



Proteomic analysis of the honey bee worker venom gland focusing on the mechanisms of protection against tissue damage

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ABSTRACT

Honey bee workers use venom for the defence of the colony and themselves when they are exposed to dangers and predators. It is produced by a long thin, convoluted, and bifurcated gland, and consists of several toxic proteins and peptides. The present study was undertaken in order to identify the mechanisms that protect the venom gland secretory cells against these harmful components. Samples of whole venom glands, including the interconnected reservoirs, were separated by two-dimensional gel electrophoresis and the most abundant protein spots were subjected to mass spectrometric identification using MALDI TOF/TOF-MS and LC MS/MS. This proteomic study revealed four antioxidant enzymes: CuZn superoxide dismutase (SOD1), glutathione-S-transferase sigma 1 isoform A (GSTS1), peroxiredoxin 2540 (PXR2540) and thioredoxin peroxidase 1 isoform A (TPX1). Although glutathione-S-transferase (GST) has also been associated with xenobiotic detoxification, the protein we found belongs to the GST Sigma class which is known to protect against oxidative stress only. Moreover, we could demonstrate that the GST and SOD activity of the venom gland was low and moderate, respectively, when compared to other tissues from the adult honey bee. Several proteins involved in other forms of stress were likewise found but it remains uncertain what their function is in the venom gland. In addition to major royal jelly protein 9 (MRJP9), already found in a previous proteomic study, we identified MRJP8 as second member of the MRJP protein family to be associated with the venom gland. Transcripts of both MRJPs were amplified and sequenced. Two endocuticular structural proteins were abundantly present in the 2D-gel and most probably represent a structural component of the epicuticular lining that protects the secretory cells from the toxins they produce.

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1. Introduction

The Hymenoptera venom gland has an epidermal origin and evolved from the female accessory reproductive gland. The sting apparatus is derived directly from the ovipositor of their ancestral Clitogastra. In Aculeata it functions as an effective weapon for defence and its morphological

organization follows the same general pattern throughout this taxonomic entity. The venom gland is composed of one or more secretory filaments that open into a reservoir in which venom is stored. In the honey bee, *Apis mellifera*, it consists of a long thin, convoluted, and bifurcated tubule (Bridges and Owen, 1984). The venom gland is present in both the worker and the queen castes, but queens have significantly larger glands than the workers (Cruz-Landim et al., 1967). Queens use venom during fights with other rival queens, an event that occurs as soon as the imago emerges, while workers use venom for the defence of the

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colony and themselves when they are exposed to dangers and predators.

Honey bee venom consists of several toxic proteins and peptides. Melittin is a water-soluble toxic peptide comprising about 50% of the venom's dry weight. This helical amphipathic peptide consists of 26 amino acid residues and is capable of interacting with and disrupting cell membranes (Gauldie et al., 1976). Phospholipase A2 (PLA2), the second most abundant bee venom constituent – representing 10–12% of its dry weight – hydrolyzes the 2-acyl bonds of phosphatidylcholines, phosphatidylethanolamines, phosphatidylinositols and phosphatidylserines, disrupting membrane integrity and releasing lysophospholipids and fatty acids which themselves may further damage the membrane (Six and Dennis, 2000). Apamin is the smallest neurotoxin in bee venom and is composed of 10 amino acids containing two disulfide bridges. Apamin has long been known as a highly selective inhibitor of the Ca^{2+} -activated K^{+} channels (Banks et al., 1979). Mast cell degranulating (MCD) peptide is a 22 amino acid bicyclic peptide containing two disulfide bridges between Cys³ and Cys¹⁵, and Cys⁵ and Cys¹⁹, respectively. It stimulates mast cell degranulation at low concentration, and also inhibits histamine release in the presence of IgE at high concentration (Buku and Price, 2001). Finally, bee venom hyaluronidase is a hydrolytic enzyme that specifically cleaves hyaluronic acid, a large linear polymer (hyaluronan) consisting of simple repeats of a disaccharide composed of the β -1,4 linked D-glucuronic acid and N-acetyl-glucosamine. Hyaluronan and chondroitin sulfates are the most abundant glycosaminoglycans of vertebrate extracellular matrix and their cleavage by hyaluronidase facilitates the penetration of venom constituents into the body (Kreil, 1995).

The venom gland and reservoir have a number of morphological adaptations to protect the secretory cells from cytolytic activity of the chemical mixture they produce. These include the epicuticular lining of the venom gland and reservoir and, in the proximal region of the venom gland, the funnel-like valve between the duct and the end apparatus (Bridges and Owen, 1984). In addition, melittin is produced as promelittin in the venom gland and the membrane-bound enzyme dipeptidyl peptidase IV sequentially cleaves dipeptides ending at proline or alanine after its release into the venom duct and sac (Kreil et al., 1980). The melittin-precursor behaves completely different in terms of membrane binding and insertion, making it much less toxic (Wolfe et al., 1998). Besides, citrate has been identified as a major component of honey bee venom (Fenton et al., 1995) and has been demonstrated to inhibit metalloproteases, PLA2 and phosphodiesterase from venoms of several different species of reptiles and arthropods. Citrate can form strong complexes with divalent metal ions and thus may inhibit Ca^{2+} -dependent PLA2 activity by chelation of metals (Francis et al., 1992).

The present study was undertaken in order to identify other mechanisms involved in the protection of the venom gland secretory cells against the venom toxins. Samples of whole venom glands, including the interconnected reservoirs, were separated by two-dimensional gel electrophoresis and the most abundant protein spots were subjected to mass spectrometric identification using MALDI TOF/

TOF-MS and LC MS/MS. In order to describe how the found gene products putatively behave in a cellular context, we assigned to each detected protein its associated molecular functions, cellular components and biological processes using the controlled vocabularies offered by the Gene Ontology (GO) project (<http://www.geneontology.org>). Two terms of GO molecular functions received our full attention: the 'antioxidant activity' and the 'structural constituent of chitin-based cuticle'.

