

Extending the honey bee venom with the antimicrobial peptide apidaecin and a protein resembling wasp antigen 5

M. Van Vaerenbergh*, D. Cardoent†, E. M. Formesyn*, M. Brunain*, G. Van Driessche‡, S. Blank§, E. Spillner§, P. Verleyen†, T. Wenseleers,¶ L. Schoofs†, B. Devreese‡ and D. C. de Graaf*

*Laboratory of Zoophysiology, Ghent University, Ghent, Belgium; †Research Group of Functional Genomics and Proteomics, University of Leuven, Leuven, Belgium; ‡Laboratory of Protein Biochemistry and Biomolecular Engineering, Ghent University, Ghent, Belgium; §Institute of Biochemistry and Molecular Biology, University of Hamburg, Hamburg, Germany; and ¶Laboratory of Socioecology and Social Evolution, University of Leuven, Leuven, Belgium

Abstract

Honey bee venom is a complex mixture of toxic proteins and peptides. In the present study we tried to extend our knowledge of the venom composition using two different approaches. First, worker venom was analysed by liquid chromatography-mass spectrometry and this revealed the antimicrobial peptide apidaecin for the first time in such samples. Its expression in the venom gland was confirmed by reverse transcription PCR and by a peptidomic analysis of the venom apparatus tissue. Second, genome mining revealed a list of proteins with resemblance to known insect allergens or venom toxins, one of which showed homology to proteins of the antigen 5 (Ag5)/Sol i 3 cluster. It was demonstrated that the honey bee

Ag5-like gene is expressed by venom gland tissue of winter bees but not of summer bees. Besides this seasonal variation, it shows an interesting spatial expression pattern with additional production in the hypopharyngeal glands, the brains and the midgut. Finally, our immunoblot study revealed that both synthetic apidaecin and the Ag5-like recombinant from bacteria evoke no humoral activity in beekeepers. Also, no IgG4-based cross-reactivity was detected between the honey bee Ag5-like protein and its yellow jacket paralogue Ves v 5.

Keywords: venom, honey bee, *Apis mellifera*, apidaecin, antigen 5.

Introduction

Honey bee venom contains several toxic compounds that cause death or inflict pain, which enables them to defend the hive against predators and external threats. In man, early exposure to bee venom evokes IgG1, IgG2 and to a lesser extent IgG4 antibody responses, whereas long-term exposure, often found in beekeepers, drives the immunity to an IgG4 type of humoral response (Müller, 2005; Larché *et al.*, 2006). Allergy to a bee sting is mediated by IgE antibodies and, so far, 12 honey bee venom allergens have been listed by the International Union of Immunological Societies (IUIS; <http://www.allergen.org/Allergen.aspx>), representing most of the compounds that are immunologically meaningful.

Following the sequencing of the honey bee genome (Weinstock *et al.*, 2006), venom protein maps became available with newly discovered proteins, some of which were subsequently studied in detail and classified as new allergens (Peiren *et al.*, 2005, 2006a, 2006b; Hoffman, 2006; Blank *et al.*, 2010; de Graaf *et al.*, 2010). Remarkably, the venom protein composition could also be further completed by whole venom gland tissue mass spectrometry (MS), a study initially performed in order to understand why the toxic compounds are not self-destructive

Correspondence: Matthias Van Vaerenbergh, Laboratory of Zoophysiology, Ghent University, K.L. Ledeganckstraat 35, 9000 Ghent, Belgium. Tel.: +32 9264 5151; fax: +32 9264 5242; e-mail: matthias.vanvaerenbergh@ugent.be

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(Peiren *et al.*, 2008). The previous venom and gland proteomic studies combined two-dimensional (2D) gel electrophoresis with matrix-assisted laser desorption/ionization (MALDI)-tandem time of flight (TOF/TOF) and/or liquid chromatography (LC)-tandem MS (MS/MS), and these gel-based approaches have some disadvantages: very low abundant compounds are not visible on the gel and low molecular weight fractions are lacking because of their higher electrophoretic mobility which allows them to migrate out of the gel. To overcome these issues related to gel-based proteomics, and to gain deeper insights into venom and venom gland proteomes/peptidomes, we extended our search for the venom constituents with a MS study of in-liquid digested LC fractions from venom and venom gland tissue.

In addition, we focused on a remarkable peculiarity of honey bee venom: the lack of an antigen 5 (Ag5) homologue, an issue that was contested by a genome-mining study that revealed multiple protein predictions with resemblance to the proteins of the Ag5/Sol i 3 cluster (Weinstock *et al.*, 2006). Ag5 is a common venom allergen of the vespid group that includes wasps, yellow jackets and hornets of the genera *Vespula*, *Vespa*, *Dolichovespula* and *Polistes*. In fact, according to the IUIS allergen list, Ag5 has been discovered in almost every species of the above-listed vespid genera and for some of them it seems to be the only known venom allergen. Moreover, the ants of the genus *Solenopsis* (*Solenopsis invicta*, Sol i 3; *Solenopsis richteri*, Sol r 3; *Solenopsis saevissima*, Sol s 3) all have a major allergen that shows strong resemblance to vespid Ag5. Although immunologically characterized in detail, the function of Ag5 proteins within wasp and fire ant venom remains largely unexplored (Gibbs *et al.*, 2008). Proteins belonging to the Ag5/Sol i 3 cluster form a major and distinct clade of the cysteine-rich secretory proteins, antigen 5 and pathogenesis-related 1 proteins (CAP) superfamily, whose members are found in a broad range of organisms spanning each of the animal

kingdoms (Gibbs *et al.*, 2008). Remarkably, Ag5 homologues have so far been discovered in the venoms of none of the hymenopteran species belonging to the Corbiculate bees, such as honey bees and bumble bees. In this second part, we focused on a honey bee Ag5-like protein [National Center for Biotechnology Information (NCBI) RefSeq: XP_001122516.2] showing the highest sequence similarity with the venom Ag5 of the yellow jacket, *Vespula vulgaris*. We relied on gene expression studies in order to verify whether this protein was produced by the honey bee venom glands and determined its phylogenetic relationship with other Hymenopteran Ag5s.

Finally, the immunological significance of this Ag5-like protein and of new venom/venom gland compounds derived from the proteomic study was determined by Western blot using sera of highly exposed, immune beekeepers.

