

## Identification of honeybee peptide active against *Paenibacillus larvae larvae* through bacterial growth-inhibition assay on polyacrylamide gel

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**Abstract** – The inhibition bands showing activity against Gram-positive bacteria were detected by analyses of acidic extracts of honeybee heads, thoraxes, and royal jellies (RJs) using a bacterial growth-inhibition assay on polyacrylamide gel. The presence of antibacterial peptide royalisin and another unknown peptide was found in two detected RJ inhibition bands by N-terminal sequencing. The data suggested that royalisin was the peptide responsible for the activity against *Paenibacillus larvae larvae* and other tested Gram-positive bacteria. The analyses of RJs collected from individual colonies at two apiaries, one of which showed incidence of American foulbrood, revealed differences in the content of the antibacterial peptide. The results suggest that the differences might be associated with genetic variability between colonies.

***Apis mellifera* / royal jelly / *Paenibacillus larvae larvae* / American foulbrood / antibacterial peptide**

### 1. INTRODUCTION

Distinct honeybee (*Apis mellifera* L.) colonies and lines can possess different levels of resistance to the most serious larval honeybee disease American foulbrood (AFB), caused by a Gram-positive bacterial

pathogen *Paenibacillus larvae larvae* (for reviews see Rothenbuhler, 1958; Spivak and Gilliam, 1998; Hansen and Brødsgaard, 1999). Two mechanisms of AFB resistance are thought to be associated with larval food. One is associated with microorganisms present in pollen that act as

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antagonists of *P. l. larvae* (Rinderer and Rothenbuhler, 1969; Rinderer et al., 1974; Reiche et al., 1996). The second is associated with the antibacterial components secreted by the adult honeybee into the larval diet. Fatty acids, primarily 10-hydroxy-*trans*-2-decenoic acid, are thought to be such substances (Blum et al., 1959; Yatsunami and Echigo, 1985; Bonvehí and Jordá, 1991).

It has been shown that royal jelly (RJ) contains a peptide royalisin, composed of 51 amino acid residues (Fujiwara et al., 1990). Royalisin belongs to the family of insect antibacterial peptides, defensins, which were shown to be active against a wide range of Gram-positive bacteria (for review see Hetru et al., 1998; Dimarcq et al., 1998). Other honeybee antibiotic peptides – apideacins, abaecin, hymenoptaecin, bee defensin (royalisin homolog) – are produced by the well-developed immune system in the hemolymph and possess different activity spectra against Gram-positive and Gram-negative bacteria (reviewed by Casteels, 1998; Bulet et al., 1999). However, there was no prior evidence in the literature whether royalisin was active against *P. l. larvae* and whether RJ contained other antibacterial peptides and proteins active against this pathogen. Obtaining such knowledge is important since antibacterial peptides/proteins in the larval diet may be potential factors of resistance of honeybee colonies against AFB.

Therefore, we have started investigations on the identification of antibacterial peptides/proteins secreted by honeybees into RJ. We have used two approaches in this research. The first one is based on fractionation of RJ followed by identification of the antibacterial peptides/proteins in the individual fractions by a diffusion test on agar plate. By this approach, the royalisin fraction of RJ was isolated and its activity against *P. l. larvae* and its antifungal properties were determined (Bíliková et al., 2001). The second approach is based on

electrophoretic separation of peptides and proteins of honeybee and RJ acidic extracts in native acidic polyacrylamide gel followed by antibacterial assay on top of the gel. This assay was used for the first time by Hultmark et al. (1980) and was used to identify, isolate, and to characterize several basic insect antibacterial peptides (Hoffmann et al., 1981; Flyg et al., 1987) and attacin-like proteins (Kaaya et al., 1987). The potential of this assay for identification of antibacterial peptides/proteins is high because acidic polyacrylamide gel electrophoresis is suitable for separation of basic peptides and proteins. A common feature of known insect antimicrobial peptides and several proteins is their positive net charge at physiological pH (for review see Bulet et al., 1999).

