

## Rapid report

The protein composition of honeybee venom reconsidered  
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**Abstract**

Pure honeybee venom samples were submitted to two-dimensional gel electrophoresis. A total of 49 excised spots were analyzed by mass spectrometry; 39 of them resulted in the identification of 6 different known bee venom proteins and of 3 proteins that have not been described in such samples before. The first new venom protein has a platelet-derived and vascular endothelial growth factor family domain, the second protein shows no homologies with any known protein and the third matches a hypothetical protein similar to major royal jelly protein 8.

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Due to vasoactive and neurotoxic components of the venom, most people that are stung by a honeybee experience a local reaction consisting of redness, swelling, tenderness and pain at the site of the sting. A large percentage of the general population (20.7%) develops a hypersensitivity reaction of type 1 [1], and sensitization may result in a broad range of clinical signs, going from a local oedematic swelling of the skin up to a life threatening systemic anaphylactic shock. The allergens responsible for these hypersensitivity reactions are listed by the Allergen Nomenclature Sub-Committee of the International Union of Immunology Societies (IUIS) ([http://www.allergen.org/](http://www.allergen.org/List.htm)

[List.htm](http://www.allergen.org/List.htm)). At present, five bee venom allergens can be found on this list: Api m 1 (phospholipase A2; PLA2), Api m 2 (hyaluronidase), Api m 4 (melittin), Api m 6 and Api m 7 (CUB serine protease).

Modern biotechnology has provided several of these allergens in a recombinant form (reviewed in [2]). The implementation of recombinant allergens in immunotherapy is already at the phase I stage of clinical trials and was proven to be more specific and safe than the use of bee-derived venom [3]. Also, in the diagnostic field, promising results were obtained using single recombinant/synthetic allergens or mixtures of them [2,4] and it was suggested that further progress lies in the completion of the repertoire of recombinant allergens [2]. In fact, the development of an artificial recombinant/synthetic bee venom preparation for clinical practice necessitates a good knowledge of the allergic components that occur in natural venom in order to refine the composition of the recombinant cocktail.

In order to identify new venom components with allergic potential, the protein composition of honeybee venom was reconsidered by a proteomic approach. The combination of two-dimensional gel electrophoresis and mass spectrometric identification has been proven to be a powerful tool for

**Abbreviations:** 2-DE, two-dimensional gel electrophoresis; ESI, electrospray ionization; EST, expressed sequence tag; IEF, isoelectric focusing; IPG, immobilized pH gradient; IUIS, International Union of Immunology Societies; LC, liquid chromatography; MALDI, matrix-assisted laser desorption ionization; MRJP, major royal jelly protein; MS, mass spectrometry; PDGF/VEGF, platelet-derived and vascular endothelial growth factor; PLA2, phospholipase A2; TOF, time of flight