Results

Proteomic analysis of pure worker venom

The chromatogram of the reversed phase (RP)-high-performance liquid chromatography (HPLC) separated worker honey bee venom is shown in Fig. 1. MALDI-TOF/TOF analysis of 26 separated protein peaks resulted in the identification of nine known venom proteins as well as of the antimicrobial peptide apidaecin, which up to now had not been detected in honey bee venom. The results of the MS identifications are summarized in Table 1.

Peptidomic analysis of venom apparatus tissue

The GNNRPVYIPQPRPPHP peptide was also found in our analysis of the venom apparatus tissue peptidome (Fig. S1), which suggests the expression of apidaecin by the venom gland. Additional peptides of melittin, phospholipase A₂, PDGF/VEGF-like protein and secapin were also found, all representing compounds that are known to

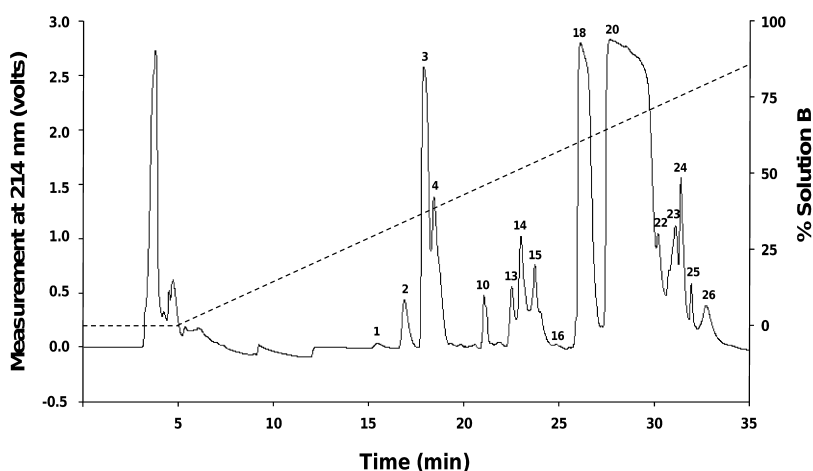


Figure 1. Reversed-phase high-performance liquid chromatography chromatogram of 2 mg of separated pure honey bee venom. The linear gradient of 0–100% buffer B over a 35-min period is represented by the dotted line. More information about the evaluated peaks, which are numbered on the chromatogram, can be found in Table 1.

Table 1. Protein identification on the high-performance liquid chromatography peaks of honey bee venom

Name	Acc. N°	MW in Da	pI	Peptide mass	Peptide sequences	Sequence coverage	Score	Peak
Apamin	gil58585166l	5220 (1928)	8.77 (8.83)	726.33 987.48	RCQQH APETALCAR	34 (82)	86	3, 4
Mast cell-degranulating peptide	gil58585162l	5777 (2477)	9.87 (10.28)	1158.64 1314.72	HVIKPHICR RHVIKPHICR	20 (45)	79	3, 4
Apidaecin*	gil58585168l	19 368 (17 359)	11.29 (11.30)	1837.97 1994.07	GNNRPVYIQPRPPHP GNNRPVYIQPRPPHPR	9 (11)	17	10, 12, 13
Secapin	gil58585180l	8674 (2868)	9.51 (10.05)	971.54	YIIDVPPR	10 (32)	44	13, 14, 15
Venom allergen Api m 6	gil94400907l	10 015 (7598)	10.06 (9.89)	1091.40 1108.48 1184.57	ICAPGCVCR CPSNEIFSR FGGFGGFGGLGGR	35 (45)	143	13, 15
Icarapin	gil60115688l	24 773 (22 697)	4.51 (4.40)	1293.59 1003.51 1072.53 1282.67 1581.91 1606.74	GKCPSEIFSR EQMAGILSR EEQGVVWNK IPEQGVVWNK KNDTVLVLSIER SVESVEDFDNEIPK	21 (23)	113	16, 22, 23
Phospholipase A ₂	gil58585172l	19 058 (15 149)	7.55 (8.07)	918.30 1034.46 1125.56 1193.69 1253.56 1663.64 1837.73	HTDACC CLHYTVDK VYQWFDLR HGLTNTASHTR LEHPVTGCGER THDMCPDVMSAGESK LSCDCDDKFYDCLK	43 (53)	256	17, 19
Melittin	gil58585154l	7580 (5119)	4.69 (4.51)	1510.91 1667.01	VLTTGLPALISWIK VLTTGLPALISWIKR	31	97	20, 22, 23, 24, 25
Hyaluronidase	gil58585182l	44 232 (40 470)	8.82 (8.82)	798.45 1191.62 1228.53	HLQVFR DHLINQIPDK EHPFWDQQR	6 (7)	77	23
Venom acid phosphatase	gil61656214l	45 360 (43 905)	5.63 (5.83)	888.52 1015.62 1152.59 1379.74 1752.80 1982.99 2068.00	QINVIFR KLYGGPLL EYQLGQFLR IVYYLGIPSEAR DPYLYYDFYPLER LQQWNEDLNWQPIATK FVDESANNLSIEELDFVK	28 (29)	85	26

The molecular weight (MW), pI (isoelectric point) and sequence coverage of the mature protein (without signal peptide) are represented between brackets. *Based on the found peptides no differentiation of the apidaecin precursor isoform was possible. Data are presented for apidaecin 14 (GI:58585168). Other apidaecin precursors containing the found peptide sequences were present in the database: apidaecin 22 (GI:58585226), apidaecin 73 (GI:4539289).

occur in honey bee venom and/or venom apparatus tissue (Peiren *et al.*, 2005, 2008).

Reverse transcription PCR confirmation of apidaecin expression by honey bee worker venom gland tissue

Reverse transcription PCR (RT-PCR) on honey bee worker venom gland tissue, using primers at the extreme ends of the coding sequence of prepro-apidaecin, generated a pool of amplicons with different sizes (Fig. 2). An identical band pattern was seen in RT-PCR of haemocyte-derived cDNA and confirmed earlier observations of Casteels-Josson *et al.* (1993). Moreover, in the latter study it was proven that all generated PCR fragments contained genuine apidaecin sequences. Sequencing of the smallest amplicon band revealed its apidaecin precursor identity and confirmed the apidaecin expression by the venom gland.