The aim of this work was to identify and partially characterize antibacterial peptides/proteins in acidic extracts of different samples of honeybee heads, thoraxes and RJs using an antibacterial assay we developed on polyacrylamide gel. The assay also was used to estimate the amount of identified antibacterial peptide showing activity against *P. l. larvae* in RJs collected from different honeybee colonies.

## 2. MATERIALS AND METHODS

### 2.1. Honeybee and royal jelly samples

Honeybees derived from *Apis mellifera carnica* Pollmann and RJs were obtained from various locations in Slovakia. Random samples of honeybees from two colonies, one with and the other without symptoms of AFB were obtained from a private apiary (Slatina) in June 1998. Honeybees were collected into perforated Falcon tubes, immediately frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$ .

RJs were collected from individual colonies at a healthy apiary (Institute of

Apiculture, Liptovský Hrádok) and from colonies at another apiary with incidence of AFB (private apiary, Valaska) in May 1999. At the apiary in Valaska several colonies had clinical symptoms of AFB in 1997. The sick colonies were burned, except for the queens, which were used to establish new colonies using honeybees imported from the healthy apiary in Liptovský Hrádok. Clinical AFB symptoms occurred in this apiary in one colony in 1998 and in two colonies in 1999. RJs were obtained from colonies that did not have AFB. The individual RJ samples represented RJ collected from one to three queen cells within one colony (samples from Liptovský Hrádok) or one queen cell containing a 2–3 day old larva (samples from Valaska). Other miscellaneous RJ samples represented a mixture of RJs collected from more than one colony obtained at the apiary of Institute of Apiculture (Liptovský Hrádok) in 1997 and in 1998. The RJs were collected into microtubes or larger vials (miscellaneous RJ samples) and stored at  $-20^{\circ}\text{C}$ .

## 2.2. Bacteria and culture media

A spore culture of *Bacillus subtilis* and bacterial strains of *Sarcina lutea*, *Micrococcus luteus*, *Escherichia coli*, *Serratia marcescens* were obtained from the collection of microorganisms of Biotika Inc. (Slovenská L'upča, Slovak Republic). Strains of *P. l. larvae* 5084 and 5086 originated from ATCC (USA). Overnight cultures (vegetative cells) of bacterial strains were prepared in BP medium (1% beef extract, 1% bacto peptone, 0.5% NaCl, pH 7.2) by incubation at  $30^{\circ}\text{C}$  or  $37^{\circ}\text{C}$  in the case of *P. l. larvae*.

## 2.3. Preparation of honeybee and RJ acidic extracts

Frozen heads or thoraxes of twenty-five honeybees were reduced to powder in a mortar in the presence of liquid nitrogen

and stored in a freezer at  $-80^{\circ}\text{C}$ . The frozen powder was weighed into microtubes kept on dry ice (19 mg of heads; 28 mg of thoraxes). Extractions were performed with succinate buffer (50 mM succinic acid, 100 mM sodium chloride, 5 mM ethylenediaminetetraacetic acid, 0.5 mM dithiothreitol) adjusted to pH 4 with 2 M NaOH. Before use, 1 mM phenylmethylsulfonyl fluoride was added to the buffer from 0.2 M stock solution in isopropyl alcohol. Buffer was added to the powder (2  $\mu\text{l}$  per mg) and the microtubes were kept in an ice-water bath for 30 min. During this period, the extraction was performed by repeated short vortexing every 3 min. The extracts were centrifuged at 17 000  $g$  for 5 min at  $4^{\circ}\text{C}$  and the supernatant was collected. RJs (at least 8 mg for an extraction) were first well suspended in succinate buffer containing 50 mM succinic acid adjusted to pH 4 with 2 M NaOH (3  $\mu\text{l}$  per mg of RJ) and then extracted using the same procedure as with head and thorax extracts.

