REVIEW OF THE EXPRESSION OF ANTIMICROBIAL PEPTIDE DEFENSIN IN HONEY BEES *APIS MELLIFERA* L.

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S u m m a r y

Honey bees defensin have a high level of polymorphism and exist as two peptides - defensin 1 and 2. Defensin 1 is synthesized in the salivary glands and is responsible for social immunity. Defensin 2 is synthesized by cells of the fat body and hemolymph is responsible for individual immunity. Defensins are inducible and controlled by the interaction of Toll and Imd signaling pathways and have a broad spectrum of antimicrobial action. The use of chitosan as an immunomodulator has been shown to lead to an increase in the expression levels of defensin and abaecin in the honey bee organism. Stimulation of the transcriptional activity of the defensin genes will allow for the control of a honey bee colony's immunity level, and reduce the using of antibiotics and other chemicals.

Keywords: honey bee, *Apis mellifera*, defensin, evolution, immunity.

INTRODUCTION

Antimicrobial peptides (AMP) are important components of the honey bee immune system (Hoffmann et al., 1999). This is one of the evolutionarily ancient and important protector mechanisms of invertebrates. In a short period of time, AMP can be activated and delivered to the site of infection (Aerts et al., 2008). At present four types of AMP are found in bees: apidaecin presented by twelve isoforms (Casteels et al., 1989), abaecin (Casteels et al., 1990), hymenoptaecin (Casteels et al., 1993) and defensin presented by two isoforms - defensin 1 and 2 (Casteels et al., 1993; Casteels-Josson et al., 1994; Qu et al., 2008).

Several varieties of antimicrobial protein molecules are found in honey bee royal jelly: major royal jelly protein (MRJP) has five isoforms (MRJP1-5) (Casteels-Josson et al., 1994; Klaudiny et al., 2005; Qu et al., 2008), jelleine has four isoforms (Jelleine-I-IV) (Fontana et al., 2004), defensin has two isoforms called royalisin (Fujiwara et al., 1990).

Kwakman et al. (2010) showed antimicrobial properties of honey in relation to *Bacillus subtilis*, methicillin resistant *Staphylococcus aureus*, β-lactamase producing *Escherichia coli*, ciprofloxacin resistant *Pseudomonas aeruginosa*, and vancomycin resistant *Enterococcus faecium*. It was shown that antimicrobial properties of honey are provided by the content of hydrogen peroxide, methylglyoxal and defensin-1.

On the power of their actions, AMP are comparable with antibiotics and can be used in the development of drugs with antifungal and antibacterial properties (Bulet et al., 1999). The use of fungicides and antibiotics in the treatment of honey bees for diseases leads to the suppression of the bees' immune system, the emergence of resistant pathogens, and the contamination of bee products (Miyagi et al., 2000). Enhancement of the honey bees immune

Journal of Apicultural Science

system by increasing of the expression level of AMP in bees themselves, is the solution to these problems (Bilikova et al., 2001).

Unfortunately, data on the defensins having the highest polymorphism and spectrum of activity of all honey bee AMP, are scattered. The data do not give a complete picture of the role of these defensins in the life and anti-infectious protection of *Apis mellifera* L.

Activity of defensins against pathogens of honey bees

Bacteria. Honey bee defensins have enough broad-spectrum antibacterial activity. The cytotoxic activity of defensins against gram-positive bacteria (Casteels-Josson et al., 1994; Bulet and Stocklin, 2005) and several species of gram-negative bacteria, is known (Mandrioli et al., 2003). One of the social immunity factors is the allocation of royalisin with the royal jelly for larvae feeding and antibacterial protection. It has been shown that royalisin is effective primarily against gram-negative bacteria and some gram-positive (Klaudiny et al., 2005). In particular, royalisin is active against American Foulbrood *Paenibacillus larvae larvae* (Bilikova et al., 2001; Bachanova et al., 2002; Yoshiyama and Kimura, 2010).

The bacteria *Lactobacillus* is non-pathogenic to bees. This bacteria also stimulates an increase in the level of gene expression of abaecin and defensin. The use of abaecin and defensin as probiotics to enhance honey bee immunity is then possible (Arbia and Babbay, 2011). Yoon et al. (2009) showed induction of defensin gene expression in body fat of workers from other members of *Apidae*: three species of bumblebees *Bombus terrestris, B. ardens ardens* and *B. hypocrita sapporoensis,* in response to injection of lipopolysaccharides simulating the action of bacterial infection. Similarly, chitosan also stimulates the defensin gene expression in the honeybee, simulating an invasion of microorganisms (Saltykova et al., 2010 a,b).