2. Materials and methods

2.1. Sample preparation

The whole sting apparatus was removed from 25 Carniolan honey bees (*Apis mellifera carnica*) after anaesthesia by chilling. Venom glands and their reservoirs were cut free and immersed in 250 μl lysis buffer (40 mM Tris-base, 8 M urea, 4% CHAPS and 5 mM DTT). The supernatant was collected after centrifugation. Protein concentration was determined by the method of Bradford.

2.2. Two-dimensional gel electrophoresis

Approximately 1 mg of protein was loaded on 17-cm immobilized pH gradient (IPG) strips, pH range 3–10 (Bio-Rad), and iso-electric focusing (IEF) was performed as described elsewhere (Vanrobaeys et al., 2003). The strips were then placed on lab-casted SDS-PAGE gels (15% T and 2.6% C). The second dimension electrophoresis was carried out on a Protean Plus System (Bio-Rad) and performed for 15 min at 10 mA/gel, followed by approximately 5 h at 20 mA/gel until the blue bromophenol front reached the bottom of the gel. The gels were stained overnight with Coomassie Brilliant Blue G-250.

2.3. In-gel digestion

Proteins were digested in-gel mainly as in Rosenfeld et al. (1992). Briefly, the excised spots were washed, air-dried and then digested overnight with 150 ng modified trypsin (Promega) in 50 mM ammonium bicarbonate at 37 °C. Peptides were extracted with 60% acetonitrile/0.1% formic acid in twice distilled water, then dried in a SpeedVac (Thermo Savant) and redissolved in 10 μl 0.1% formic acid.

2.4. Mass spectrometry and protein identification

Two mass spectrometric methods for protein identification were used: MALDI TOF/TOF-MS and LC MS/MS. MALDI TOF/TOF-MS was performed on a 4700 Proteomics Analyzer (Applied Biosystems). One microliter of the tryptic digest, mixed with an equal volume of 100 mM α -cyano-4-hydroxy-cinnamic acid in 50% acetonitrile/40% ethanol and 0.1% trifluoroacetic acid in water, was applied on a MALDI target plate. When the peptide mass fingerprint analysis was not conclusive, several peptides were subjected to further MS/MS analysis using collision-induced dissociation. Protein spots that were still not identified were subjected to LC MS/MS. The samples were loaded on an automated nano-HPLC system (Dionex Corporation)

and the separated peptides were detected on-line by a hybrid triple quadrupole/linear ion trap MS system (Q-TRAP, Applied Biosystems). More detailed technical information on the mass spectrometry analyses can be found in Vanrobaeys et al. (2003).

Beside the on-line protein NCBI database, our output files were searched against GLEAN3 with genome-derived protein predictions and two EST databases of the honey bee; they all were formatted to make them accessible for MASCOT searching (<http://www.matrixscience.com>). One missed cleavage was permitted and carbamidomethyl and methionine oxidation were selected as variable modifications. The searches were carried out with a tolerance of 100 ppm in the MS mode and 0.4 Da in the MS/MS mode for data obtained by MALDI MS, whereas the tolerance of 0.4 Da (MS mode) and 0.8 Da (MS/MS mode) were permitted for data obtained by LC MS/MS.

2.5. Comparative sequence analysis

Sequence similarity was investigated using the protein–protein BLAST algorithm (Tatusova and Madden, 1999) available at <http://www.ncbi.nlm.nih.gov>. The conserved domain architecture was predicted with CDART (Geer et al., 2002) and multiple sequence alignment was performed with the Clustal-W program (Thompson et al., 1994). The classification of the extended R&R motif was determined using profile hidden Markov models by a web-based tool at <http://bioinformatics2.biol.uoa.gr/cuticleDB/index.jsp> (Karouzou et al., 2007). The presence of a signal peptide was verified using the Signal P 3.0 Server at <http://www.cbs.dtu.dk/services/SignalP/>.

The identified proteins (bee venom components excluded) were putatively assigned the Gene Ontology (GO) terms of their associated molecular functions, cellular components and biological processes, using the on-line web tool (at <http://www.geneontology.org/index.shtml>). As none of the found proteins is included in the GO database, this was inferred via the best BLASTP matches.

2.6. Glutathione-S-transferase and superoxide dismutase assays

Glutathione-S-transferase (GST) and superoxide dismutase activity was determined using the GST Assay kit (Sigma) and the SOD Assay Kit-WST (Dojindo Molecular Technologies, Inc.), respectively. Next to the venom gland, tests were run (in duplicate) with other tissues from adult honey bees for comparison. The tissues (70 venom glands, 2 thoracic muscles, 10 midguts, 15 brains and 15 hypopharyngeal glands) were dissected separately on ice in phosphate buffered saline (pH 7.2) and homogenized by grinding and ultrasonication. Protein concentrations were adjusted to 5 mg/ml and the assays were performed in accordance to the manufacturer's instructions.

2.7. Reverse transcriptase-polymerase chain reaction (RT-PCR) and DNA sequencing

Venom gland cDNA was prepared from 50 glands as described in our previous study (Peiren et al., 2006) with the only exception that RNA was extracted using the RNeasy Mini Kit (Qiagen). Transcripts of major royal jelly protein 8 (MRJP8) and 9 (MRJP9) were amplified using the primer sets given in Table 1. The obtained amplicons were purified using the Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare) and subsequently sequenced on an ABI Prism 377 automated DNA sequencer (Perkin Elmer), using the ABI Prism BigDye V 3.1 Terminator Cycle Sequencing Kit (Applied Biosystems).