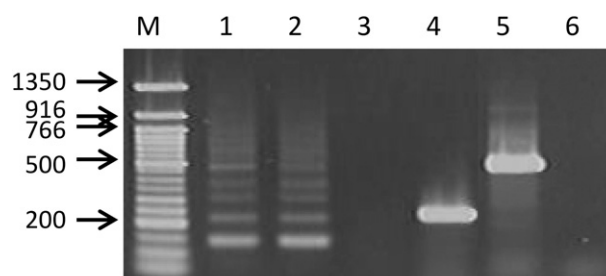


Figure 2. Reverse transcription PCR confirmation of apidaecin expression by honey bee worker venom gland tissue. 1 = venom gland cDNA with apidaecin coding sequence primers, 2 = haemocyte cDNA with apidaecin coding sequence primers, 3 = venom gland gDNA control with profilin intron primers, 4 = venom gland cDNA control with profilin exon primers, 5 = venom gland cDNA control based on the honey bee venom PLA₂ sequence, 6 = no template control with PLA₂ primer set, M = 50 bp DNA marker (New England Biolabs, Ipswich, MA, USA). Base pair lengths of major marker bands are shown.

	Venom gland			Brain		
	+	Ag5 ORF	Ag5 MAT	+	Ag5 ORF	Ag5 MAT
Winter bees						
Summer bees				ND	ND	ND
BFR bees						

Figure 3. Seasonal variation of antigen 5 (Ag5)-like gene expression in venom gland and brain tissue of honey bee workers. Expression patterns of the Ag5-like gene were determined by reverse transcription PCR. In winter honey bees the Ag5-like gene is expressed by venom glands and brain tissue. Summer and bee flight room (BFR) venom glands lack Ag5-like gene expression, while its expression was demonstrated in brain tissue of BFR bees. Ag5-like gene expression in brain tissue of summer bees was not determined (ND). ORF = open reading frame based on the Ag5-like prediction (XM_001122516.2). MAT = mature Ag5-like prediction without signal sequence. + = profilin cDNA control.

Spatial and seasonal variation of Ag5-like gene expression

The RT-PCR analysis on venom gland tissue from winter bees with primers developed in conserved regions of the Ag5-like gene prediction generated an amplicon of the expected length (252 bp, result not shown). In contrast, RT-PCR on different tissues of summer bees using the same conserved primer set demonstrated the expression of the Ag5-like gene in the hypopharyngeal glands, the brains and the midgut only, and not in the venom glands and the other tissues (haemocytes, salivary glands, drone mucus glands, deviscerated abdomen and muscle tissue). The Ag5-like gene seemed to be expressed most abundantly in the brains (results not shown).

To confirm these results, RT-PCR analysis was carried out on venom gland tissue of winter, summer and bee flight room (BFR) honey bees using primers developed at the 5'- and 3'-terminal ends of the predicted coding sequence. In contrast to winter bee venom glands, no Ag5-like gene expression was demonstrated in summer and BFR bee venom glands. Additionally, the Ag5-like gene was shown to be expressed abundantly in brains of winter and BFR bees. The same results were obtained with primers for amplification of the Ag5-like fragment without signal peptide (Fig. 3). The mature winter venom gland fragment was cloned and sequenced (NCBI accession number JX310326), which confirmed its Ag5 similarity. Six nucleotide substitutions were found between the cloned fragment and the predicted NCBI sequence (XM_001122516.2), but none of them influenced the amino acid sequence (sequence alignments are shown in Fig. S2).

Ag5 sequence analysis and phylogenetics

Sequence alignment of Hymenopteran Ag5-like sequences shows that Ag5 has a complex evolutionary history, with frequent gene duplications and losses. Within the Hymenoptera alone, at least six orthologous groups of sequences can be distinguished (Fig. 4, groups A–F). Among these, the studied *Apis mellifera* Ag5-like

sequence (Fig. 4, highlighted) is orthologous to other predicted Ag5-like sequences from the bumble bees *Bombus terrestris* and *Bombus impatiens*, the leafcutter bee *Megachile rotundata* and the jewel wasp *Nasonia vitripennis* (Fig. 4, group D). The sequence, however, is clearly paralogous to previously reported Ag5 allergen sequences in Vespidae wasps (*Vespula*, *Vespa*, *Dolichovespula*, *Polistes*, *Polybia* and *Rhynchium*) and ants (e.g. *Solenopsis*; Fig. 4, group A).

Immunoblotting

We were able to produce recombinant honey bee Ag5-like protein and equine uterocalin (irrelevant protein) in *Escherichia coli* and Ves v 5 in Sf9 insect cells and purify them by affinity chromatography (Fig. S3). The apidaecin peptide sequence GNNRPVYIPQRPHPRL was produced synthetically.

Enzyme-linked immunosorbent assay (ELISA) revealed very high titers of IgG4-antibodies specific for honey bee venom, PLA₂ and melittin in all beekeepers' sera (results shown in Table S1).

Western blots showed a lack of IgG4 recognition of purified bacterial recombinant Ag5-like protein and synthetically produced apidaecin in all sera (see Fig. 5). In addition, several sera of beekeepers with a history of yellow jacket stings (sera 2–8 and 10) recognize the recombinant Ves v 5 by IgG4 (strips 5–8 and 10). Thus, no cross-reactivity was observed between the Ag5-like protein from bacteria and Ves v 5 from insect cells. Anti-His staining revealed multiple bands, probably resulting from dimerization and/or degradation of the recombinants. One of the negative controls (strip 12) contained IgG4-antibodies responsive to the blocking milk proteins.