Fungi. Of the entire AMP of honey bees, only defensin has cytotoxic activity against fungi - fungus of chalkbrood *Ascosphaera apis*, fungi of aspergillosis *Aspergillus flavus* Link and *Aspergillus niger* Tieghem, yeast-like fungi *Candida albicans* and *Aurobasidium pullulans* (Chernysh et al., 1999; Aronstein et al., 2010). In particular, Aronstein and Saldivar (2005) showed an increase in levels of defensin gene expression in the five-day larvae *A. mellifera* experimentally infected with *A. apis*. This experiment confirmed the universal mechanism of defensin action against fungal infections, since components of both signaling pathways - Toll and Imd were involved (Evans and Spivak, 2010).

Protozoa. Microsporidia *Nosema* is an obligate parasite in the intestines of the honey bee. Until recently, *Nosema ceranae* was known as a parasite of the chinese wax bee *Apis cerana*. Since 2005, there has been information about microsporidia *N. ceranae* parasitizing on *A. mellifera* (Higes et al., 2009), which is more pathogenic to honey bees than *Nosema apis* (Klee et al., 2007). This is probably a consequence of long-term coevolution of *A. mellifera* with *N. apis*. Such a coevolution contributed to the emergence of certain immune mechanisms in the honey bees to this parasite, including defensin gene expression (Antunez et al., 2009). The microsporidian pathogen *N. ceranae* is a new parasite for the honey bee causing a major breach in the gut and suppressing immunity as a whole, expression of defensins in particular (Klee et al., 2007).

Mites and viruses. The weakening of the immunity with ectoparasites is caused not only by parasitization, but also by the transmission of viral infections. The most common ectoparasites of honey bees are the mites. Among the many species of mites, four species are the most dangerous for honey bees: *Varroa destructor*, *Varroa jacobsoni*, *Acarapis woodi, Tropilaelaps clareae* (Grobov and Lihotin, 1989).

Bees infected with *V. destructor* die when exposed to the bacteria *Esherichia coli*. These infected bees differ from healthy bees in the large number of hemolymph damaged cells and content of viral particles (Yang et al., 2004). It is shown that immunosuppressive effect and severe clinical symptoms increase with a rise in the mite infestation level (Williams et al., 2009).

The humoral immune system of bees is affected by *V. destructor*, reducing the defensin transcription level (Yang et al., 2004; Gregory, et al., 2005). Perhaps, *V. destructor* causes immunosuppression of bees by means of replication of the deformation of wing virus DWV which is carried by this mites (Genersch and Aubert, 2010).

Induction of defensin expression in the honey bee organism

Defensins represent a large family of cysteine-rich AMP. Honeybee defensins are homologous to formicins of fly *Phormia terranovae* and differ from other peptides by having a peculiar structure as well as selective activity against gram-positive bacteria and some filamentous fungi (Klaudiny et al., 1994; Chernysh et al., 1999).

At present, four types of AMP were detected in honeybee: apidaecin (Casteels et al., 1989), abaecin (Casteels et al., 1990), hymenoptaecin (Casteels et al., 1993) and defensins. The defensins are represented by two peptides, defensin 1 and defensin 2 with a molecular weight of 5.5 and 4.8 kDa, respectively, and encoded by two genes, *defensin 1 and defensin 2*. Defensin 1 is presented by three isoforms - defensin of hemolymph and 2 isoforms, found in royal jelly and named royalisin (Ro-F) with a molecular weight of 5525,1 kDa and 5515,5 kDa, respectively (Klaudiny et al., 2005). Royalizin differs from the hemolymph defensin by two amino acid substitutions (Klaudiny et al., 2005). Honey has been shown to possess antibacterial properties against *Bacillus subtilis*, methicillin-resistant *Staphylococcus aureus*, β-lactamase-producing *Escherichia coli*, ciprofloxacin-resistant *Pseudomonas aeruginosa*, and vancomycin-resistant *Enterococcus faecium* (Kwakman et al., 2010). These antimicrobial properties of honey are provided by the content of hydrogen peroxide, methyl-glyoxal and the antimicrobial peptide defensin-1.

