE-gel is obscured due to high abundant compounds, such as phospholipase A₂ (PLA₂; 10–12% of the venom dry weight [24]), that mask the detection of low abundant compounds with similar molecular weight and pI.

Several studies have shown that the use of a combinatorial peptide ligand library (CPLL) can significantly improve the coverage of proteomic analyses as this allows to access many low abundant compounds in complex proteomes [25]. The method has been used in studies of the 'deep' venom proteome of two snake species, namely the Western diamondback rattlesnake (*Crotalus atrox*) [25] and the African puff adder (*Bitis arietans*) [26,27] and led to the discovery of a large number of proteins previously undetected in these proteomes. In this work, we now adopted CPLL that has so far not been used in Hymenoptera venomomics.

The modular arrangement of MALDI and ESI ionization with different types of mass analyzers has resulted in a wide variety of mass spectrometric instrumentation [28]. Many of them have been used in honeybee venom research, except FTMS, although this equipment provides the highest performance in mass resolution and mass accuracy [29]. In the context of venom research, only three research groups used this technology for protein identifications within the entire venom [30–33]. The discovery of a high number of new venom constituents in these studies points to a highly effective technology for identification purposes in these complex protein mixtures.

The present study aimed an in-depth analysis of the honeybee venom proteome by merging CPLL sample pre-treatment and nanoLC FT-ICR MS/MS. The CPLL flow-through and elution samples were separated using 1D-SDS-PAGE. Then, proteins and peptides were identified by a complete slice-by-slice LC-ESI-LTQ-FT-ICR MS/MS analysis of tryptic peptides. Such a sample decomplexation/fractionation before mass spectrometry is the best approach for maximum protein coverage [33]. Functions of the identified compounds were predicted using bioinformatics.

2. Material and methods

2.1. Venom collection

Mid July 2011, adult worker honeybees (*Apis mellifera carnica*) were collected at the hive entrance. Pure venom was collected as

previously described [18]. Venom of 150 honeybees was pooled to a protein concentration of 69.54 mg/ml, as determined by Bradford protein assay (Thermo Scientific Pierce, Hudson, NH, USA).

2.2. Protein enrichment

The dynamic range of protein concentrations in the honeybee venom sample was compressed by a CPLL approach (ProteoMiner protein enrichment small-capacity kit, Bio-Rad Laboratories, Hercules, CA, USA). This experiment was performed according to the instructions of the manufacturer. In brief, 200 µl of venom sample was added to the beads for 2 h at room temperature on a rotational shaker. Subsequently, the non-binding fraction was collected (=flow-through). Non-specific binding components were removed by 3 rounds of washing (150 mM NaCl, 10 mM NaH₂PO₄, pH 7.4). Bound proteins were eluted with 3 steps of 20 µl elution buffer (8 M urea, 2% CHAPS, 5% acetic acid) and pooled.

2.3. 1D-SDS-PAGE

Approximately 100 µg of both flow-through and eluted protein fractions were loaded on a 10% Tris-glycine-SDS-PAGE gel and separation was carried out at 140 V. In addition, 25 µg of flow-through proteins and 50 µg of elution proteins were separated at 100 V on a 16.5% Tris-tricine-SDS-PAGE gel. Gel separation was carried out on a Mini protean 3 system (Bio-Rad Laboratories, Hercules, CA, USA) and was continued until the blue bromophenol front reached the bottom of the gels. Tricine gel fixation was performed in 0.3% TCA for 30 min. Both gels were stained with Coomassie Brilliant Blue G250 and the background was destained with 30% MeOH. Proteins were reduced in-gel by adding 10 mM DTT/25 mM NH₄HCO₃ (56 °C for 45 min) and alkylated in 55 mM iodoacetamide/25 mM NH₄HCO₃ (RT for 45 min). Subsequently, the gel was washed in 25 mM NH₄HCO₃. All flow-through and elution protein bands larger than 40 kDa were cut out of the 10% glycine gel, while those smaller than 40 kDa were cut out of the 16.5% tricine gel. Also gel parts without any visible protein bands were excised and analyzed. Residual Coomassie staining was removed by washing the gel pieces in 150 µl of 200 mM NH₄HCO₃/50% ACN for 30 min at 37 °C. Gel pieces were dried in a speedvac (Thermo Savant, Holbrook, NY, USA).