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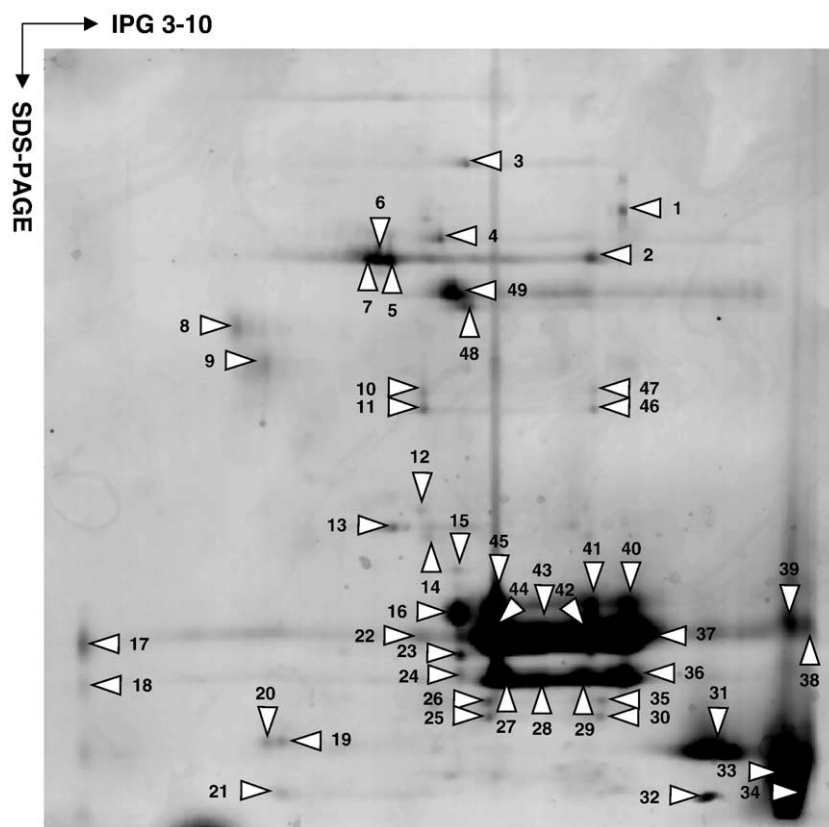


Fig. 1. Representative 2D-gel of the honeybee venom, out of three, stained with Coomassie Brilliant Blue G-250. More information about the different spots can be found in Table 1.

proteome mapping and was retained for the present study. In addition, the honeybee *Apis mellifera* is now one of the few insects of which man can benefit to have its entire genome sequence available, being a considerable surplus value for the protein spot identification. No such information is presently available of any other stinging Hymenoptera.

Venom was collected from Carniolan honeybees (*Apis mellifera carnica*) by dissecting the whole sting apparatus and emptying the reservoir by softly compressing. The droplet of venom that appeared at the tip of the sting was immediately collected in 250  $\mu$ l of sample buffer (40 mM Tris-base, 8 M urea, 4% (w/v) CHAPS and 5 mM

dithiothreitol). Three samples of pure venom, pooled from 35 bees each, were collected and were submitted to two-dimensional gel electrophoresis (2-DE), as previously described [5]. Briefly, approximately 1 mg of protein was applied on 18 cm immobilized pH gradient (IPG) strips (pH 3–10L) and isoelectric focusing (IEF) was carried out on a Multiphor II system (Amersham Biosciences, Uppsala, Sweden). After completion of the IEF and reduction and alkylation steps, the IPG strips were placed on home casted SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gels. The second dimension electrophoresis was performed in a Protean Plus Dodeca Cell system (Bio-

#### Notes to Table 1:

- <sup>a</sup> Entry code of NCBI (National Center for Biotechnology Information): <http://www.ncbi.nlm.nih.gov>.
- <sup>b</sup> PS=protein sequencing; RS=deduced sequence from mRNA/cDNA sequencing; GS=hypothetical sequence from genome sequencing.
- <sup>c</sup> C=complete; I=incomplete.
- <sup>d</sup> 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](http://au.expasy.org/cgi-bin/pi_tool)); data between brackets are of the mature protein/peptide.
- <sup>e</sup> M=MALDI TOF/TOF; L=LC MS/MS.
- <sup>f</sup> Theoretical molecular mass of peptide.
- <sup>g</sup> Deduced peptide sequences obtained after LC-MS/MS analysis of the indicated spot (number between brackets). • Unique peptide sequences obtained in other spots, containing the same protein or in replicate runs of the same spot picked from another gel. \* Peptide sequence found in the vicinity of the in silico predicted gene on AmeL2.0[GroupUn.1029].
- <sup>h</sup> Total Mascot score of the indicated spot in footnote g.
- <sup>i</sup> Data between brackets are of the mature protein/peptide.
- <sup>j</sup> Numbers refer to the spot numbers given in Fig. 1. Spot numbers are in italic when more than one protein was identified in the corresponding spot.
- <sup>k</sup> A peptide mass fingerprint search of spot 44 matched 18 of the experimental peptides to the protein predicted peptides with a Mascot score of 117 and a sequence coverage of 88%. The peptide mass fingerprint search of the other 23 protein spots resulted in similar results.