Discussion

First, the present study aimed to unravel the complex honey bee venom mixture using a gel-free proteomic analysis. Our previous approaches were based on 2D gel-separation of venom proteins followed by a MS

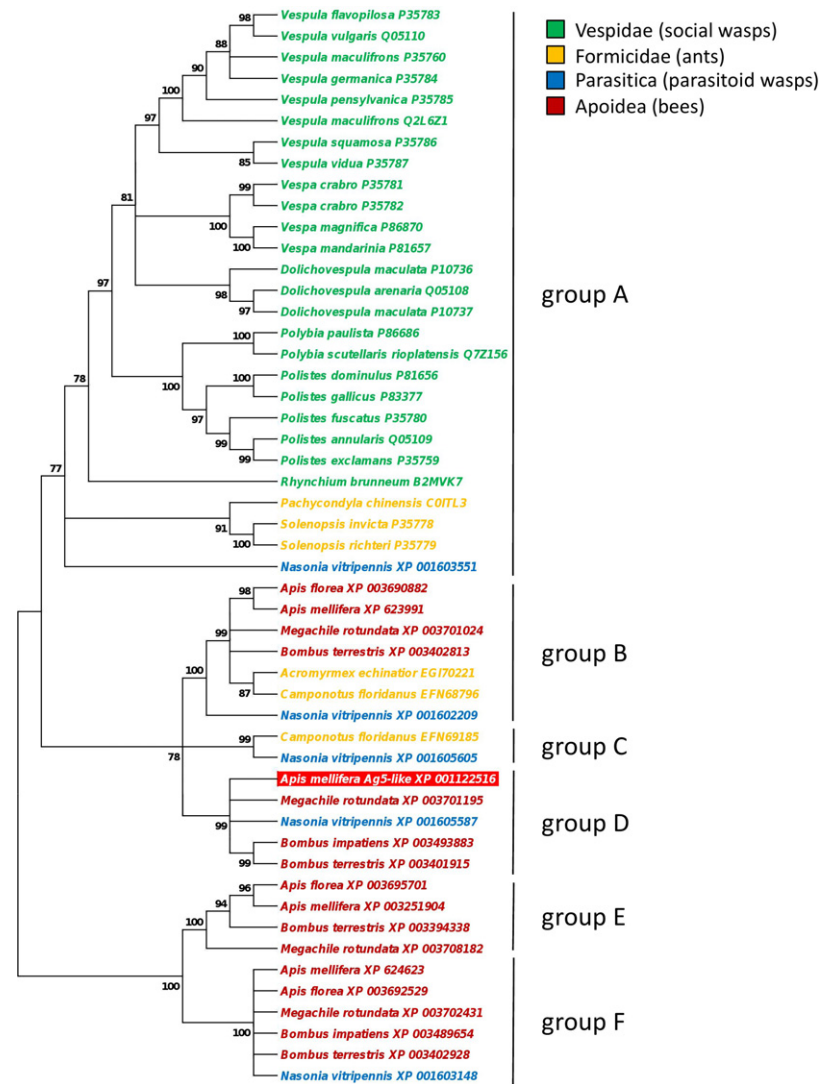


Figure 4. Neighbour-joining phylogenetic tree of Hymenopteran Ag5s. Phylogenetic placement of the *Apis mellifera* Ag5-like protein sequence (highlighted) in comparison with other known or predicted Hymenopteran Ag5-like proteins, as indicated based on a neighbour-joining analysis with a Jones *et al.* (1992) (G+) substitution model. Tentative orthologue groups are indicated with letters A-F. The numbers at the nodes indicate bootstrap support. Branches with <70% bootstrap support were collapsed.

analysis of excised spots (Peiren *et al.*, 2005; de Graaf *et al.*, 2010). Although those analyses successfully identified four new venom compounds, that technology is limited to the detection of compounds with low molecular weight (<10 kDa) and in low abundance. The present liquid-based venom proteome analysis confirmed the presence of multiple honey bee venom compounds, but also revealed the presence of three additional peptides which were not found in our preceding gel-based analyses: while apamin and mast-cell degranulating peptide (MCD) were discovered previously (Stöcklin & Favreau, 2002; Baracchi & Turillazzi, 2010; Matysiak *et al.*, 2011), the 18 amino acid peptide apidaecin had never been described in venom samples.

For venom research, a venom sample collected by manual milking is preferred to samples collected by electrostimulation, as the latter may contain contaminants derived from saliva or digestive tract fluids (de Graaf *et al.*,

2009). Moreover, the protein composition of a venom sample collected by manual milking may closely resemble that of venom injected during a natural honey bee sting: in addition to the release of venom proteins which are produced by the venom glands, proteins originating from other tissues such as the sting apparatus cell lining, stinger lancets and/or stinger lubricant, which has been hypothesized to be generated by the Dufour gland (Martin *et al.*, 2005), may also be released. As such, we were uncertain about the tissue origin of the apidaecin peptide detected in the venom sample. This issue was resolved by the detection of apidaecin transcripts (by RT-PCR) and an apidaecin peptide (by peptidomics) in the venom gland tissue, which indicates that this peptide is produced by the venom glands.

Apidaecins were first discovered by Casteels *et al.* (1989) in honey bee lymph upon bacterial infection, and were described to be expressed by haemocytes