## 2.4. Bacterial growth-inhibition assay on polyacrylamide gel

Peptides and proteins present in the acidic extracts of honeybee heads, thoraxes or RJs were separated by a slightly modified method of native discontinuous acidic polyacrylamide gel electrophoresis (Reisfeld et al., 1962; Hames, 1981). The 15% separating polyacrylamide gel ( $9.5 \times 16 \times 0.1$  cm) was prepared using acrylamide-bisacrylamide (30:0.2) solution and gel stock buffer (0.1 M KOH adjusted by acetic acid to pH 4.05) diluted 8 $\times$  at gel preparation. The gel was prepared in advance. After polymerization, it was overlaid with gel buffer containing added ammonium persulphate and let stand overnight covered with Saran wrap to avoid evaporation. The 4% stacking polyacrylamide gel ( $2 \times 16 \times 0.1$  cm) was prepared using acrylamide-bisacrylamide

(30:0.8) solution and gel stock buffer (0.1M KOH adjusted by acetic acid to pH 5.2) diluted 4× at gel preparation. Photopolymerization of stacking gel under a daylight fluorescent lamp was performed for 1 hour. Two parallel sets of samples were loaded on the gel. Before loading, acidic extracts were mixed with 1/5 volume of loading solution containing 30% glycerol and 0.01% methyl green. Electrophoresis was run in a buffer containing 3.12 g β-alanine and 0.8 ml acetic acid per liter at a constant current of 18 mA for 5.5 hours. After electrophoresis, a portion of the separation gel with one set of samples was stained with 0.05% Coomassie Brilliant Blue R250 in a solution containing 5% acetic acid and 45% methanol for 1.5 hour and destained overnight in a solution containing 10% acetic acid and 5% methanol. Another portion of the separation gel with a parallel set of samples was incubated for 10 min in rich bacterial medium RBM (2% beef extract, 2% peptone, 0.5% NaCl), which was buffered with 0.2 M sodium phosphate, pH 7.2. The gel was transferred to a sterile square Petri plate (10 × 10 cm; Bibby Sterilin, UK) and bacterial growth-inhibition assay was performed with small modification of a procedure described by Grenier et al. (1993). The gel was dried in a laminar flow air cabinet for 5 min. During this time, a 3MM paper (Whatman, UK) was impregnated (0.09 ml/cm<sup>2</sup>) with a bacterial suspension of vegetative cells (1 × 10<sup>8</sup> cfu/ml) or with *B. subtilis* spore suspension (1 × 10<sup>7</sup> cfu/ml) diluted with RBM medium and then transferred on the surface of the gel. After 3 min, the paper was removed and the sealed Petri plate was incubated at 30 °C or 34 °C in the case *P. l. larvae* for 16–20 h. The Petri plates were photographed against a black background to help visualize inhibition bands (clear spots) in the bacterial lawn on the gel surface. The pictures were taken with a DC 120 Zoom Digital Camera (Kodak, USA) and processed by Kodak Digital Science 1D Image Analysis Software.

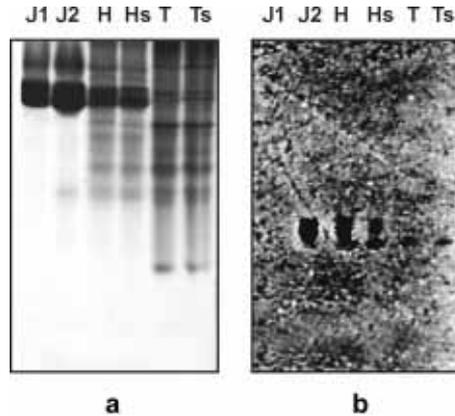
## 2.5. N-terminal sequencing of peptides

Peptides and proteins present in acidic extract of RJ were separated by discontinuous acidic PAGE as described above. The gel was subsequently incubated two times for 7 min in electroblotting buffer containing 10 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) in 10% methanol adjusted with NaOH to pH 10.7 and electroblotted for 45 min onto a ProBlott membrane according to the procedure recommended by manufacturer (Applied Biosystems, USA). Peptide bands were visualized with Coomassie Brilliant Blue R 250 staining, excised and sequenced (first 5 amino acid residues of N-termini of peptides) by automated Edman degradation on a LF 3600D Protein Sequenator (Beckman, USA).