3. Results

The 2D-gel revealed approximately 350 protein spots (Fig. 1). The 62 most dominant spots were retained for further mass spectrometric analysis. A detailed list of the identified proteins is given in Table 2. More than one third (23 of 62) of them correspond to known bee venom proteins: PLA2 (spots 41, 43, 50–62), hyaluronidase (spot 42), acid

Table 1
Primers used to amplify the transcripts of MRJP8 and MRJP9 from venom gland tissue

Gene	Primer	Sequence	Expected length (bp)
MRJP8	MRJP8_P3_FWD	ACCGATCAATGGGGTGATAA	918
	MRJP8_P3_REV	TTCAAGAGGAAATCCATGCAC	
MRJP8	MRJP8_ORF_FWD	ATGATAAGATGGTTGCTGCTGA	1248
	MRJP8_ORF_REV	ACGAGATATGCAACGAGTATTC	
MRJP8	MRJP8_UTR_REV ^a	TGATTCAAGGAGATATGCAACGA	1256
MRJP9	MRJP9_P3_FWD	TTCGCAGATTGCTGCTTTTG	1134
	MRJP9_P3_REV	CCTCTATTTCGGAACGCAAG	
MRJP9	MRJP9_ORF_FWD	ATGTCITTTCAATATCTGGTGGT	1269
	MRJP9_ORF_REV	AAGGAAAATTGAGAAAAATTGTGT	
MRJP9	MRJP9_UTR_FWD	ATGTCITTTCAATATCTGGTGGTGTG	1586
	MRJP9_UTR_REV	CCTCTATTTCGGAACGCAAGA	

^a MRJP8_UTR_REV was used in combination with MRJP8_ORF_FWD.

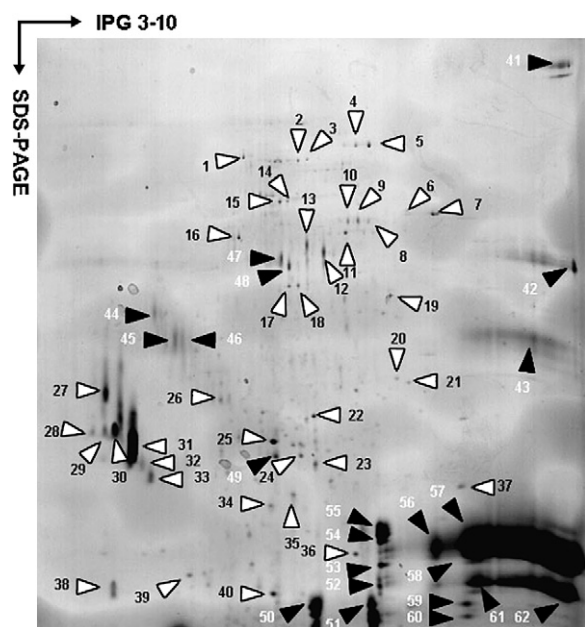


Fig. 1. 2D-gel of the honey bee venom gland stained with Coomassie Brilliant Blue G-250. Known venom proteins are marked with a black arrow. More information about the different spots can be found in Table 2.

phosphatase (spots 47–48), PVF-1 (spots 25 and 49) and icarapin (spots 44–46). The remaining 39 spots represent 27 proteins from venom gland and reservoir tissue. Spots 12 and 13 could unambiguously be identified as MRJP8, which belongs to the same major royal jelly protein family as the bee venom component MRJP9 (Peiren et al., 2005). The latter was not found in the examined spots of the present proteomic study. However, we were able to confirm the *in vivo* expression of both MRJP8 and MRJP9 by the venom gland using RT-PCR. The obtained transcript sequences of venom gland MRJP8 (GenBank accession number EU703874) and venom gland MRJP9 (GenBank accession number EU703875) correspond (99% identity) to the cDNA sequences obtained from the nurse head (MRJP8, AY398690) (Albert and Klaudiny, 2004) and the hypopharyngeal gland (MRJP9, DQ000307) (Albert and Klaudiny, 2007), respectively. Further, it was found that the deduced protein sequences of both venom gland MRJPs are characterized by the presence of a signal peptide.

BLASTP searching using the GO database gave a match for every venom gland/sac protein, with the exception of XP_624767 (spot 34; hypothetical protein), and the *P*-values varied between $3.6\text{e-}13$ and $5.5\text{e-}278$. The best matches were mostly *Drosophila melanogaster* proteins but the BLASTP search yielded also one human (SAP_HUMAN), one silk worm (IDGFL_BOMMO) and one honey bee (MRJP1) protein. Although the latter was strongly related to the query protein (MRJP8), it has several unique biological features. For instance, its C-terminal proteolytic fragment was identified as an antimicrobial peptide jelleine in royal jelly (Fontana et al., 2004). Consequently, the 3 GO biological processes that were assigned to MRJP8 but inferred via MRJP1 ('defence response to fungus', 'defence

response to Gram negative bacterium' and 'defence response to Gram positive bacterium') are probably not applicable and therefore eliminated from Table 3.

In general, 20 GO molecular functions were found, next to 12 GO cellular components and 17 GO biological processes (Table 3). Most abundant were proteins involved in 'antioxidant activity/response to oxidative stress' with four hits: AAP93581, BI513885, XP_624361 and XP_393445, corresponding to CuZn superoxide dismutase (SOD1), glutathione-S-transferase sigma 1 isoform A (GSTS1), peroxiredoxin 2540 (PXR2540) and thioredoxin peroxidase 1 isoform A (TPX1), respectively. The latter two belong to the same peroxiredoxin gene family. Based on the presence of the full consensus sequence (PVCT) at the N-terminus, XP_624361 corresponds to the 1-cys peroxiredoxin subgroup, whereas XP_393445, with the same conserved Cys in another motif (FYPLDFTFVCPTET) and the additional Cys in GEVCPA at the C-terminus, corresponds to the 2-cys peroxiredoxin subgroup, also known as the TPXs.

Since antioxidants are ubiquitously expressed, the finding of several corresponding GO terms does not necessarily mean that the venomous glands are especially rich in this kind of proteins when compared to other tissues. To address this concern, the GST and SOD activities of the venom gland and four other adult bee tissues were determined using commercialized assays. It was found that the venom gland GST activity was even lower when compared to that of the brains, the midgut and the hypopharyngeal glands (Fig. 2A), whereas the SOD activity was similar to that of the same three tissues (Fig. 2B).