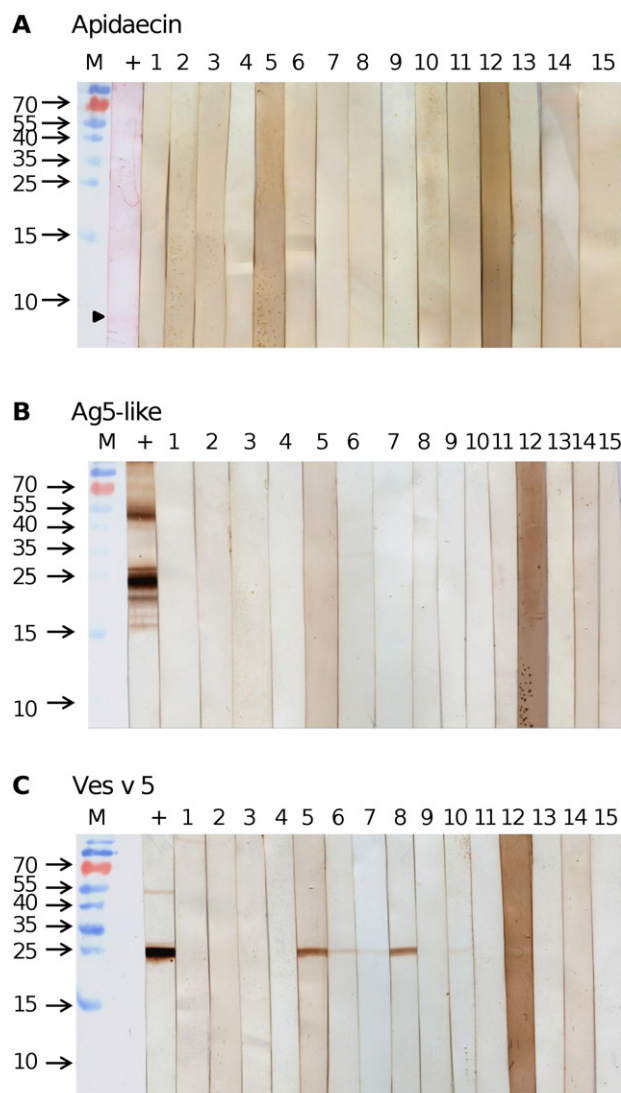


Figure 5. IgG4 responses to apidaecin, honey bee antigen 5 (Ag5)-like protein and yellow jacket Ves v 5 in beekeepers. Pure proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis under reducing and denaturing conditions and transferred to PVDF. Membranes were incubated with sera of beekeepers (strip 1–10) and negative control sera of subjects never stung by Hymenopterans (strip 11–15), followed by enzyme-linked anti-human IgG4. M = PageRuler™ Prestained Protein ladder (Fermentas). Molecular weights (kDa) of the marker bands are shown. Positive controls (+) are executed by Ponceau (apidaecin band indicated by an arrowhead) and anti-His staining (Ag5-like protein and Ves v 5).

(Casteels-Josson *et al.*, 1993). They are small, proline-rich antibacterial peptides which are generated by the processing of single precursor proteins. This multi-peptide precursor structure allows the amplification of the insects' immune response upon bacterial challenge (Casteels-Josson *et al.*, 1993). Moreover, its genomic structure enables the development of a pathogen-specific response by splice variation (Evans *et al.*, 2006). Different isoforms are described which all derive from a single prepro-

protein. The peptides detected in the venom and venom glands correspond to the apidaecin isoforms Ia or Ib (Casteels *et al.*, 1994) and may also play important antimicrobial roles. As antimicrobial peptide expression by barrier epithelial cell linings of multiple tissues seems to be a general feature of host defence in multicellular organisms (Tzou *et al.*, 2000), apidaecin expression by venom apparatus epithelial cells may protect the individual honey bee against invading pathogens. Alternatively, its presence in the venom may also have a function in the social immunity of the hive. Indeed, as honey bee venom is present on the cuticle of adult bees and on comb wax, it has been suggested that it may act as a social antiseptic device (Baracchi & Turillazzi, 2010; Baracchi *et al.*, 2011). Unlike the reported lytic antibacterial activity of other venom peptides such as melittin and possibly also apamin and MCD (Li *et al.*, 2006; Baracchi & Turillazzi, 2010), apidaecin kills bacteria through a bacteriostatic process. It is predominantly active against many Gram-negative bacteria by special antibacterial mechanisms (Li *et al.*, 2006). Consequently, apidaecin may be one of the peptides playing an important role in the protection of the hive against these pathogenic bacteria.

Second, the present study focuses on the identification of an Ag5-like gene transcript expressed by the honey bee venom glands. Ag5s are important and highly abundant venom allergens within the Vespidae and Formicidae families. Remarkably, an Ag5 protein has never been detected in venom proteome analyses of honey bees or any other member of the Apidae family. Based on an NCBI prediction, we were able to clone, sequence and recombinantly produce an Ag5-like sequence expressed by the venom gland of winter honey bees; however, it seems that venom gland tissue of summer and BFR honey bees do not express this protein, whereas its expression is maintained in the brain tissue. So far, most proteomic studies have focused on the venom of summer bees, which may explain why it remained undetected. Because of the putative presence of a signal peptide, we hypothesize that the honey bee Ag5-like protein is secreted by the venom gland of winter bees. A proteomic study focusing on the venom of winter bees should further confirm this hypothesis.

Additionally, we demonstrated Ag5-like expression in the hypopharyngeal glands and the midgut of summer bees, while expression is lacking in haemocytes, salivary gland, mucus glands (drones), deviscerated abdomen and muscle tissue. Midgut expression has also been reported in *Drosophila* (Gibbs *et al.*, 2008). On the other hand, honey bee Ag5-like protein is not expressed in salivary glands, while Ag5s have been found in the saliva of blood feeding Diptera such as ticks, sand flies, stable flies and mosquitoes (Gibbs *et al.*, 2008). Unfortunately, the function of any of the Ag5s remains unknown (Gibbs

Figure 6. Protein sequence alignment of yellow jacket Ves v 5 (Ves_v_5; Q05110.1) and detected honey bee Ag5-like (XP_001122516.2) by ClustalW. They have a sequence identity of 25%.

Third, our analysis revealed the lack of IgG4 recognition of both apidaecin and honey bee Ag5-like protein by the beekeepers' sera. Beekeepers are regularly stung in summer time, which is known to cause a strong venom-specific IgG4 response (Müller, 2005). We are unable to conclude whether this lack in humoral response against both compounds is the result of low immunogenicity, low abundance in the venom and/or low exposure. In the case of the antimicrobial peptide apidaecin, a low immunogenicity can be explained by its short length (Chiarella *et al.*, 2010). For the honey bee Ag5-like protein, its restricted expression in winter time certainly lowers the exposure to this venom compound significantly, as beekeepers are then hardly stung; however, we cannot exclude the possibility that the immunogenicity of the natural protein differs from that of the tested recombinant owing to a possible incorrect folding of the latter. An incorrect folding of bacterial recombinants may be caused by a lack of post-translational modifications such as glycosylations and disulphide bridges (Sahdev *et al.*, 2008). The absence of glycosylations cannot be responsible for an incorrect folding of the Ag5-like recombinant as glycosylation sites are absent (inferred by NetNGlyc 1.0 and NetOGlyc 3.0). In contrast, four disulphide bridges are predicted to be present (inferred by DISULFIND). As the bacterial recombinant was produced in the cytoplasm, it lacks these disulphide bridges (Sahdev *et al.*, 2008). Moreover, for the immunoblot experiment, electrophoretic separation has been conducted under reducing and denaturing conditions, which generally disrupts the protein conformation. As such, the lack of IgG4 recognition of the