## 3. RESULTS

### 3.1. Identification of antibacterial peptides/proteins in miscellaneous RJ samples, heads and thoraxes of honeybees

Figure 1 shows the results of the analyses of acidic extracts of two miscellaneous RJ samples as well as head and thorax extracts of 25 randomly collected honeybees from the two colonies of the same apiary (Slatina), one with and the other without symptoms of AFB. A larger spot of bacterial growth inhibition, composed of two inhibition bands located close to each other, was detected on a polyacrylamide gel overlaid with *B. subtilis* in the case of one of the two extracts of RJs (Fig. 1b, lanes J1, J2) and of both extracts of honeybee heads (Fig. 1b, lanes H, Hs). Thorax extracts (Fig. 1b, lanes T, Ts) yielded one inhibition band, located at a similar position as the lower inhibition band of the head and RJ extracts. Inhibition bands of the extracts from honeybees of the colony without symptoms



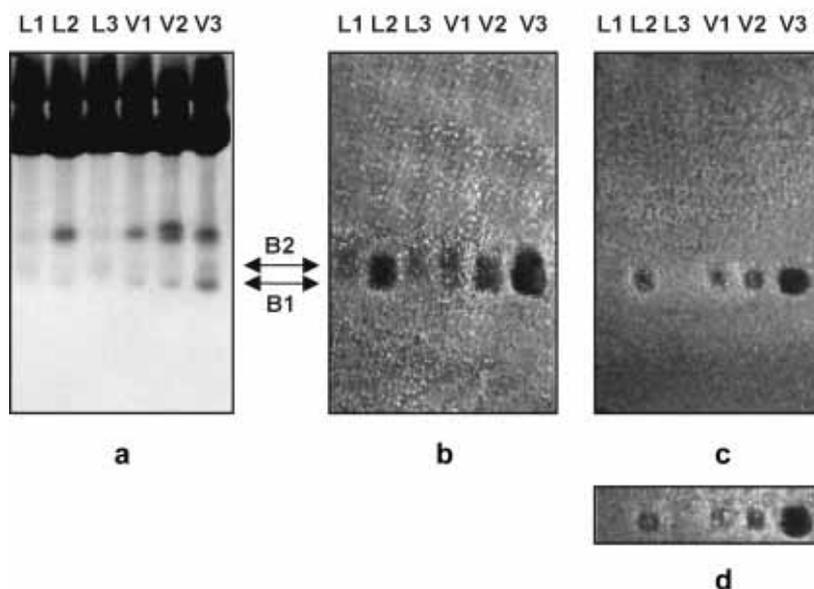
**Figure 1. Identification of antibacterial peptides in different honeybee and miscellaneous RJ samples through bacterial growth-inhibition assay on polyacrylamide gel.** (a) Separation of acidic extracts of the samples on 15% polyacrylamide gel after discontinuous acidic electrophoresis and staining of proteins with Coomassie Brilliant Blue R250. (b) The other portion of the separating polyacrylamide gel with an identical set of samples used in (a) overlaid with *B. subtilis* spores and after overnight bacterial growth showing clear spots against a black background - inhibition bands. Lanes J1, J2: acidic extracts of different miscellaneous RJs (3 mg–10 µl); lanes H and T, acidic extracts of heads (16 µl) and of thoraxes (25 µl) of 25 randomly collected honeybees from a colony without symptoms of AFB from the apiary in Slatina; lanes Hs and Ts, acidic extracts of heads (16 µl) and of thoraxes (25 µl) of 25 randomly collected honeybees from a colony with symptoms of AFB from the apiary in Slatina. The extracts loaded on gel corresponded to extraction of 0.66% of one honeybee head and 0.38% of one honeybee thorax.

of AFB (Fig. 1b, lanes H, T) were slightly larger than those from the colony with AFB symptoms (Fig. 1b, lanes Hs, Ts). The position of inhibition bands in the lower part of the polyacrylamide gel documented high mobility of the antibacterial factor. It implied that these factors represented basic antibacterial peptides, rather than proteins. The extracts contained different amounts of antibacterial factors that were low in all samples, since no peptide bands corresponding to inhibition bands were detected in the gel after protein staining (Fig. 1a). The inhibition bands were detected by other analyses of RJ and head extracts by bacterial growth-inhibition assay on the gel with Gram-positive (*B. subtilis*, *M. luteus*, *S. lutea*, *P. l. larvae*), but not with Gram-negative (*E. coli*, *S. marcescens*) bacteria. The thorax extracts yielded an inhibition band only with *B. subtilis* and *M. luteus* that

showed to be the most sensitive bacterial strains in our analyses.