Also interesting was the finding of two hits with the GO molecular function 'structural constituent of chitin-based cuticle' (GB16211-PA and GB18929-PA, corresponding to structural cuticle protein and pupal cuticle protein, respectively). Conserved domain searching revealed that both proteins have two copies of the extended form (68 AA, Pf00379) of the Rebers–Riddiford (R&R) consensus, known to establish the binding with chitin (Rebers and Willis, 2001). Detailed examination of the found R&R consensus sequences revealed that they were all of the RR-1 subgroup, typical for so called soft cuticles.

We further mention the identification of heat shock protein cognate 3 (HSC3; spots 1), heat shock protein cognate 5 (HSC5; spot 2) and Erp60 from which the two latter are involved in protein folding (GO biological process). Spot 34 was identified as a hypothetical protein (XP_624767) with a strong homology to *Nasonia vitripennis* apolipoprotein-III (third best BLASTP hit; bit score 148; E-value $2\text{e-}34$). Remarkably, we also found an imaginal disc growth factor-like protein (IDGF; spots 6 and 7) and odorant binding protein 14 (OBP14; spot 40).

4. Discussion

Protein cataloguing and profiling of the venom gland proteins of *A. mellifera* provides a general panorama of the structural elements of this very specialized secretory tissue and its physiological events. It is a requisite to gain insight into the mechanism of toxin secretion and gland cells protection against the same venomous final products.

Table 2
Protein identification on the 2-D gels of honey bee venom gland tissue

Spot no ^a	Protein name ^b	Acc. no. ^c	MW (Da) ^d	pI ^d	Ident. method ^e	Peptide mass ^f	Peptide sequences ^g	Mascot score ⁱ	Sequence coverage (%) ^j
1	Similar to heat shock protein cognate 3 CG4147-PA, isoform A	XP_393090	72,478 (70,556)	5.21 (5.14)	M & L	1505.78 1886.96 1786.98 1358.70 1944.02 1338.78	VFAPEEISAMVLGK VTHAVVTVPAYFENDAQR IINEPTAAAIAYGLDKK DVDEIVLVGGSTR DNHPLGKFDLTGIPPAPR ELTDIVQPIIAK	243	14 (14)
2	Similar to heat shock protein cognate 5 CG8542-PA	XP_392147	75,404	6.38	L	1461.75	SDIGEVLVVGGMTR	46	2
4–5	Transferrin	NP_001011572	78,657 (75,760)	6.77 (6.60)	M & L	1469.75 1044.54 2236.06 1532.79 1237.68 1131.67 1456.68 2477.27 1073.58 1773.75 1942.05 2388.12 2667.25 1747.77 1054.52 2217.15	IFTICVPEIYSK (5) GIPVSCISGR (5) EADVVAVDPEDMYLAVKDNK (5) LASNAGYNVIEQVR (5) DLPINNVLQGLR (5) NVGYKIPITK (5) CLAHNGGEIAWTK (5) RFFGLPVGVTAAIPTSENADYR (5) NSGATDKIIR (5) YDCTLEKSQDDCLK (5) AIKENNADLTVVSGSVLR (5) SCHSGYKDSFAGWTAPIYTLK (5) GLIKSENEAADFFSGSCAPGLDSK (5) LCQQCVGNLASNNDRIR (5) CNLGLPPR (5) SPTVLEELTHGTLAASTLYSK (5)	1099	36 (37)
6–7	Similar to imaginal disc growth factor 4 CG1780-PA, isoform A	XP_396769	48,741 (46,226)	8.06 (7.25)	M & L	1615.72 1087.64	EGDYPAPIYESYGR (7) IVVGIPTYAR (7)	116	6 (6)
8–10	Similar to CG4572-PB, isoform B [serine carboxypeptidase]	XP_393931	53,720 (51,579)	6.88 (6.35)	L	1468.74 1230.68 986.55	VWVFGNELAGYSK (9) TVDSLTEVLVR (9) WALDLITR (9)	139	7 (7)
11	Similar to ERp60 CG8983-PA, isoform A	XP_623282	55,857 (53,704)	5.57 (5.49)	M & L	1716.86 1626.87	AAEMLLDNDPSITLAK NPLVVAYYAVDYIK	144	6
11	Enolase	GB15039-PA	35,711	5.33	M & L	1845.91 1851.93 1595.89 2322.06 2236.06 1227.71 1345.62 1426.65 1609.84 2279.15	GNPTVEVDLVTDDGLFR SEVPSGASTGIHEALELR AISNINNTIGPELIK LAMQEFMILPTGASSFSAMK FGLDATSVGDEGGFAPNILDNK EALNLIIDSIK IGMDVAASEFYK SDPSTYLDSDSLK VNQIGTVTESINAHK ^h SGETEDTFIADLVVGLSTGQIK ^h	384	42