2.4. In-gel digest

An in-gel tryptic digest was performed by adding 12 µl of trypsin solution (0.002 µg/µl in 50 mM NH₄HCO₃; sequencing grade modified trypsin, Promega, Madison, WI, USA) to each gel piece. After overnight incubation at 37 °C, the solution with hydrophilic tryptic peptides was collected. Hydrophobic tryptic peptides were extracted from the gel by two subsequent incubation steps (15 min at 30 °C) with respectively 60 and 40 µl of 60% ACN/0.1% formic acid. Hydrophilic and hydrophobic peptides of each gel piece were pooled, dried by speedvac and dissolved in 15 µl 2% ACN/0.1% formic acid.

2.5. LC-ESI-LTQ-FT-ICR-MS

Five µl of the tryptic peptide fractions was analyzed, with the exception of fractions obtained from heavily stained gel bands

for which only 2 µl was injected. LC-ESI-FT MS analysis was performed as described in previous research [32]. Subsequently, raw LC-MS/MS data were analyzed using the Mascot v2.3 search engine (Matrix Science, London, UK). MS/MS data were searched against the Amel4.5 NCBI Refseq (available at ftp://ftp.ncbi.nih.gov/genomes/Apis_mellifera/protein/; database contains 10570 sequences and 5504336 residuals) and Augustus9 (available at ftp://ftp.hgsc.bcm.edu/Amellifera/Amel_4.5GenePredictions/Augustus/; database contains 11,560 sequences and 7,698,817 residuals) protein prediction databases [34]. An automatic decoy database search was performed to enable false discovery rate (FDR) determination. The significance threshold was adapted to 0.001 to reach a FDR < 1% for the identity threshold of both database searches. Searches were executed with carbamidomethylation of cysteines as a fixed modification and oxidation of methionines as a variable modification. One tryptic miscleavage was permitted and peptide mass tolerance and MS/MS tolerance were set to 10 ppm and 0.3 Da, respectively. Precursor peptide charge states were set to 2+ and 3+.

2.6. Criteria for positive identifications

Setting the significance threshold at $p < 0.001$ led to a FDR of 0.32% for the Amel4.5 NCBI Refseq search and 0.93% for the Augustus9 search. We defined positive protein identifications as queries detected by at least two unique, bold and red (significant and top ranking) peptides from the Mascot output with an ion score ≥ 30 . In addition, the discovery of small peptides was enabled by allowing queries with a sequence coverage higher than 10% due to the detection of only one, bold and red (significant and top ranking) peptide with an ion score ≥ 30 . All protein identifications were merged in one list and all double identifiers were removed.

2.7. Sequence analysis

As not all sequences are correct in prediction datasets, we tried to determine the correct protein sequence for each identification. First, this was done by searching for available EST data by Blast searches against the Amel 4.5 scaffolds on Beebase [35], which shows available honeybee ESTs mapped on the genome. Second, UniProt Blast searches [36] were performed to find homologs in well-annotated species such as *Drosophila melanogaster*, *Mus musculus* and *Homo sapiens*. Sequence identity of the honeybee venom predictions and their homologs was evaluated using the ClustalW software (standard parameters; [37]). The combination of honeybee EST evidence and homology-based evidence was used to determine the correct protein sequences. A database containing all correct(ed) protein sequences was constructed, which was used for performing bioinformatic analyses further described in this section.

The presence of an N-terminal secretion signal peptide was verified using the SignalP4.0 server [38]. Sequences were truncated to a length of 200 amino acids and the D-cutoff for signalP-TM networks was set to 0.350. Next, to determine a putative function for protein predictions, protein signatures were searched by InterProScan [39] and peptidase and protease inhibitor families were searched by Blast MEROPS [40]. GO-terms were assigned using Blast2GO v.2.6.0 [41]: honeybee venom

proteins were subjected to a BlastP against the Swiss-Prot database using an expect value of $E = 10^{-3}$. Mapping and GO-annotation were performed using default parameters. Proteins existing in exosomes were searched in the exosome protein database ExoCarta (<http://www.exocarta.org/>). Also, similar proteins present in venoms of other species were searched by a stand-alone Blast search of the Apis venom sequence database against a constructed database containing all venom proteins present in GenBank, but lacking predicted venom sequences, *A. mellifera* sequences and patent sequences.