Furthermore, no IgG4 cross-reactivity between the Ag5-like protein and Ves v 5 was detected. Although two expression systems with differential capacities to perform post-translational modifications were used to produce these recombinants, we believe this may not have influenced the outcome of our immunoblot experiment. As Ves v 5 also lacks glycosylation sites, both proteins were produced without carbohydrate groups. In contrast, while the bacterial system has probably not foreseen the Ag5-like recombinant with the correct disulphide bridges, Ves v 5 produced in the baculovirus system is secreted and probably contains appropriate disulphide bridges. However, this difference has been neutralized in our immunoblot experiment by the electrophoretic separation of both proteins under reducing and denaturing conditions. As such, we hypothesize that the low sequence identity between both compounds (25% sequence identity; Fig. 6) plays a more significant role. In addition, the honey bee Ag5-like sequence shows low sequence identity (maximum of 27%) with other Hymenopteran venom Ag5 allergens, a remarkable characteristic which was resolved by our phylogenetic analysis. It appears that Hymenopteran Ag5s have a complex evolutionary history with frequent gene duplications and losses, and that the honey bee Ag5-like protein is clearly paralogous to the group of ant and wasp venom allergens (Fig. 4, groups D and A, respectively). Sequence identity is generally much higher between the

orthologous group of venom allergens, which may be responsible for the observed IgE cross-reactivities between venom Ag5s of the *Vespula* genus and even between *Vespula* and *Dolichovespula* Ag5s and between *Vespula* and *Vespa* Ag5s (Henriksen *et al.*, 2001). Most likely, IgE-level cross-reactivity between the honey bee Ag5-like protein and these other Hymenopteran venom Ag5s may also be lacking because of this low sequence identity.

Whereas traditional diagnostic tools rely on whole-venom preparations, the so-called component resolved diagnosis (CRD) allows a patient's allergen recognition profile to be determined. Originally aimed at adapting the immunotherapy to the patient-specific profile, this approach also allows the culprit species to be determined, which is often important because the patients fail to identify or name the Hymenopteran species that stung and because presently used diagnostic tests based on whole venom often reveal a false double positivity to multiple species owing to their similar cross-reactive allergens and cross-reactive carbohydrate determinants (de Graaf *et al.*, 2009). CRD using species-specific allergens may solve this issue. In many European countries, the European honey bee (*A. mellifera*) and yellow jackets (*V. vulgaris*) are the most prevalent stinging insects. As such, several research studies have recently focused on their differential allergy diagnosis by CRD (Müller *et al.*, 2009; Mittermann *et al.*, 2010; Seismann *et al.*, 2010). One of the important differential allergens is Ag5, which is a major venom allergen of the yellow jacket, while so far no honey bee venom Ag5 homologue had been described. Our present findings of the occurrence of a paralogue of Ves v 5 in the bee venom gland and that its peculiar expression is restricted to winter time are important. Owing to the limited contact between humans and winter bees, we hypothesize that sera of patients allergic to honey bee venom lack specific IgE antibodies to the honey bee Ag5-like protein. Moreover, cross-reactivities with wasp and ant venom Ag5s may not be present owing to a low sequence identity. As such, the present findings would support a differential diagnosis of sting allergy by CRD.

Experimental procedures

Ethics statement

Blood sampling was approved by the local ethics committee (registration number: B67020072793) and all participants provided verbal informed consent. There was a verbal agreement with the participants that the samples would be used for no other purpose than the determination of the immune status against bee venom. The procedure was kept somewhat informal as the participants (beekeepers) were employed by or in some way connected with the research centre.

Animals, venom and tissue collection

Summer and winter worker honey bees (*A. mellifera carnica*) were collected from the hives of the experimental apiaries of Ghent University and University of Leuven, which were reared using standard beekeeping methods. BFR honey bees were collected during the winter from a hive that was placed for several months in a climate room that simulates summer conditions (temperature fixed at 25 °C, *ad libitum* sugar solution, plain water and pollen).

Pure honey bee worker venom was collected by 'manually milking' as described by Peiren *et al.* (2005). Venom glands and their reservoirs of 10 honey bees were dissected as described previously (Peiren *et al.*, 2008). Honey bee worker brain, haemocytes, venom gland, hypopharyngeal gland, salivary gland, drone mucus glands, midgut, deviscerated abdomen and muscle tissue for RNA extraction were dissected as described by de Graaf *et al.* (de Graaf *et al.*, 2010) and submerged in RNALater® (Ambion, Austin, TX, USA).

Proteomics/peptidomics

Proteomic analysis of pure venom. Two milligrams of pure worker honey bee venom was dissolved in 200 µl of 0.1% trifluoroacetic acid [TFA (buffer A)] and separated on a Shimadzu RP-HPLC system consisting of an SCL-10Avp system controller, LC-ADvp pump, FCV-10ALvp low pressure gradient unit, SPD-10Avp UV-VIS detector and an FRC-10A fraction collector (Shimadzu, Kyoto, Japan). Proteins were eluted from the Pathfinder 300 C18 AP column (Shimadzu) by a linear gradient from 0 to 100% buffer B containing 30% 0.1% TFA and 70% acetonitrile over a 35-min period (0.7 ml/min). Separate protein peaks were collected and dried by vacuum centrifugation. Consequently, 10 µg protein of each peak was dissolved in 10 µl of 50 mM ammonium bicarbonate. Further reduction, alkylation, tryptic digestion and guanidination were executed according to the In-Solution Tryptic Digestion and Guanidination kit protocol (Thermo Scientific, Waltham, MA, USA).

The MALDI MS analysis was carried out on a 4700 MALDI TOF/TOF Analyzer (Applied Biosystems, Boston, MA, USA); 1 µl of the guanidinated sample was mixed with 1 µl α-cyano-4-hydroxycinnamic acid (10 mg/ml) in 60% acetonitrile containing 0.1% TFA and 10% ethanol. All MALDI spectra were calibrated with 4700 Proteomics Analyzer Calibration Mixture (4700 Cal Mix, Applied Biosystems) and, prior to data collection, all instrumental parameters were tuned. Protein identification (Peptide Mass Fingerprinting) was performed by searching the extracted peaks against the SwissProt and in-house *A. mellifera* database using the MASCOT search engine [Matrixscience, London, UK (peptide mass tolerance: 0.100 Da, two missed cleavages, deamidation (NQ) and oxidation (M))]. Further confirmation of the identification of the proteins was done by selecting the highest peaks for MS/MS fragmentation spectrometry and using the above-described Mascot search engine (peptide mass tolerance: 0.250 Da, one missed cleavage, MS/MS tolerance: 0.25 Da).