Defensin 1 gene consists of a 2012 bp (number in the genebank AY496432) and contains two introns. The first intron in size 571 bp located between 773 and 1345 nucleotides, and the second - in the size of 278 bp located between nucleotides 1525 and 1804. Excluding introns, the sequence of the gene is fully aligned with the cDNA of royalisin Ro-K. Sequencing of cDNA showed a similarity of the gene of royalisin with a defensin 1 gene, except for one site in the 377 basis where there was a single nucleotide replacement (C-T) (Casteels-Josson et al., 1994).

Defensin 2 gene consists of 1950 bp (number in the genebank AY588474) and contains an intron in size 335 bp located between the 947 and 1283 nucleotides. At the DNA level there is a high level of similarity of the central module of defensins. On the other hand, the genes have a low level of similarity above the TATA box and TATA box below to the place of processing of the mature peptide as well as in 3'-noncoding region**.**

Genes *defensin 1* and *defensin 2* genes differ significantly in length, intron-exon structure and sequence of their pre-pro regions. In particular, the second intron of *defensin 1*, which is not represented at *defensin 2* is the first intron found in the encoding part of arthropod defensin gene. Short aminated C-terminal extensions are found only in the Hymenoptera defensins (Hanzawa et al., 1990; Raj and Dentin, 2002). Perhaps these extensions of 11 amino acids allow the defensin molecule to take an alpha-spiral structure, which stabilizes the aminated C-terminus (Casteels et al., 1989). The exact role of these extensions in the function of the peptide is not yet fully disclosed. Perhaps these extensions are the result of exon shuffling (Long, 2001).

Journal of Apicultural Science

Because of a recombination of two exons, the atypical defensin with an extension at C-terminus has appeared. Similar processes of the emergence of defensin C-terminal extensions also occurred during the evolution of two bumblebee species.

Studies concerning the variability of the defensin gene fragment in *A. m. mellifera* populations in the Urals showed the presence of two alleles of this locus (Ilyasov et al., 2008). Allele B of the defensin gene fragment, large in size, met with a frequency of 0.14-0.25, and allele A, with a smaller size - with a frequency of 0.75-0.86. Such changes in defensin allele frequencies may be due to the occurring micro evolutionary processes.

Many variants of defensin isoforms may be due to posttranslational modification under the influence of genes in other loci, rather than one-locus mutations (Solbrig and Solbrig, 1979). Perhaps one of the causes of the multiplicity of antimicrobial activity of bee defensins in the evolution process, is the variability in processing of defensin precursors which leads to the appearance of isoforms.

The majority of AMP is produced by the fat body cells (adipocytes) and hemolymph cells (hemocytes) with infections and injuries of the integument, and usually distinguished in the hemolymph (Hoffmann et al., 1999; Choi et al., 2008). However, using the RT-PCR method, defensin 1 has been shown to be expressed in the head and thorax of honey bees by the hypopharyngeal, mandibular and thoracic salivary glands (Lopez et al., 2003; Klaudiny et al., 2005) and released in the royal jelly (Qu et al., 2008) and honey (Kwakman et al., 2010). There is wide variability in defensin quantity and level of activity in the royal jelly of different healthy honey bees colonies. One reason may be genetic variation in the levels of defensin expression. Defensin 1 gene polymorphism may play an important role in the modification of the expression level of AMP and its antimicrobial and antifungal activity (Bilikova et al., 2001). This may be important for practical beekeeping when selecting honey bees colonies more resistant to microbial pathogens, especially colonies more resistant to *Paenibacillus larvae larvae* (Evans and Spivak, 2010). The low level of transcription in the healthy bees and the presence of regulatory elements in the promotor region of the defensin 2 gene proves that defensin 2 is induced by a pathogenic factor (Casteels et al., 1989; Klaudiny et al., 2005). Thus, we can conclude that the predominant role of defensin 1 is the formation of a social immune system of the honey bee. This is in contrast to the defensins 2 produced by adipocytes and hemolymph cells which is a component of individual immunity (Fig. 1). The wide representation of defensins in