12	Major royal jelly protein 8	NP_001011564	46,927 (45,063)	6.00 (5.81)	M & L	1506.66 2546.26 2171.17 1180.57 2777.35 2740.29 1314.75 1604.76 1224.74	YIDYDFGSDEKR IEIDMCDRLWVLDGLINNVR SVCPPQLLVFDLNTSQLLK LTSSTFASDPR SQYQANNVHYQGKENILWTQASAK GISDNGVLFFGLVGDTSACWNENR NKETLQAITGLK VIYGFDFNDVNFR ILIANVNDLIK	586	36 (37)
13	Major royal jelly protein 8	NP_001011564	46,927 (45,063)	6.00 (5.81)	L	1506.66 1942.96 1497.83 2171.17 1604.76 1224.74	YIDYDFGSDEKR TGNGGPLLAPYPDWTWAK LWVLDGLINNVR SVCPPQLLVFDLNTSQLLK VIYGFDFNDVNFR ILIANVNDLIK	344	21 (22)
16	Similar to ATP synthase- CG11154-PA, isoform A	XP_624156	55,130	5.25	M & L	1277.63 2284.22 1366.75 2837.45 1087.63 974.55 1472.83 1438.78 1434.75 1987.03 1962.00 2890.45	TIAMDGTEGLVR GQSVLDGYPPIRIPVGAETLGR IINVIGEPIDER LAPIHADAPEFVDMMSVEQEILVTGIK VVDLLAPYAK IGLFGGAGVGK TVLIMELINNVR VALTGLTVAEYFR FTQAGSEVSALLGR AIAELGIYPVAVDPLDSTSR FLSQPFQVAEVFTGHAGK ILAGDYDHLPEVAFYVMVGPIEEVVAK	723	38
17–18	Arginine kinase	NP_001011603	40,008	5.66	L	1318.70 1146.61 1796.91	VSSTLSGLEELK (17) LIDDHFLFK (17) LGLTLFCPTNLGTTVR (17)	210	11
21	[Similar to phosphoglyceromutase CG1721-PA, isoform A]	GB15052-PA	28,991	7.07	M			99	
22	Similar to peroxiredoxin 2540 CG11765-PA	XP_624361	25134	5.88	M & L	1075.59 2344.22 2334.07 2203.07 1183.63 1610.90 1103.56	LAVHQPHFK LLAHSVDKLDQHDVWVNDIK SYCQDIPGAFYPPIADHDR LDMIDEISKDDPEQALTVR ALYIISPDHR VMILPTVKDEELPK GVDKVSMPSGK	307	47
23	Similar to saposin-related CG12070-PA, isoform A isoform 1	XP_392338	99,499 (97,857)	5.29 (5.26)	M		CTILINSYGK	37	
24	Similar to thioredoxin peroxidase 1 CG1633-PA, isoform A isoform 1	XP_393445	21,787	5.65	M	1581.73 1430.79 1224.68	DYGVLDEESGVPR GLFIIDDKQNLK QITINDLPVGR	79	19

(continued on next page)

Table 2 (continued)

Spot no ^a	Protein name ^b	Acc. no. ^c	MW (Da) ^d	pI ^d	Ident. method ^e	Peptide mass ^f	Peptide sequences ^g	Mascot score ⁱ	Sequence coverage (%) ^j
25	Glutathione-S-transferase S1, isoform A	BI513885	20,368	5.15	L	2038.15 943.55 2240.06 1591.75 1593.84 2120.12 1386.67	IKPTTPFGQVPVLDVDGKK KIAQSVAISR DDWEALEIDSIVDTIHQVR LAAFHVEENEIK KIADEVVPPYLER IADEVVPPYLERLDAQ SWLDKRPHSDF	349	(49)
25, 49	Similar to PDGF- and VEGF-related factor 1 CG7103-PA [PVF-1]	XP_392204	33,147	6.06	M & L	2306.99 1548.84 2127.83	CGGCCTHSLSCQPTATEIR (25) NFEILVTVLESSGK (25) ETQECSTGFYFDQNSCR (25)	143	16 (17)
27, 31–33	[Similar to CG12330-PA; structural cuticle protein]	GB16211-PA	29,924 (27,943)	4.47 (4.50)	M & L	2398.10	ALEWNAAHPEEDDGGQPRPPGR	50	8 (9)
28–30	[Similar to CG30045-PA; similar to pupal cuticle protein 78E, putative]	GB18929-PA	26,916 (25,153)	4.19 (4.19)	M & L	1920.93 2313.11 1139.52	ALDWIAAHPSKEDQNQV (30) AEDVIPVAQSQEGPNPDGSYK (30) WSYESGNGIK (30)	85	19 (21)
34	Hypothetical protein [apolipophorine III]	XP_624767	21,348 (19,448)	5.48 (5.45)	M	1953.98 1160.66	QJQEWNIPDQDTIVK FQQGVQTLK	50	13 (15)
35	Similar to CG3153-PB, isoform B [Niemann-Pick type C2 lysosomal protein]	XP_001120140	16,794 (15,007)	6.10 (5.65)	M	1098.57	DIEYIYKR	42	5 (6)
36	CuZn superoxide dismutase	AAP93581	15,634	6.21	M	1682.82 1404.78 2049.07	GTIFFEQPESTNSVK TIQLQGPHSVIGR TLVVHADPDDLGLKGGVELSK	110	32
37	Similar to CG2852-PA, isoform A [cyclophelin type peptidyl-prolyl isomerase]	XP_624416	20,104 (18,055)	8.78 (8.60)	M			91	
40	Odorant binding protein 14	NP_001035313	15,201 (13,518)	5.71 (5.41)	M			106	
41, 43, 50–62	Phospholipase A2 (Api m 1)	NP_001011614	19,058 (15,249)	7.05 (8.07)	M & L	1837.73 1387.66 1143.56 1125.56	LSCDCDKFYDCLK (58) NSADTISSYFVGK (58) MYFNLIIDTK (58) VYQWFDLR (58)	244	25 (31)
42	Hyaluronidase (Api m 2)	NP_001011619	44,260 (40,747)	8.67 (8.72)	M	1357.66 1501.79	YGQLFMEETLK EYLNNELGPAVKR	124	6 (7)
44–46	Venom protein 2 precursor (icarapin)	NP_001012431	24,789 (22,697)	4.51 (4.40)	M & L	1581.91 1280.67	KNVDTVLVLPISIER (46) IPEQGVVNWKN (46)	205	11 (12)

47–48	Venom acid phosphatase precursor [acid phosphatase] (Api m 3)	NP_001013377	45,389 (43,905)	5.63 (5.63)	M & L	1752.80 1439.73 2862.32 1656.93 1982.99 2670.30 1718.90 1817.90 2068.00	DPVLYDFVPLER (47) MREYQLGQFLR (47) YGDFLGDIYTESVSALSFYDRTK (47) MSLQVLAALYPPNK (47) LQQWNEDLNWQPIATK (47) RYEDNIFLPEDCLLFTIELDR (47) IFTKHMILDVVSQTQK (47) ELQLPGCEVLCPLYK (47) FVDESANNLSIEELDFVK (47)	469	38 (40)
14, 19, 20, 26, 38, 39	Not identified								

^a Numbers refer to the spot numbers given in Fig. 1.