Rad, Hercules, USA). Gels were stained overnight with Coomassie Brilliant Blue G-250, and digitized by a densitometer (PDQuest, Bio-Rad, Hercules, USA). The three gels showed a high degree of identity, reflected by the high scatter plot correlation coefficient (>85%) between those 2D-gels.

The protocol for in-gel digestion has been published elsewhere [5]. Briefly, the excised spots were washed, air-dried and then digested with 0.02 µg modified trypsin (Promega, Madison, USA) per spot in 50 mM ammonium bicarbonate at 37 °C overnight. Peptides were extracted with 60% (v/v) acetonitrile/0.1% (v/v) formic acid in water,

Table 1  
Protein identification on the 2-D gels of honeybee venom

| Protein name                                                         | Acc. no. <sup>a</sup> | AA-sequence         |                   | MW<br>(Da) <sup>d</sup> | pI <sup>d</sup> | Ident.<br>method <sup>c</sup> | Peptide<br>mass <sup>f</sup>                                                                               | Peptide sequences <sup>g</sup>                                                                                                                                                                         | Mascot<br>score <sup>h</sup> | Sequence<br>coverage<br>(%) <sup>i</sup> | Spot no. <sup>j</sup>                    |
|----------------------------------------------------------------------|-----------------------|---------------------|-------------------|-------------------------|-----------------|-------------------------------|------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------|------------------------------------------|------------------------------------------|
|                                                                      |                       | Origin <sup>b</sup> | Full <sup>c</sup> |                         |                 |                               |                                                                                                            |                                                                                                                                                                                                        |                              |                                          |                                          |
| Api m 6                                                              | 34921475              | PS                  | I                 | 7598                    | 9.70            | L                             | 1369.44<br>1091.47<br>1108.50<br>1293.61<br>1570.84                                                        | FGGFGGFGGLGGRGK<br>ICAPGCVCR<br>CPSNEIFSR<br>GKCPSNEIFSR<br>FCPNVVPKPLCIK                                                                                                                              | 242                          | 80                                       | 31                                       |
| Acid phosphatase                                                     | 60652325              | RS                  | C                 | 45389<br>(43905)        | 5.63<br>(5.63)  | M & L                         | 1152.59<br>1379.74<br>1439.73<br>1752.80<br>1982.98<br>2068.00<br>2633.18<br>1817.91<br>2445.26            | EYQLGQFLR (2)<br>IVYYLGIPSEAR (2)<br>MREYQLGQFLR (2)<br>DPYLYYDFYPLER (2)<br>LQQWNEDLNWQPIATK (2)<br>FVDESANNLSIEELDFVK (2)<br>YGDFLGDIYTESVSALSSFYDR (2)<br>ELQLPGCEVLCPLYK•<br>YLQLIENVIPSNEELICDKR• | 357                          | 35 (37)                                  | 2, 5–7                                   |
| Hyaluronidase                                                        | 155680                | RS                  | C                 | 44260<br>(40747)        | 8.67<br>(8.72)  | M & L                         | 1015.57<br>1233.61<br>1357.66<br>1501.79<br>1574.79<br>2108.08<br>1191.62<br>1228.53<br>1970.88<br>2237.09 | ADLEATLRK<br>QNWASLQPYK<br>YGQLFMEETLK<br>EYLNELGPAVKR<br>SFPGVGVDFESWRPIFR<br>DHLINQIPDK<br>EHPFWDDQR<br>EFNVYWNVPTFMCHK<br>ITDLGADGFIIWGSSDDINTK<br>KVLPPYYWYK•                                      | 387                          | 36 (40)                                  | 49                                       |
| New venom<br>protein 1,<br>PDGF/<br>VEGF-like                        | 15365820              | RS                  | I                 | 15793                   | 6.91            | L                             | 1258.67<br>1534.82<br>1667.65<br>2044.91<br>2127.83<br>2292.02<br>2458.02                                  | DTDLSRADLEATLR•<br>NFEILVTVLESSGK<br>MWHPDLCSCFCR<br>RIVPIEHTQCTCDCK<br>ETQECSTGFYFDQNSCR<br>RCGGCCTHSLSCQPTATEIR<br>QSYVPEECTACNNVDEQKK                                                               | 265                          | 75                                       | 13                                       |