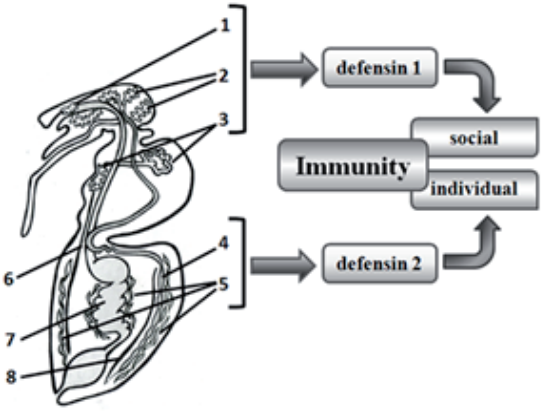

Fig. 1. Forms of defensin in individual and social immunity of honey bees.
1 - mandibular gland; 2 - hypopharyngeal gland; 3 -thoracic gland;
4 - dorsal vessel; 5 - fat body; 6 - ventral diaphragm; 7 - midgut; 8 - dorsal diaphragm.

the honey bee organisms indicates their universal and significant functions in individual and social immunity.

The share of AMP synthesized by the hemocytes is negligible. The main part of AMP is produced by the fat body cells - adipocytes (Hoffmann and Richhart, 1997). Initiation of AMP gene transcriptional activity occurs at the injury and gets into the hemolymph of inductors of a different nature and origin - bacteria, fungi and fragments peptidoglycanes and lipopolysaccharides of the bacteria cell walls (Dunn, 1990), some insecticides (Zhu and Lu, 1992), and chitin oligomers (Furukawa et al., 1999). The experimental data obtained under the single application of chitosan as an immunomodulator for honey bees showed a twofold increase of transcriptional activity of defensin and abaecin genes by PCR in real time (Saltykova et al., 2010 a,b) (Fig. 2).

Activation of AMP synthesis in insect adipocytes and hemocytes is carried out by several mechanisms (Fig. 3). It is assumed that the generation of AMP synthesis inducers - lipopolysaccharides, peptidoglycanes and β-1,3-glycans - involves phagocytic cells releasing into the hemolymph from the cell wall components of absorbed and digested bacteria (Taniani et al., 1997). Hemocyte mediators, such as prostaglandins, can enhance the activity of adipocytes and hemocytes (Stanley-Samuelson, 1994). It is shown that phagocytes with absorbed microorganisms are able to directly attach to the adipocytes, which also stimulates the synthesis of AMP (Glupov, 2001). In addition, activation of AMP synthesis in adipocytes and hemocytes is assumed under the action of mediators of the endocrine system (Glupov, 2001).

There are two major NF-kB-mediated signaling pathways - Imd and Toll - controlling the gene expression of AMP in insects (Osta et al., 2004). The Toll pathway is responsible for the protection against fungi and gram-positive bacteria, whereas the Imd pathway primarily provides protection against gram-negative bacteria (Hoffmann, 2003; Evans and Spivak, 2010). At the same time, control of defensin gene expression is carried out by the interaction of Toll and Imd pathways (Aronstein et al., 2010) demonstrating the importance of this element of the AMP system. Antimicrobial peptides are active at low concentrations. These peptides show a wide spectrum of activity interacting with the cytoplasmic membranes of pathogens and acting in concert with other agents of immune response (Osta et al., 2004).

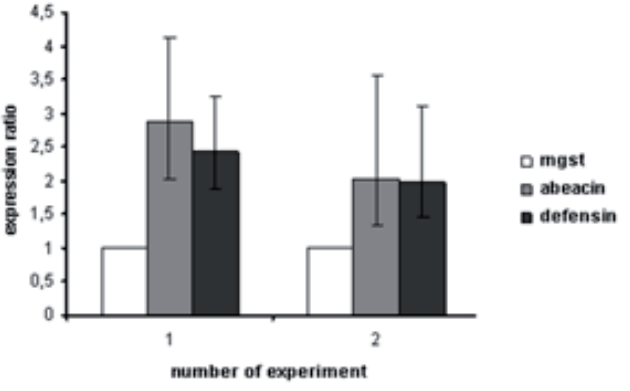

Fig. 2. Expression level changes of AMP defensin and abaecin in the honey bee organism in two experiments (biological replicates). Transcript level for a gene with low transcriptional variation mgst (microsomal glutathione-S-transferase) (Evans and Wheeler 2000) was used to normalize against variable mRNA levels (Saltykova et al., 2010 a,b).