Peptidomic analysis of venom apparatus tissue. We made a peptide extract of 10 dissected poison sacks in 100 µl methanol/water/acetic acid (90/9/1, v/v/v). Upon thoroughly sonicating, the sample was centrifuged for 10 min at 11 000 g at 4 °C. The super-

natants were transferred and the resulting pellet was resuspended in 20 µl of methanol/water/acetic acid (90/9/1, v/v/v). Sonication and centrifugation steps were repeated and both supernatants were pooled, dried (vacuum centrifuge) and stored at -30 °C until further analysis. To analyse the sample, the dry extract was dissolved in 5% acetonitrile and 0.5% formic acid and separated by nanoLC with a Dionex UltiMate™ 3000 Dual LC System (Dionex, Sunnyvale, CA, USA) device, coupled online to a MicrOTOF-Q (Bruker Daltonics, Billerica, MA, USA) mass spectrometer. We applied an acetonitrile gradient from 5 to 40% in 30 min, followed by a gradient to 90% in 3 min and back to 5% in 10 min. As many peptides as possible were fragmented in a collision cell.

The data obtained by mass spectrometry were converted to an .mgf-file and used as the input on the in-house Mascot server for an MS/MS-ion search. We performed searches with variable modifications (amidation, pyroglutamate and oxidation of methionine) and with a peptide tolerance and MS/MS tolerance of 0.2 Da in the in-house *Apis* neuropeptide precursor database. This database consists of the 39 neuropeptide precursors, including two precursors for apidaecin (Audsley & Weaver, 2006; Hummon *et al.*, 2006).

RNA isolation, cDNA synthesis, primer development and RT-PCR

Tissue RNA isolation, cDNA synthesis, RT-PCR and amplicon sequencing was done as described by de Graaf *et al.* (2010). DNA elongation in PCR was adapted to 1 min at 72 °C. For amplification of transcripts from various cDNA sources, primers were developed with a melting temperature (T_m) of ~60 °C, using the formula $T_m = 2(A + T) + 4(G + C)^\circ C$. The different primer sets are listed in Table 2. Primer sequences for amplification of apidaecin were developed as used by Casteels-Josson *et al.* (1993); primers 5SB6-2 and 3(S)B6. Profilin (NM_001098167) primers to control for the presence of genomic DNA were developed within its first intron, while exon primers were used to control for the presence of cDNA. Honey bee venom phospholipase A₂ (PLA₂, NM_001011614.1) primers and primers for amplification of the predicted honey bee antigen 5-like sequence (Ag5-like, XM_001122516.2) were developed at the extreme 5' and 3' ends

of the coding sequence. Additionally, primers were developed for amplifying the secreted mature Ag5-like sequence which lacks a signal sequence (determined using the SignalP 4.0 Server: <http://www.cbs.dtu.dk/services/SignalP-4.0/>) (Emanuelsson *et al.*, 2007). For directional cloning of the mature Ag5-like sequence in the prokaryotic expression vector, the forward primer was preceded by four bases (CACC). Also a set of primers in conserved regions of the Ag5-like sequence was determined by aligning a set of Ag5 homologous sequences.

Production and purification

Recombinant baculovirus expression. Recombinant yellow jacket Ves v 5 was produced in Sf9 insect cells as described by Seisemann *et al.* (2010). The cellular supernatant containing the secreted recombinant protein was dialysed to phosphate-buffered saline (PBS) pH 8.0 and supplied to a Hi-Trap column (Sigma-Aldrich, St. Louis, MO, USA) for His-tag purification at a flow rate of 1 ml/min (ÄKTAprime™ plus system, GE Healthcare Life Sciences, Waukesha, WI, USA). The column was washed by a four step-gradient with elution buffer (PBS pH 8.0 containing 300 mM imidazole): 10 min gradient to 3% elution buffer followed by 10 min at constant 3% elution buffer, which was repeated with elevating concentrations of elution buffer (to 6%, 10% and 15%). The recombinant protein was eluted from the column with 100% elution buffer. Protein dialysis to PBS was executed by desalting (PD MidiTrap G-25, GE Healthcare) and sample purity was determined by Coomassie Brilliant Blue R-250 staining of an sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) gel run under reducing (2x Laemmli sample buffer with 10% of β-mercaptoethanol) and denaturing conditions (sample at 100 °C for 5 min and SDS added to PAGE gel and running buffer). The protein concentration of the sample was estimated by comparison of band thickness on a Coomassie-stained SDS-PAGE gel with a dilution series of albumin standard.

Recombinant bacterial expression. Equine uterocalin and the mature honey bee Ag5-like sequence were cloned, sequenced and expressed according to the procedures described by de Graaf *et al.* (2010). For denaturing purification of recombinant proteins by His-tag, cell pellets were dissolved in 8 ml of lysis

Table 2. Primer sets used in reverse transcription PCR

Gene	Primer sequence	Amplicon length
Apidaecin (CDS)	5'-CCAACCTAGATCCGCCTACTCGACCT-3' 5'-TATTTACGTGCTTCATATTCTTC-3'	multiple isoforms
Profilin (gDNA_CTL)	5'-GCGACAAGAGGGAAAGTACG-3' 5'-CGGTGGACAAAATTCTGGAG-3'	685 bp
Profilin (cDNA_CTL)	5'-GGCTTCGAAGTAAGTAAAGAGGA-3' 5'-AGTTTTTCAACGACCGATGC-3'	248 bp
PLA ₂ (cDNA_CTL)	5'-ATGCAAGTCGTTCTCGGATC-3' 5'-ATACTTGCGAAGATCGAACCA-3'	501 bp
Ag5-like (CONS)	5'-CTGACCTGGGACGATGAAC-3' 5'-GCCATTAAATAACTATTAGCCCAGAA-3'	252 bp
Ag5-like (ORF)	5'-ATGGCGCGGGAGGGAATAA-3' 5'-TTAACATCGAGTTCCCAGATAA-3'	672 bp
Ag5-like (MAT)	5'-CACCGATGTTATTTCTGCATCGGC-3' 5'-TTAACATCGAGTTCCCAGATAA-3'	609 bp