3. Results and discussion

3.1. Identification of honeybee venom proteins

Our in-depth analysis revealed an unexpectedly rich composition of the honeybee worker venom. The detection of 705 unique tryptic peptides provides biological evidence for 102 venom proteins and peptides (Supplementary Tables 1 and 2). This list includes 19 compounds found in preceding honeybee venom proteome analyses and 6 additional compounds described in a study of the honeybee venom gland proteome (see Tables 1 and 2). Interestingly, we also present the sequence data of three venom enzymes, yet only identified by enzymatic activity tests in studies published more than 30 years ago: the glucosidase 2, β -galactosidase and group XV PLA₂ sequences may represent the enzymes catalyzing the α -glucosidase [22], β -galactosidase [14] and lysophospholipase [15,16] enzymatic reactions, respectively. In addition, this study detected 83 new venom compounds, which was enabled by the combined use of the CPLL technology, the high performance mass spectrometric instrument (LC-ESI-FT-ICR-MS) and the improved honeybee gene prediction sets [34]. Indeed, the CPLL pretreatment clearly decreased the dynamic range of the venom sample (see Fig. 1). PLA₂ and melittin contents, comprising 10–12% and 50% of the total venom dry weight respectively [24], were diminished in the elution fraction while also a broad range of bands appeared which are not visible upon separation of an untreated venom sample. Besides, while previous honeybee venom mass spectrometry studies focused on a specific molecular weight range ([6]: 950–4000 Da; [10]: 750–15000 Da; [18–20]: SDS-PAGE gel lacks low molecular weight fraction), this study extended its search towards the complete molecular weight range.

Despite the high number of identifications, some of the previously reported honeybee venom compounds are missing in this study. The absence of the antigen5-like wasp venom paralog is not surprising as it has only been described as a venom gland transcript in winter bees [17], while this study focused on venom of summer bees. Also, four compounds described long ago (cardiopep [4], minimine [21], adolapin [5] and β -acetylaminodeoxyglucosidase [14]) are missing, as their sequence information has so far not been determined. In addition, small peptides, such as tertiapin [12], procamine [11], apidaecin [17] and mast cell degranulating peptide (MCDP) [10] are difficult to detect using our approach, although a MCDP tryptic peptide (HVIKPHICR) with an ion score of 26 was detected at less stringent search parameters ($p < 0.01$ and FDR of 4.05%). Finally, two high molecular weight proteins,

Table 1 – Identified putative venom toxins.

Name	Acc. N°	Allergen	HBV	HBV GL	Exosome	Similar venom compounds		
						Species	Evidence	Ref.
Esterases								
Phospholipase A ₂ -1	gi 58585172	Api m 1	x	x				
Phospholipase A ₂ -2	gi 110758297							
Group XV phospholipase A ₂	gi 328791555		x					
Acid phosphatase 1	gi 301601654	Api m 3	x	x	x	<i>Nasonia vitripennis</i>	P	[32]
Acid phosphatase 2	gi 328790726				x	<i>Pteromalus puparum</i>	T + EA	[65]
Acid phosphatase 3	gi 110768981				x	<i>Pteromalus puparum</i>	T + EA	[65]
5'-Nucleotidase	gi 66523706				x	<i>Gloydius blomhoffi</i>	P	[57]
Carboxylesterase	gi 187281550	Api m 8	x		x	<i>Nasonia vitripennis</i>	P	[32]
Proteases and peptidases								
CLIP serine protease	gi 66507455					<i>Bombus ignitus</i>	P	[51]
CUB serine protease 1	gi 58585116	Api m 7	x			<i>Apis cerana</i>	U	gi 146395065
CUB serine protease 2	gi 48101366					<i>Nasonia vitripennis</i>	T	[32]
Putative trypsin	au9.g8903.t1					<i>Nasonia vitripennis</i>	P	[32]
Serine protease snake	gi 328783264							
Dipeptidyl peptidase IV	gi 187281543	Api m 5	x		x	<i>Vespula vulgaris</i>	P	[44]
Serine carboxypeptidase	gi 226533687	Api m 9	x	x	x	<i>Crotalus adamanteus</i>	T	[66]
Prolylcarboxypeptidase	gi 328778095				x			
Metalloprotease	gi 110748908				x	<i>Eulophus pennicornis</i>	T	[67]
Protease inhibitors								
Api m 6	gi 94400907	Api m 6	x					
Serpin 1	gi 328793022				x	<i>Crotalus adamanteus</i>	T	[66]
Serpin 2	gi 328791596				x	<i>Crotalus adamanteus</i>	T	[66]
Serpin 3	gi 328780925				x	<i>Crotalus adamanteus</i>	T	[66]
Carbohydrate metabolism								
Hyaluronidase	gi 58585182	Api m 2	x	x		<i>Apis cerana</i>	T	[68]
N-sulfoglucosamine sulfohydrolase	gi 328793712					<i>Crotalus adamanteus</i>	T	[66]
Endochitinase	gi 66511507					<i>Nasonia vitripennis</i>	T	[32]
Growth factors								
Platelet-derived growth factor	gi 328789531		x	x	x	<i>Bitis gabonica gabonica</i>	P	[69]
Imaginal disc growth factor 4	gi 66514614			x		<i>Chelonus inanitus</i>	P	[70]
Major royal jelly proteins								
MRJP8	gi 58585070	Api m 11	x	x		<i>Chelonus inanitus</i>	P	[70]
MRJP9	gi 67010041	Api m 11	x			<i>Chelonus inanitus</i>	P	[70]
Peptides								
Melittin	gi 58585154	Api m 4	x			<i>Vespula maculifrons</i>	T	[71]
Apamin	gi 58585166		x					
Secapin	gi 58585180		x			<i>Vespa velutina nigrithorax</i>	U	gi 33321084
Other toxins								
C-type lectin	gi 328792562							
Icarapin	gi 60115688	Api m 10	x	x		<i>Apis cerana</i>	T	[72]