Journal of Apicultural Science

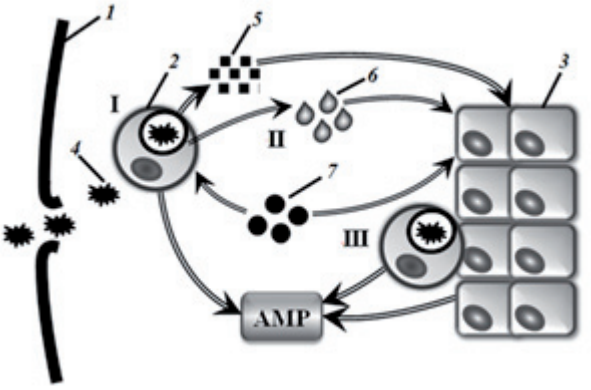

Fig. 3. Activation of AMP synthesis in insect adipocytes and hemocytes.
I - generation of inductors of AMP synthesis; II - secretion of hemocyte mediators; III - attachment of hemocyte to the fat body. 1 - the surface of the body; 2 - phagocyte, 3 - adipocyte; 4 - microorganism; 5 - cell wall components of the microorganism; 6 - hemocyte mediators; 7 - mediators of the endocrine system.

The general principle of the AMP is reduced not only to inconsistency of membranes of pathogenic cells, but it is mediated by other mechanisms. Defensins kill pathogenic cells, penetrating through their cytoplasmic membrane (Cociancich et al., 1993). Defensins cause the formation of channels in the plasma membrane of target cells through which cytoplasmic K^+ outflows, and consequently, there is a partial depolarization of the plasma membrane, reduction of ATP content in the cytoplasm and inhibition of respiratory processes. Experiments in vitro demonstrated that defensins of *Phormia* and *Aeschna* also cause a disturbance of membrane permeability of *Plasmodium gallinaceum* sporozoites followed by a change in morphology and loss of the parasitoid mobility (Shahabuddin et al., 1998).

CONCLUSIONS

Honey bee defensins have a high level of polymorphism, and exists in two forms - defensin 1 and 2. Defensin gene expression is inducible and regulated by the Toll and Imd signaling pathways, which lead to its versatility action against pathogens of the honey bees (*Paenibacillus larvae*, *Melissococcus pluton, Ascosphaera apis, Nosema apis*). The uniqueness of this peptide of Hymenoptera among other arthropods as well as the multiplicity of antimicrobial activity of honey bee defensins is determined by the variability in the processing of defensin precursors leading to the appearance of multiple molecular forms. The action of the defensin mechanism is reduced to a breach of the integrity and permeability of the cytoplasmic membrane of pathogenic organisms and provides a wide range of action.

Defensin 1, contained in royal jelly and honey and synthesized in the salivary glands, is involved in the formation of the social immunity of colonies. Defensin 2, synthesized by cells of the fat body and hemolymph, is responsible for individual immunity of the honey bee. The wide multifunctional activity of defensins is characterized by principles of social life that emerged during the evolution of some Hymenoptera species.

The use of immunomodulators leads to a marked increase in the level of expression of antimicrobial peptides in the organism of the honey bee. This allows for the control of the level of honey bee colony immunity and allows for a reduction in the use of antibiotics and other chemicals.

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EKSPRESJA PEPTYDU PRZECIWBAKTERYJNEGO - DEFENSYNY U PSZCZOŁY MIODNEJ *APIS MELLIFERA* L. - PRACA PRZEGLĄDOWA

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S t r e s z c z e n i e

Pszczoła miodna posiada w wysokim stopniu zróżnicowaną defensynę występującą w postaci dwóch peptydów - defensyny 1 oraz 2. Defensyna 1 jest syntetyzowana w gruczołach ślinowych i odpowiada za odporność społeczną pszczół, natomiast defensyna 2 syntetyzowana jest przez komórki ciała tłuszczowego oraz w hemolimfie i odpowiada za odporność indywidualną. Defensyny są indukowalne. Regulowane są poprzez współoddziaływanie szlaków sygnalizacyjnych Toll i Imd. Defensyny posiadają szerokie spektrum działania przeciwbakteryjnego. Wykazano, że zastosowanie chitozanu jako immunomodulatora prowadzi do podniesienia poziomu ekspresji defensyny i abaecyny w organizmie pszczoły miodnej. Stymulowanie aktywności transkrypcyjnej genów defensynowych pozwoli kontrolować poziom odporności rodzin pszczelich oraz zmniejszyć zastosowanie antybiotyków i innych leczniczych środków chemicznych.

Słowa kluczowe: pszczoła miodna, *Apis mellifera*, defensyna, ewolucja, odporność.