CDS, Primer set to amplify the coding sequence; gDNA_CTL, Primer set to control the presence of genomic DNA; cDNA_CTL, Primer set to control the presence of cDNA; CONS, Primer set to amplify a conserved domain; ORF, Primer set to amplify the entire open reading frame; MAT, Primer set to amplify the mature fragment (=ORF without the signal sequence).

buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazol and 8 M ureum) and sonicated on ice with five 10-s pulses at high intensity. After centrifugation (12 000 g, 4 °C, 30 min) supernatant was supplied to the Profinity IMAC Ni-charged resin (Bio-Rad, Hercules, CA, USA) which was equilibrated with lysis buffer. The resin was washed with 24 ml of wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole and 8 M ureum) and recombinant protein was eluted in elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 500 mM imidazol and 8 M ureum). After dialysis to lysis buffer (SnakeSkin dialysis tubing, 3.5 MWCO, Thermo Scientific) recombinant Ag5-like protein was further purified by a second identical purification step. Dialysis to PBS, sample purity and protein concentration determination was carried out as mentioned previously.

Patient sera

The sera of 10 highly exposed beekeepers with no allergy against bee venom were collected. Information about whether or not they had a history of yellow jacket stings was also available. In addition, we collected five negative control sera from persons that had never been stung by Hymenopteran insects.

Enzyme-linked immunosorbent assay

Honey bee venom-, honey bee venom PLA₂- and melittin-specific serum IgG4 titers were determined for all sera using ELISA. Nunc MaxiSorp® flat-bottomed 96-well plates were coated with 150 µl of honey bee venom (2 µg/ml, Sigma-Aldrich), purified honey bee venom PLA₂ (4 µg/ml; Latoxan, Valence, France) and purified melittin (1 µg/ml; Latoxan) in coating buffer (100 mM bicarbonate/carbonate buffer, pH 9.6) at 4 °C overnight. Subsequently, wells were washed three times with PBS Tween 20 (PBST), blocked with 50 mg/ml skimmed milk powder in PBS at room temperature for 2 hours and washing was repeated. For each serum sample a two-fold dilution series from 1:40 to 1:20480 was performed using blocking buffer. Plates were incubated for 45 minutes at 37 °C and washed three times before bound IgG4 was detected with 150 µl of HRP-conjugated mouse anti-human IgG4 (Southern Biotech) diluted in 1:10000 in blocking buffer (1 hour at 37 °C). Wells were washed three times with PBST and 200 µl of substrate solution (SIGMAGAST OPD, Sigma-Aldrich) was added to each well. After 30 minutes, the reaction was stopped with 100 µl of stop solution (3 M HCl) and the plates were read at 490 nm. Antibody titer was defined as the highest dilution with a reading above the mean of the negative controls plus 1.96 SDs.

Western blotting

Twelve microgram of each protein fraction was separated by 15% SDS-PAGE under reducing and denaturing conditions in a discontinuous system (Bio-Rad) and blotted to polyvinylidene difluoride membrane. After blotting, the membrane was cut into strips, blocked for 2 hours and incubated overnight at 4 °C with 2 ml of diluted serum (1/16 diluted in blocking solution). Subsequently, strips were washed three times with blocking solution and incubated in 2 ml of 1:1000 diluted HRP-conjugated mouse anti-human IgG4 antibody (Southern Biotech) in blocking solution. Finally, blots were washed 3 times with PBST and once with PBS before DAB staining. Anti-His staining of His-tagged recombinant proteins was performed as described previously (Peiren *et al.*, 2006a; de Graaf *et al.*, 2010). Ponceau S staining was used for

determination of blotting success of non-His-tagged proteins. PBS buffer and 1 µg of recombinant equine uterocalin were spotted to serve as negative controls (Suire *et al.*, 2001).

Phylogenetic analysis

Sequences related to our honey bee Ag5-like sequence in other Hymenoptera were retrieved based on a protein blast against all non-redundant protein and predicted protein sequences in the NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins>) using default search parameters. Low-quality or incomplete sequences, or sequences that showed unusually high sequence divergence, were removed. Subsequently, sequences were aligned using MUSCLE (Edgar, 2004), after which a neighbour-joining phylogenetic tree was estimated using the Jones–Taylor–Thornton (Jones *et al.*, 1992) model (+G). A discrete Gamma distribution with three categories was used to model differences in the substitution rate among sites. Mean evolutionary rates in these categories were estimated at 0.34, 0.84, 1.82 substitutions per site and the shape parameter of the gamma distribution was estimated at 1.9. In all calculations, positions with <50% site coverage were eliminated. This resulted in a total of 51 sequences of 208 amino acid positions each in the final dataset. The reliability of the phylogenetic placement of the sequences was assessed using the bootstrap method using 500 bootstrap replicates. A tentative classification of sequences into orthologue groups was made based on the repeated appearance in the tree of sequences from species with known phylogenetic placement and fully sequenced genomes (e.g. *Nasonia*, *Apis*). All evolutionary analyses were conducted using MEGA5 (Tamura *et al.*, 2011). maximum likelihood or Bayesian trees, obtained using Mr. Bayes, resulted in very similar phylogenetic patterns to the ones obtained using a neighbour-joining approach (results not shown).

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Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/imb.12013

Figure S1. Peptide mass fingerprint and tandem mass spectrometry fragmentation spectrum of the identified apidaecin peptide in venom gland tissue.

Figure S2. Nucleotide and protein sequence alignments of the cloned honey bee Ag5-like sequence with the National Center for Biotechnology Information prediction (by CLUSTALW).

Figure S3. Coomassie blue staining of sodium dodecyl sulphate polyacrylamide gel electrophoresis-separated synthetic apidaecin peptide and purified recombinants uterocalin, Ves v 5 and honey bee Ag5-like protein.

Table S1. Information about beekeeper sera.