Table 1: All discovered putative toxins are classified according to their function, and GenBank/Augustus accession numbers (Acc No) and allergen names (Allergen) are shown (derived from <http://www.allergen.org/>). All of them contain a secretion signal peptide (derived by SignalP). Their finding in preceding honeybee venom (HBV) and venom gland (HBV GL) proteomic studies is marked with “x”. Also proteins which exist in the exosome protein database ExoCarta (derived from <http://www.exocarta.org/>) are indicated with “x” (Exosome). In gray, the results are shown of standalone blasts against a venom sequence database, which reveals the existence of similar venom proteins and venom gland transcripts (Evidence) of other species (Species). Type of evidence: P = venom protein; T = venom gland transcript; EA = enzymatic activity; U = unknown.

hexamerin [19] and vitellogenin [42], may be lacking due to venom sample variation (different collection method, spatial and/or seasonal venom variation) or technological variation (liquid versus gel-based proteomics).

3.2. Categorization of venom proteins

As low abundant compounds are enriched by the CPLL pretreatment, we expected that the extended venom protein list would contain many compounds which probably have no function once they are injected into the victim. These

so-called venom trace molecules only have a local function in the venom duct or reservoir or are released by leakage of the gland tissue [1]. In contrast, toxins are typically high abundant and are actively secreted by the venom glands to contribute to the venom defense or social immunity function. As such, we categorized the detected compounds in those two groups (Tables 1 and 2) based on their predicted subcellular location and protein function. As the CPLL treatment shifts relative protein abundances, we were unable to use protein abundance as a distinguishing parameter.

Table 2 – Identified venom trace molecules.