### 3.2. Identification of antibacterial peptides in RJs from different honeybee colonies

The RJs collected from individual colonies in one healthy apiary, and RJs from healthy colonies in another apiary with incidence of AFB (see material and methods) were analyzed by bacterial growth-inhibition assay on polyacrylamide gel. Differences in the number, the intensity and the size of inhibition bands were found between RJ samples from different colonies employing *B. subtilis* spore culture and vegetative bacterial cells of two ATCC strains of *P. l. larvae* (Fig. 2). Extracts of almost all analyzed RJs (except for two



**Figure 2. Analysis of some RJ samples collected from individual colonies at the two apiaries employing bacterial growth-inhibition assay on polyacrylamide gel.** (a) Separation of acidic extracts of the RJ samples on 15% polyacrylamide gel after discontinuous acidic electrophoresis and staining of proteins with Coomassie Brilliant Blue R250. (b), (c), (d) The other portions of the separating polyacrylamide gels with an identical set of samples used in (a) (only with lower amounts) after growth of *B. subtilis*, *P. l. larvae* ATCC strain 5086, and *P. l. larvae* ATCC strain 5084, respectively, on the gel surface. In (d) only the portion of the gel with inhibition bands is shown. Lanes L1, L2, L3, acidic extract of RJs from colonies from the healthy apiary (Liptovský Hrádok); lanes V1, V2, V3, acidic extracts of RJs from colonies from other apiary with incidence of AFB (Valaska). Acidic extracts of 9 mg RJ – 30  $\mu$ l (a) and 4.7 mg RJ – 16  $\mu$ l (b, c, d) were analyzed. B1 and B2 mark the position of lower and upper inhibition band, respectively.

samples each taken from a different apiary) yielded one or two inhibition bands (marked B1 and B2) with *B. subtilis* (Fig. 2b). The peptide bands corresponding to inhibition bands and detected with protein staining are shown in Figure 2a. Only one of the five extracts of RJs from colonies of the healthy apiary and five out of six extracts of RJs from the colonies of an apiary with incidence of AFB, yielded one inhibition band (B1) with vegetative bacterial cells of two ATCC strains of *P. l. larvae* (Fig. 2c and 2d). Other analysis of the RJs that did not yield inhibition bands (one miscellaneous RJ sample - Fig. 1, lane J1 and the two RJs taken from individual colo-

nies), led to detection of a weak inhibition band by using a greater amount of RJ (9 mg instead of 4.7 mg) and sensitive bacterial cells of *B. subtilis* (data not shown). The performed analyses demonstrated that tested RJs from individual colonies contained different amounts of antibacterial peptides.

### 3.3. Characterization of antibacterial peptides in inhibition bands

The sample of RJ that contained the highest amount of peptides in inhibition bands (Fig. 2, sample V3) was used for

characterization of antibacterial peptides by microsequencing. N-terminal amino acid sequences of the peptides present on the polyacrylamide gel at the position of the two inhibition bands (B1 and B2 in Fig. 2) were determined after electrophoresis of the acidic extracts of RJ by acidic PAGE and transfer of the peptides on a ProBlott membrane. Two different N-terminal sequences for each antibacterial band have been determined. The sequence of the peptide of each antibacterial band, which was determined on the basis of sequencing a major component of the band, was VTCDL. It corresponded to a known N-terminal sequence of antibacterial peptide royalisin - VTCDL (Fujiwara et al., 1990). The sequence of a minor peptide in band B1 was XEDNT. Only a partial sequence, XEXNX, of a minor peptide in the weak band B2 was determined. A sequence similarity search of the XEDNT sequence in Swiss-Prot database using BLAST program for short amino acid sequences did not reveal any peptide or protein with such N-terminal sequence.