^b Name between square brackets was derived from BLASTP searching. Synonyms are between round brackets.

^c Entry code of NCBI (National Center for Biotechnology Information): <http://www.ncbi.nlm.nih.gov>.

^d Theoretical molecular weight (MW) and pI are calculated using the on-line Expasy's Compute pI/MW program (http://au.expasy.org/cgi-bin/pi_tool); data between brackets are of the mature protein/peptide.

^e M = MALDI TOF/TOF; L = LC MS/MS.

^f Theoretical molecular mass of peptide.

^g Deduced peptide sequences obtained after LC MS/MS analysis of the indicated spot (number between brackets).

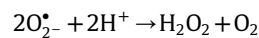
^h Peptide sequence found on the in silico translated genome sequence in the vicinity of the given partial protein.

ⁱ Total Mascot score of the indicated spot in g.

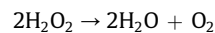
^j Data between brackets are of the mature protein/peptide.

Several studies focussed already on the proteome of the venoms from stinging Hymenoptera (honey bee: Peiren et al., 2005; yellow jacket: Kolarich et al., 2007), but their gland tissue proteome remained largely undiscovered.

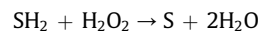
The present study demonstrated the prominent presence of protein spots matching the GO terms 'antioxidant activity/response to oxidative stress'. However, when antioxidant enzyme activity was measured, it turned out that the values of the venom gland were comparable or even lower to those of others tissues. Antioxidant enzymes are involved in several vital functions including the reduction of life-shortening oxidative damage. Reactive oxygen species (ROS) are constantly produced as by-products of aerobic metabolism, and insects are similar to other animals in possessing a suite of enzymes dedicated to their removal. This antioxidant defence system is conserved throughout evolution, but there are unique adaptations among different taxonomic groups. Genes encoding functional glutathione reductase and glutathione peroxidase are lacking in insects, but homologous genes for thioredoxin reductase (Kanzok et al., 2001) and thioredoxin peroxidase activities (Radyuk et al., 2001), respectively, act in their place. SOD catalyzes the reaction:



Two types of enzymes are known that remove toxic H_2O_2 ; they include catalases (CATs) which catalyze the reaction:



and general peroxidases which catalyze the reaction:



in which SH_2 is a substrate that becomes oxidized, normally reduced thioredoxin or glutathione (Corona and Robinson, 2006). Our survey evidences the presence of SOD, GST, PXR and TPX in venom gland tissue of the honey bee. The three latter enzymes have peroxidase activity in insects and thus may be involved in the reduction of hydrogen peroxide. The enzyme CAT was not found in the present study, possibly reflecting its relatively low abundance. In mated queen spermatheca and drone semen the antioxidant enzymes, particularly CAT, SOD and GST, contribute to the protection of the spermatozoa against oxidative stress and thereby help to facilitate their long-term survival (Weirich et al., 2002; Collins et al., 2004). The extended longevity of queen honey bees compared to workers seems to be associated with lower levels of peroxidisable fatty acids in membranes (Haddad et al., 2007).

Seven classes of insect proteins with GST-like subunits have been defined (Ranson et al., 2001). One of the best characterized functions of the Delta and Epsilon classes is their role in insecticide detoxification. Hence, the complete absence of the Epsilon class in the honey bee and the presence of only a single Delta class GST may partially account for the extreme sensitivity of this species to certain insecticides (Claudianos et al., 2006). In contrast to the scarcity of Delta and Epsilon GSTs in the honey bee, the species has

Table 3

Assignment of Gene Ontology terms of the associated molecular functions, cellular components and biological processes inferred via the best BASTP matches