Name	Acc. no	SignalP	EX	ER	HBV	HBV GL	Similar venom compound		
							Species	Ev.	Ref.
Secreted proteins									
C1q-like protein	gi 221325614	x	x		x				
Lysozyme c-1	gi 328779578	x	x						
Peptidoglycan-recognition protein SA	gi 254910928	x	x						
Transferrin	gi 58585086	x	x			x			
Modular serine protease	gi 328780689	x					Nasonia vitripennis	P	[32]
Cathepsin F	gi 328788558	x					Crotalus adamanteus	T	[66]
Cathepsin K	au9.g225.t1	x					Mesobuthus eupeus	U	gi 148970410
Peptidylglycine α -hydroxylating monooxygenase	gi 328787622	x	x				Crotalus adamanteus	T	[66]
Apolipoporphins	gi 328780886	x	x						
	gi 328780884								
Dorsal-ventral patterning protein Sog	gi 328791019	x							
Laminin subunit γ -1	gi 328776171	x	x						
Endoplasmic reticulum									
Glucosidase 2, subunit α	gi 66500170	x	x		x		Crotalus adamanteus	T	[66]
Glucosidase 2, subunit β	gi 328789473	x	x	x					
Calreticulin	gi 66545506	x	x	x			Nasonia vitripennis	T	[32]
UDP-glucose:glycoprotein glucosyltransferase	gi 328786702	x	x	x					
Protein disulfide-isomerase A3	gi 66546657	x	x	x		x	Crotalus adamanteus	T	[66]
Peptidyl-prolyl cis-trans isomerase B	gi 335892796	x	x			x	Crotalus adamanteus	T	[66]
Hsc70-3	gi 229892214	x	x	x		x	Crotalus adamanteus	T	[66]
Hypoxia up-regulated protein 1 (Hsp70)	gi 328784616	x	x	x			Crotalus adamanteus	T	[66]
Endoplasmic (Hsp90)	gi 110758921	x	x	x			Crotalus adamanteus	T	[66]
dnaJ/Hsp40/ERdj3	gi 328790510	x	x				Crotalus adamanteus	T	[66]
Endoplasmic reticulum lectin 1	gi 328787701	x					Crotalus adamanteus	T	[66]
Protein disulfide-isomerase	gi 328790461	x		x			Crotalus adamanteus	T	[66]
Endoplasmic reticulum resident protein 29	gi 110751310	x		x			Crotalus adamanteus	T	[66]
Endoplasmic reticulum resident protein 44	gi 328777360	x	x	x			Crotalus adamanteus	T	[66]
Calumenin	gi 66509518	x		x					
Procollagen-lysine,2-oxoglutarate 5-dioxygenase 3	gi 328784759	x	x						
ERGIC-53	gi 328785297	x	x				Crotalus adamanteus	T	[66]
Golgi complex									
α -Mannosidase 2	gi 66514147	x							
Mannosyl-oligosaccharide	gi 328782017			x			Crotalus adamanteus	T	[66]
α -1,2-mannosidase isoform A									
Mannosyl-oligosaccharide	gi 328781530			x			Crotalus adamanteus	T	[66]
α -1,2-mannosidase isoform B									
Glycoprotein 3- α -L-fucosyltransferase A	gi 328785366	x	x						
Metallocarboxypeptidase	gi 328785691	x	x				Crotalus adamanteus	T	[66]
Nucleobindin	gi 328789222	x	x				Crotalus adamanteus	T	[66]
Calsyntenin-1	gi 328778301	x	x				Crotalus adamanteus	T	[66]
Lactosylceramide 4- α -galactosyltransferase	gi 328793424	x							
Lysosomes									
α -L-Fucosidase	gi 328793281	x	x				Crotalus adamanteus	T	[66]
β -Galactosidase	gi 110764149	x	x		x				
Aspartic protease (cathepsin D)	gi 66560290	x	x				Crotalus adamanteus	T	[66]
γ -Interferon-inducible lysosomal thiol reductase	gi 328785531	x					Microctonus hyperodae	T	[73]
Glucosylceramidase	gi 66511554	x	x						
Proactivator polypeptide	gi 328782499	x	x			x	Crotalus adamanteus	T	[66]
Plasma membrane proteins									
Renin receptor	gi 328776891	x	x				Crotalus adamanteus	T	[66]
V-ATPase subunit S1	gi 328783393	x					Crotalus adamanteus	T	[66]
Multiple inositol polyphosphate phosphatase	gi 328778827	x	x				Nasonia vitripennis	P	[32]
Mitochondrial proteins									
Phospholipid hydroperoxide glutathione peroxidase	gi 110756698	x	x				Bufo gargarizans	U	gi 89515096
Kynurenine-oxoglutarate transaminase	gi 328789112						Crotalus adamanteus	T	[66]
Proteins without signal peptide									
Hsp70Ab	gi 229892265		x				Crotalus adamanteus	T	[66]
Hsc70-4	gi 229892210		x				Crotalus adamanteus	T	[66]
Histone H4	gi 328789054		x				Pelinobius muticus	T	[74]
Ras-related protein Rab-1A	gi 328784309		x				Crotalus adamanteus	T	[66]
Ras-related protein Rab-11A	gi 328778735		x				Crotalus adamanteus	T	[66]

(continued on next page)

Table 2 (continued)