| New venom<br>protein 2                                               | 15353106              | RS                  | I                 | 24387                   | 4.71            | M & L                         | 1581.59<br>1258.68                                                                                         | VREQMAGILSR (8)<br>KNVDTVLVLPISIER (8)                                                                                                                                                                 | 165                          | 12                                       | 8, 9, 19,<br>20                          |
| New venom<br>protein 3, similar<br>to major royal<br>jelly protein 8 | 48137209              | GS                  | C                 | 26323                   | 6.81            | M & L                         | 2438.18<br>1998.00<br>2273.24<br>1040.53<br>1370.65<br>1539.47                                             | YTINDEFSLQDGILGMALSHK<br>IGNGGPLLEPYPNWSWAK<br>TFVTILRNDGVPSLNVISNK<br>NNVPIDVDR*<br>QAAIQSGEYNYK*<br>YFDYNFGSDEKR*                                                                                    | 152                          | 26                                       | 4                                        |
| Phospholipase A2 <sup>k</sup>                                        | 16904372              | RS                  | C                 | 19058<br>(15249)        | 7.05<br>(8.07)  | M & L                         | 1125.56<br>1143.56<br>1704.77<br>1387.66                                                                   | VYQWFDLR (44)<br>MYFNLIIDTK (44)<br>CYKLEHPVTGCGER (44)<br>NSADTISSYFVGK (44)                                                                                                                          | 156                          | 26 (33)                                  | 15–18,<br>22–30,<br>35–45                |
| Prepromelittin                                                       | 5622                  | RS                  | C                 | 7585<br>(2847)          | 4.69<br>(12.02) | M & L                         | 1667.01<br>1597.83                                                                                         | VLTTGLPALISWIKR (34)<br>YIIDVPPRCPPGSK (32)                                                                                                                                                            | 128                          | 21 (58)                                  | 32, 33,<br>34                            |
| Preproscapin                                                         | 72280                 | RS                  | C                 | 8689<br>(2869)          | 9.51<br>(9.84)  | L                             |                                                                                                            |                                                                                                                                                                                                        | 75                           | 18 (56)                                  | 32, 33                                   |
| Not identified                                                       |                       |                     |                   |                         |                 |                               |                                                                                                            |                                                                                                                                                                                                        |                              |                                          | 1, 3, 10,<br>11, 12,<br>14, 21,<br>46–48 |

thereafter dried in a SpeedVac (Thermo Savant, Holbrook, USA) and dissolved in 10  $\mu$ l of 0.1% (v/v) formic acid.

For further analysis of the samples, two mass spectrometric strategies were used. In a first attempt, we tried to identify the protein spots by MALDI TOF/TOF-MS (matrix-assisted laser desorption ionization time of flight/time of flight-mass spectrometry) performed on a 4700 Proteomics Analyzer (Applied Biosystems, Framingham, USA). One microliter of the digest sample was added to an equal volume of matrix solution (100 mM  $\alpha$ -cyano-4-hydroxycinnamic acid in 50% acetonitrile (v/v) / 0.1% (v/v) trifluoroacetic acid in water) and 1  $\mu$ l of the resultant mix was applied on the MALDI target.