Acc. no.	BLASTP			Molecular function	Cellular component	Biological process
	Best match	Species	P-value			
AAP93581	Sod, superoxide dismutase	<i>Drosophila melanogaster</i>	5.7e-54	Antioxidant activity	Cytoplasm	Aging Determination of adult life span Removal of superoxide radicals Superoxide metabolic process Response to oxidative stress
BI513885	GstS1, glutathione-S-transferase	<i>Drosophila melanogaster</i>	1.9e-42	Copper, zinc superoxide dismutase activity Glutathione peroxidase activity Glutathione transferase activity		
GB15039-PA	Eno, enolase	<i>Drosophila melanogaster</i>	1.3e-125	Phosphopyruvate hydratase activity		Glycolysis
GB15052-PA	Pglym78, phosphoglyceromutase	<i>Drosophila melanogaster</i>	3.7e-105	Phosphoglycerate mutase activity		
GB16211-PA	Cpr65Az, cuticular protein 65Az	<i>Drosophila melanogaster</i>	2.4e-23	Structural constituent of chitin-based cuticle		
GB18929-PA	Cpr65Az, cuticular protein 65Az	<i>Drosophila melanogaster</i>	1.1e-27	Structural constituent of chitin-based cuticle		
NP_001011564	MRJP1: major royal jelly protein 1 precursor	<i>Apis mellifera</i>	4.9e-135		Extracellular region	Not applicable
NP_001011572	Tsf1, transferrin 1	<i>Drosophila melanogaster</i>	1.1e-101	Ferric ion binding	Cellular iron ion homeostasis Defence response Iron ion transport	
NP_001011603	Argk, arginine kinase	<i>Drosophila melanogaster</i>	2.8e-155	Iron ion transmembrane transporter activity Arginine kinase activity		Phosphorylation
NP_001035313	Obp56d, odorant binding protein 56d	<i>Drosophila melanogaster</i>	3.1e-07	Odorant binding	Extracellular region	Olfactory behaviour Response to pheromone Sensory perception of chemical stimulus Protein folding response to heat
XP_392147	Hsc70-5, heat shock protein cognate 5	<i>Drosophila melanogaster</i>	2.2e-258	ATPase activity Unfolded protein binding	Mitochondrion	
XP_392338	SAP_HUMAN, PSAP, GLBA, SAP1: proactivator polypeptide precursor [contains: saposin-A]	<i>Homo sapiens</i>	6.0e-47	Enzyme activator activity	Extracellular space integral to membrane	Glycosphingolipid metabolic process Lipid transport
XP_393090	Hsc70-3, heat shock protein cognate 3	<i>Drosophila melanogaster</i>	5.5e-278	Lipid binding ATPase activity	Endoplasmic reticulum Lipid particle	Response to heat RNA interference sleep
XP_393445	Jafrac1, thioredoxin peroxidase 1	<i>Drosophila melanogaster</i>	7.5e-84	Antioxidant activity Glutathione peroxidase activity Peroxidase activity Thioredoxin peroxidase activity Serine carboxypeptidase activity	Cytosol	Cell redox homeostasis
XP_393931	CG4572	<i>Drosophila melanogaster</i>	1.3e-111			
XP_396769	IDGFL_BOMMO, ENO3: Chitinase-like protein ENO3 precursor	<i>Bombyx mori</i>	1.2e-99			
XP_623282	ERp60	<i>Drosophila melanogaster</i>	3.2e-163	Protein disulfide isomerase activity	Lipid particle	Protein folding
XP_624156	ATPsyn-beta, ATP synthase-beta	<i>Drosophila melanogaster</i>	8.9e-223	Hydrogen-exporting ATPase activity, phosphorylative mechanism	Lipid particle Mitochondrial proton-transporting ATP synthase complex, catalytic core F	Proton transport
XP_624361	Prx2540-2, peroxiredoxin 2540	<i>Drosophila melanogaster</i>	3.7e-89	Antioxidant activity Glutathione peroxidase activity Peroxidase activity		
XP_624416	CG2852	<i>Drosophila melanogaster</i>	8.0e-62	Peptidyl-prolyl <i>cis-trans</i> isomerase activity		
XP_624767	None					
XP_001120140	NPC2, Niemann-Pick Type C-2	<i>Drosophila melanogaster</i>	3.6e-13		Extracellular region	

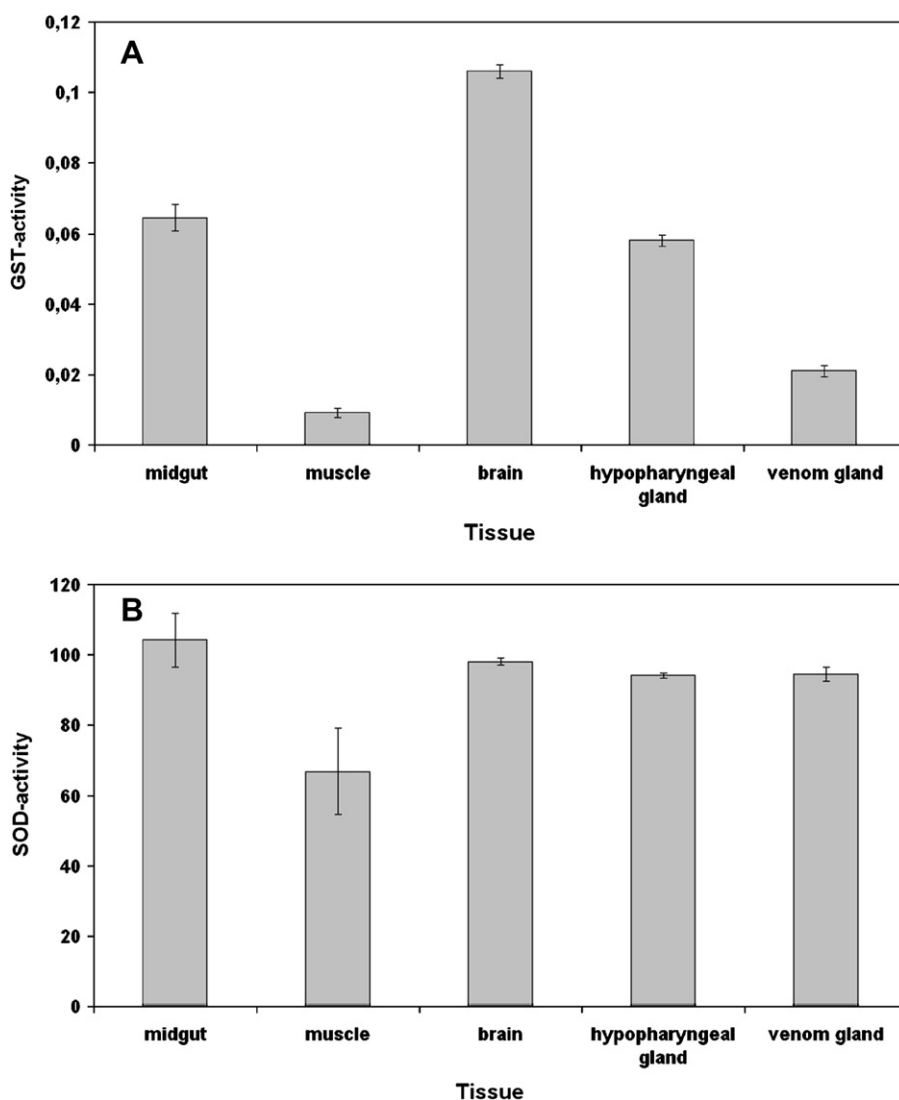


Fig. 2. Antioxidant enzyme activity of different tissue homogenates from adult honey bees. (A) Glutathione-S-transferase (GST) activity and (B) superoxide dismutase activity (SOD).

more members of the Sigma class (four genes vs. a single Sigma GST gene in the Diptera) (Claudianos et al., 2006). In general, Sigma GSTs show low levels of activity with the typical GST substrates, but they have a high affinity for the lipid peroxidation product 4-hydroxynonenal, and their localization in metabolically active tissues in flies, such as the flight muscles, has been suggested to be instrumental in protecting these tissues from by-products of oxidative stress (Singh et al., 2001). The GST that we found in the venom gland belongs to this Sigma class.