Name	Acc. no	SignalP	EX	ER	HBV	HBV GL	Similar venom compound		
							Species	Ev.	Ref.
Proteins without signal peptide									
Actin related protein 1	gi 297591985		x				<i>Crotalus adamanteus</i>	T	[66]
Tubulin β -1 chain	gi 48095525		x				<i>Crotalus adamanteus</i>	T	[66]
Tubulin β -chain-like	gi 110762983		x				<i>Crotalus adamanteus</i>	T	[66]
Moesin/ezrin/radixin homolog 1	gi 328784401		x				<i>Crotalus adamanteus</i>	T	[66]
Ubiquitin-60S ribosomal protein L40	gi 110756311		x				<i>Ophiophagus hannah</i>	T	[75]
Elongation factor 1- α	gi 58585198		x				<i>Crotalus adamanteus</i>	T	[66]

Table 2: All discovered venom trace molecules are classified according to their subcellular localization. GenBank/Augustus accession numbers (Acc No) are shown. The presence of a secretion signal peptide (derived by SignalP), ER-retention signal peptide (ER) and their finding in preceding honeybee venom (HBV) and venom gland (HBV GL) proteomic studies is marked with “x”. Also proteins which exist in the exosome protein database ExoCarta (derived from <http://www.exocarta.org/>) are indicated with “x” (EX). In gray, the results are shown of standalone blasts against a venom sequence database, which reveals the existence of similar venom proteins and venom gland transcripts (Ev.) of other species (Species). Type of evidence: P = venom protein; T = venom gland transcript; U = unknown.

The subcellular location of each identified compound was predicted by several parameters. Many compounds (86/102) contain an N-terminal secretion signal peptide, which allows to target proteins to the secretory pathway [endoplasmic reticulum (ER), Golgi complex, lysosomes]. Generally, proteins lacking this signal peptide are not actively secreted and were therefore assigned to the group of venom trace molecules (Table 2). The subcellular location of homologs of well annotated species (fruitfly, mouse and human) and the ‘cellular component’ GO-terms were chosen as additional parameters (Supplementary Table 3). In contrast to putative toxins which have secreted homologs and assigned GO-terms linked to the extracellular space, venom trace molecules are often found in intracellular compartments of the secretory pathway. Also, multiple compounds contain a C-terminal ER-retention signal (XDEL) which is indicative for ER-retained proteins (Table 2).

To derive a function for all identified compounds, we used different levels of information, i.e. known functions of homologs, the ‘molecular function’ and ‘biological process’ GO-terms, and the predicted functional domains (Supplementary Table 3). Only compounds with a (putative) function in defense or social immunity were assigned to the list of putative toxins (Supplementary Table 4). In addition, we searched for venom homologs of other species, found by venom gland transcriptome and venom proteome studies (Tables 1 and 2 and Supplementary Table 5).

This approach allowed us to identify 33 putative toxins and 58 trace molecules, which are listed according to their function (Table 1) and subcellular location (Table 2), respectively. At present, no function could be attributed to eleven compounds. They were categorized separately (Table 3) because they lack functional domains and/or similar annotated sequences.

3.2.1. Putative toxins

This study confirmed the presence of multiple toxins found in preceding honeybee venom analyses. Toxic functions have

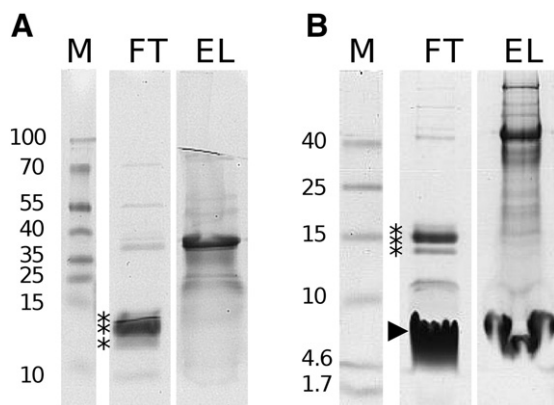


Fig 1 – Electrophoretic separation of a combinatorial peptide ligand library (CPLL)-treated honeybee 765 venom sample. CPLL flow-through (=FT) and elution (=EL) samples are separated on a 10% Tris-glycine-766 SDS-PAGE gel (A) and a 16.5% Tris-tricine-SDS-PAGE gel (B). Molecular weight regions which are known 767 to contain high amounts of PLA2 (*) and melittin (▶) are indicated. Molecular weights (in kDa) of the 768 markers (Thermo Scientific, Rockford, IL, USA) are indicated in the figure: A) PageRuler Prestained 769 Protein Ladder; B) Spectra Multicolor Low Range Protein Ladder.