Samples that could not be identified by MALDI analysis were loaded on an automated nano-HPLC system (Dionex Corporation, Hercules, USA) coupled to a hybrid triple quadrupole/linear ion trap (Q-TRAP LC-MS/MS system, Applied Biosystems, Framingham, USA). In this hyphenated set-up, an automated MS to MS/MS switching protocol was used. For more detailed technical information about the mass spectrometric analysis, we refer to a previously published paper [6].

A nucleotide sequence database (72243 sequences) and a protein sequence database (6681 sequences) for *Apis mellifera* was downloaded from the World Wide Web (<http://www.ncbi.nlm.nih.gov>) and adapted to the right format for the database searching program MASCOT (<http://www.matrixscience.com>). The acquired MS and MS/MS data from both strategies were submitted to our local MASCOT server v1.9 (<http://www.matrixscience.com>). If mass spectral data fitted a translated expressed sequence tag (EST), this sequence was loaded into a BLAST query for annotation.

The spot pattern obtained after 2-DE and Coomassie Brilliant Blue G-250 staining from one of the samples is depicted in Fig. 1. The result of the mass spectrometric identification is summarized in Table 1. A total of 49 different spots were analyzed, 39 of them resulted in the identification of 6 different known bee venom proteins and of 3 proteins that have not been described in such samples before. Although mass spectrometric data is present for the ten unidentified protein spots, the data did not allow characterizing the proteins unambiguously.

Melittin and PLA2 are the two most predominant proteins in bee venom, representing, respectively, 50 and 10–12% of its dry weight [7]. Therefore, we loaded approximately 1 mg total protein on the IPG strips in order to detect the less abundant proteins. PLA2 was identified in no less than 24 spots. The heterogeneity of PLA2 on gel electrophoresis has already been reported and was assigned to either photooxidation of the histidine imidazoles or to variations in the carbohydrate content [8]. However, in present study, no distinction could be made between the different spots on the basis of the MALDI MS and ESI MS (electrospray ionization mass spectrometry) data.

Three other bee venom allergens were identified: Api m 6, hyaluronidase and melittin. The latter, a peptide of 26 residues, was found at the edge of the 2-D gel, just above the front marker dye. This was also the case for secapin, a peptide of 25 residues with no clear biological activity. Both peptides are expressed as a prepro-polypeptide that is processed extracellularly by endopeptidase cleavage. In Table 1, the accession numbers of both peptides refer to the unprocessed form, although the peptide sequences gained by LC-MS/MS match the mature chain. Other known bee venom peptides like mast cell degranulating peptide, apamin and tertiapin could not be identified in this study because their molecular weight drops below the lower limit of the technique. The recently detected CUB serine protease, an allergic protein of approximately 39 kDa noted in bee venom by two other laboratories [9], could also not be found. It is not unlikely that this allergen corresponds to one of the minor unidentified spots.

The present study provided 8 internal peptide sequences originating from 4 different spots that match a recently found cDNA sequence of honeybee venom acid phosphatase [10]. A few decades ago this protein was considered to be an important bee venom allergen [11]. However, because of incomplete IgE binding and sequence data, it was removed from the IUIS list of allergens.

This proteome analysis revealed also 3 new honeybee venom proteins, representing a substantial relative increment in number of proteins. For two of them, a partial nucleotide sequence was already known as an EST of the honeybee brain [12]. The deduced amino acid sequence of the first sequence corresponds to a 15.78-kDa protein with a platelet-derived and vascular endothelial growth factor (PDGF/VEGF) family domain (gnl|CDD|16535; E-value:  $1e-06$ ). The second shows no homologies with any known protein. The third protein matches a hypothetical protein similar to major royal jelly protein (MRJP) 8, derived from an annotated genomic sequence using a gene prediction method. MRJPs occur in the hypopharyngeal and mandibular glands as the major component of the larval bee queen food, also known as royal jelly. The exact function of MRJP 8 is still unsure [13]. In the near future, the physiological function and potential allergic characteristics of these 3 new venom proteins will receive our full attention.

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