Beside the antioxidant enzymes, we found several proteins involved in other forms of stress. Heat shock proteins were initially described as chaperones that facilitate the folding of other proteins. Recent studies prove that HSPs also play a role in cell protection, anti-apoptosis, developmental regulation and signal transduction. The heat shock response can result in stress tolerance and protection

against stress induced molecular damage (Morimoto et al., 1997). In most of the species, several proteins belong to the HSP70 family (Pf00012). Some are only expressed under stress conditions (HSP), while the HSCs are not induced by heat and are produced in cells under normal growth conditions (Snutch et al., 1988). XP_393090 and XP_392147 could be identified as members of this HSC subgroup. Erp60 is an endoplasmic reticulum (ER)-resident eukaryotic protein involved in oxidative protein folding. Among others, it contains a domain that belongs to the protein disulfide isomerase a (PDla) family, subfamily PDI related (PDIR), and exhibits isomerase and chaperone activities. PDIR-proteins are preferentially expressed in cells actively secreting proteins, and its expression is induced by stress (Ferrari and Söling, 1999). Apolipoprotein III is an important component of insect hemolymph lipid transport processes. Besides, it plays a role in immunity, pattern recognition,

programmed cell death and detoxification of lipopolysaccharide endotoxins (Whitten et al., 2004). Transferrins are glycoproteins with a high affinity for iron and ferrous ions. Like other organisms, insects must balance two properties of ionic iron, that of an essential nutrient and that of a potent toxin. Particularly iron must be acquired to provide catalysis in oxidative metabolism, but it must be controlled to avoid destructive oxidative reactions. Insects have evolved distinctive forms of the iron transport protein transferrin, and the storage protein ferritin. These proteins may serve functions in insects that differ from those in other organisms (Nichol et al., 2002).

Also two endocuticular structural proteins were found. These proteins form a matrix in which the chitin fibers of the cuticula are anchored. Many insect cuticular proteins have a conserved domain known as the 'R&R consensus'; a more constrained version of this consensus was first reported by Rebers and Riddiford (1988). Two major forms of this motif can be distinguished: RR-1 and RR-2. The former occurs in proteins from soft, flexible, hydrated and little hardened cuticula, whereas the latter occurs in proteins from hard cuticula. The two identified proteins from the venom reservoir belong to the soft type, as expected. In contrast to most of these proteins, the ones that we found have not one but two RR-1 motifs. Such double RR-1 motif has so far only been detected in the fruit fly (*Drosophila melanogaster*) and the malaria mosquito (*Anopheles gambiae*). However, BLASTP searching for each of the identified endocuticular proteins revealed a match with high identity (97%) at the N-terminal end and with only one RR-1 motif. This aspect needs our further attention.

There are also a number of proteins for which the presence was not within our expectation. MRJP8, the name of this protein refers to 'royal jelly', the secreted from the mandibular and hypopharyngeal glands of young worker bees that serve to feed the larvae and the queen. Among the different MRJPs, only MRJP8 is not produced in the hypopharyngeal glands (Santos et al., 2005). So far, it was only found in an EST database of the brain of worker bees (Albert and Kludiny, 2004), but the present paper demonstrates unambiguously that MRJP8 is also expressed by the venom gland. Because of the presence of a signal peptide, it seems reasonable to believe that MRJP8, like MRJP9 (Peiren et al., 2005), represents a new constituent of the honey bee venom. Both proteins have several striking common features: (i) phylogenetic analyses put them at the basis of the MRJP tree (Drapeau et al., 2006), meaning that they are the most ancient members of the MRJP protein family and (ii) both MRJP8 and MRJP9 are devoid of later evolved repetitive regions, which has been suggested to embody the novel nutritious function of MRJP proteins as major components of royal jelly (Albert and Kludiny, 2007). These facts, together with appearance of both proteins in the venom proteome/transcriptome implicate that the original "pre-royal jelly" function of MRJP proteins might be the as yet unknown function in the bee venom.

The insect OBPs belong to a class of small soluble proteins which mediate the chemical communication at the peripheral level in insects (Rubin et al., 2000). The mechanism has not yet been entirely clarified and it is possible that these proteins also fulfil other functions. The OBP

that we found in the venom gland belongs to the PBP/GOBP subfamily (Pf01395). This family contains both pheromone binding and general odorant binding proteins. Finally, we identified an imaginal disc growth factor, more specifically IDGF4, referring to its homology with IDGF4 from *Drosophila* (47% sequence identity). The name 'imaginal disc growth factor' was given by Kawamura et al. (1999), because these proteins were for the first time isolated from *Drosophila* imaginal disc cell cultures. IDGFs were the first soluble polypeptide growth factors isolated from invertebrates. They belong to the glycosyl hydrolases family 18 (Pf00704).

In conclusion, the proteomic study of the honey bee venom gland tissue revealed the presence of the antioxidant enzymes SOD1, GSTS1, PXR2540 and TPX1. Although GST has also been associated with xenobiotic detoxification, the protein we found belongs to the GST Sigma class which is known to protect against oxidative stress only. Moreover, we could demonstrate that the GST and SOD activity of the venom gland was low and moderate, respectively, when compared to other tissues from the adult honey bee. Several proteins involved in other forms of stress were likewise found but it remains uncertain what their function is in the venom gland. MRJP8 was identified as second member of the MRJP protein family to be associated with the venom gland. Two endocuticular structural proteins were abundantly present in the 2D-gel and most probably represent a structural component of the epicuticular lining that protects the secretory cells from the toxins they produce.

Conflict of interest

I confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. I further confirm that the order of authors listed in the manuscript has been approved by all of us.

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