Table 3 – Detected venom proteins with an unknown function.

Name	Acc. no.	SignalP	ER
Unknown function protein 1	gi 110748765	x	
Unknown function protein 2	gi 328778365	x	
Unknown function protein 3	gi 48132776	x	
Unknown function protein 4	gi 66546405	x	
Unknown function protein 5	gi 328781452	x	
Unknown function protein 6	gi 66518899	x	
Unknown function protein 7	gi 328783315		x
Unknown function protein 8	gi 328778397	x	
Unknown function protein 9	gi 328782411	x	
Unknown function protein 10	gi 110776960	x	
Unknown function protein 11	gi 328780111		

Table 3. The function of 11 detected proteins could not be revealed. GenBank/Augustus accession numbers (Acc. no) are shown and the presence of a signal peptide (SignalP) and ER-retention signal (ER) is indicated with ‘x’.

been proposed for most of them: phospholipase A₂-1, melittin, apamin, hyaluronidase, major royal jelly proteins (MRJPs) [43], dipeptidyl peptidase IV [44], Api m 6 [45] and CUB serine protease [46]. For others, such as icarapin and secapin, the function remains elusive, but we hypothesize that they have a toxic function as they have been detected with less sensitive technologies [8,10,17,18] and may therefore be moderately/highly abundant. Additionally, for some well-known but functionally non-characterized honeybee venom compounds we propose a function based on similar venom compounds from other species: first, in snake venom, acid phosphatase has been suggested to play a role in liberating purines (mainly adenosine). It acts as a multitoxin and potentiates venom-induced hypotension and paralysis [47]. Second, the platelet-derived growth factor may act similarly to snake venom VEGF-like molecules, which are the most potent vascular permeability factors known and which can facilitate venom spreading [48]. Two other enzymes, carboxylesterase and serine carboxypeptidase could play a role in degradation of insect neurotransmitters [49] and a wide range of proteins [50], respectively.

In addition, our study identified 17 new putative toxins. They belong to the classes of esterases, proteases, protease inhibitors, carbohydrate-degrading enzymes, growth factors and C-type lectins. Their putative functions are presented in Supplementary Table 4. All newly detected toxins may allow spreading of the venom and/or cause tissue damage. Despite the diminished amounts of the melittin peptide in the CPLL elution (Fig. 1), no new small peptides were discovered. As such, besides melittin and apamin (and also MCDP and apidaecin), no additional antimicrobial peptides playing a role in social immunity [2,6] were found.

Interestingly, our study revealed that honeybee venom contains multiple toxins belonging to the same protein class: five S1 serine endopeptidases, three acid phosphatases, three serpins and two group III PLA₂s were identified. Their combination with unique binding domains, as is seen in the group of serine proteases (no domain/CUB/CLIP), may allow a similar catalytic activity, but each directed towards a specific target. This activity may even be directed towards species-specific targets, as similar toxins could have evolved because of the biochemical arms race with specific attacker species belonging to the distinct classes of arthropods (wasp and robber bees stealing honey) and vertebrates (birds, mice). Moreover, the CLIP serine protease showed to be similar to a bumblebee (*Bombus ignitus*) CLIP serine protease, which was demonstrated to play a distinct role in insects and mammals [51]. Alternatively, similar proteins may perform variable catalytic functions due to sequence differences in the active site, as is seen in the newly detected acid phosphatases (APH2 and APH3) which contain an amino acid substitution in the active site septapeptide compared to APH1 (RHGXKXP → RHGXRXRP). Consequently, although multiple proteins belong to the same protein class, they may act upon a wide range of targets and/or exert different functions, which broadens the panel of toxins and allows the honeybees to efficiently defend the hive.

Multiple honeybee venom toxins show resemblance to toxins found in other Hymenopteran venoms. As honeybees are closely related to bumblebees and wasps, and as their venoms share the same functions, homologs are likely to

share similar functions. In contrast, venom homologs of parasitoid wasps may serve different functions as their venom is not used for defense, but influences the arthropod host's immunity, physiology, mobility, reproductive capacity and behavior to keep them alive and serve as food for their offspring [32]. Also multiple snake venom analogs (growth factors [48], hyaluronidases [52], C-type lectins [53], serine proteases [54], metalloproteases [55,56], acid phosphatases [47], nucleotidases [57,58] and PLA₂s [59]) were found, supporting evidence for convergent evolution.

3.2.2. Venom trace molecules

Preceding honeybee venom proteome analyses presumably found mostly abundant compounds using technologies with a lower sensitivity. Therefore, the C1q-like protein was so far the only honeybee venom trace molecule found in a mass spectrometric study [20]. de Graaf and coworkers already indicated that further digging in the venom proteome would yield additional low abundant venom trace molecules [1]. Indeed, more than half of the proteins (58/102) identified in this study appears to belong to this category. Their putative functions are briefly summarized in Supplementary Table 4.

We believe that some of them are actively secreted as they may have a local function in the venom duct or reservoir, playing a role in maturation (e.g. peptidylglycine α -hydroxylating monooxygenase) or stabilization (e.g. heat shock proteins) of the secretes. Others are secreted by diverse tissues for exerting essential functions in the extracellular space (e.g. immunity-related proteins and apolipoproteins). However, most of the venom trace molecules play roles in secretory pathway processes, such as protein folding, degradation and post-translational modification, N-glycan maturation and degradation, and sphingolipid metabolism. As several compounds contain an ER-retention signal, their presence in the venom may be explained by their unintentional release due to an inefficient retrieval and retrograde transport within the secretory pathway of the highly active secreting venom gland tissue. Other secretory pathway proteins may remain bound to toxins during their transfer through the secretory pathway and may be unintentionally co-secreted. Finally, the release of large secretory pathway-localized multiprotein complexes (e.g. the BiP complex) may contribute to the high number of detected trace molecules. The identification of few plasma membrane compounds in the venom may be explained by the release of their often large extracellular domains.

Remarkably, the venom contains two mitochondrial compounds and 16 compounds which lack a secretion signal peptide. First, both the phospholipid hydroperoxide glutathione peroxidase (GTPX) and kynurenine-oxoglutarate transaminase 1 (KAT) show the highest similarity to the *Apis cerana* [60] and *Aedes aegypti* [61] mitochondrial homologs, respectively. However, other GTPXs and KATs have been found in different subcellular compartments. As such, more research is needed to reveal the exact subcellular location of these honeybee proteins. Second, most of the compounds without a signal peptide are in a way connected to the secretory pathway, which might explain their presence in the venom. In contrast, the cytoskeletal proteins are typical cytoplasmic compounds, suggesting that they are released by a different process. Although apoptosis of cells of the

venom apparatus cell lining may contribute to their presence within the venom, we would expect to find a higher number of cytoplasmic proteins and even some nuclear compounds. However, all detected cytoskeletal proteins have been found in exosomes, which are small membrane vesicles of endocytic origin that are secreted in various extracellular fluids, including in the venoms of the snake *Gloydius blomhoffii blomhoffii* [62] and two solitary wasps [63]. As such, we propose that the selective, active process of exosome-mediated secretion [64] is responsible for their release. Moreover, as about 64% (58/91) of the identified proteins can be found in exosomes (Tables 1 and 2), it seems that this represents an important way of secretion in the honeybee venom gland.

3.3. Consequences for honeybee venom allergy

Systemic allergic reactions after a honeybee sting have been reported to occur in 0.8–5% of the general population and may be life-threatening [1]. Nowadays, already 12 honeybee venom allergens are immunologically characterized (Table 1). This mass spectrometric study analyzed venom of *A. mellifera carnica*, a subspecies which is the second most popular among beekeepers. Moreover, venom of adult worker honeybees was collected during the summer, when they actively forage. As the general human population is mainly stung by these foraging individuals, this proteomic study offers an extended list of potential new venom allergens.

4. Conclusions

The combination of the CPLL approach with FT-ICR MS/MS allowed us to explore the hidden honeybee venom. In total, 102 compounds were detected, which were categorized according to their putative function and/or subcellular localization. The 33 putative toxins belong to the classes of esterases, proteases, protease inhibitors, carbohydrate-degrading enzymes, growth factors, MRJPs and antimicrobial peptides. Their (predicted) biological function provides insights into the venom toxicity. In addition, our highly sensitive approach yielded a long list of low abundant venom trace molecules. As preceding studies described eleven honeybee venom compounds which remained undetected in this analysis, the honeybee venom is now largely extended to 113 compounds. Finally, this study offers a long list of potential new venom allergens.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jprot.2013.04.039>.

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