#### 4. DISCUSSION

The antibacterial assay on polyacrylamide gel for the analyses of extracts of honeybee heads, thoraxes, and RJ was developed. To design a suitable assay procedure, we tested the application of various buffers, extraction conditions and two different activity assays on polyacrylamide gel (Hultmark et al., 1980; Grenier et al., 1993). Better resolution and detection of antibacterial bands was obtained by assay procedure of Grenier et al. (1993) using discontinuous gel system of Reisfeld et al. (1962) than that of Hultmark et al. (1980) employing continuous gel system. However, optimization of the discontinuous gel system was needed to obtain good assay performance and to eliminate an artifact inhibition bands profile that could be observed on the polyacrylamide gel after

performing electrophoresis in non-optimized gel system. The artifact profile was characterized by partial or complete loss of inhibition bands (B1 and B2) in the lower part of the gel and appearance of the new larger inhibition spot at the position of dominant proteins in the upper part of the gel. The developed bacterial growth-inhibition assay on polyacrylamide gel represents a sensitive method, which enables identification of honeybee antibacterial peptides in analyzed samples. It also allows for comparison of the content of antibacterial peptide in the samples using small amounts of RJs and single honeybees.

Using the assay, the antibacterial bands, detected by analyses of RJ and honeybee head extracts, had identical positions on the acidic polyacrylamide gel. This indicated that both RJ and honeybee heads contained the same antibacterial peptides. N-terminal sequencing was used to characterize the peptides localized in two antibacterial bands in the gels for the honeybee head and RJ samples. The sequencing revealed that the major peptide in each band was royalisin, a previously characterized antibacterial peptide of RJ (Fujiwara et al., 1990). Another peptide was a minor component. In the identified N-terminal sequence of major peptide it was not possible to precisely determine the amino acid in position 3 probably because the native peptide was sequenced with undisturbed disulfidic bonds. Cystein in position 3 in royalisin participates in one of the three disulfidic bonds of this molecule (Bulet et al., 1999). The determined sequences of the minor peptide in each band, especially the one in the weak antibacterial band B2, were only partial, because the amounts of the peptides in analyzed samples were low. To obtain larger amounts of the peptides to determine their primary structure, the use of HPLC for separation and preparation of the peptides will be necessary. However, the similarity of determined sequences of the minor peptides suggests that they correspond to the

same peptide. No peptide or protein with such sequence on N-terminus was found by a search in Swiss-Prot database. Although it is not possible to completely exclude an antibacterial property of the unknown peptide, several other facts suggest that royalisin is the peptide that acts against *P. l. larvae*. Firstly, activity against *P. l. larvae* was observed only in the antibacterial band B1 containing larger amount of peptides. Secondly, the inhibition bands on gels were detected only with Gram-positive but not Gram-negative bacterial strains. It correlates with a characteristic property of insect defensins to which royalisin belongs (Hetru et al., 1998; Bulet et al., 1999). Finally, another study carried out in our laboratory showed that the peptide fraction isolated by dual dialysis of RJ which contained royalisin as a dominant component inhibited growth of *P. l. larvae* (Bíliková et al., 2001).

Royalisin could be the factor in RJ that caused significant mortality of vegetative cells of *P. l. larvae* in the study by Hornitzky (1998). The author observed complete mortality of the vegetative cells within 5 min by RJ, whereas it took 20 min to observe a similar effect when the saline, adjusted to pH 4 simulating acidity of RJ, was used. That royalisin may be the killing factor in the Hornitzky experiment is supported by the knowledge that insect defensins have almost immediate lytic effect on Gram-positive bacteria (Bulet et al., 1999). Recently, the inhibition effect on growth of *P. l. larvae* in cultivation medium was demonstrated using fractions of RJ and two worker jellies (WJs) prepared by an extraction employing water/ethanol solution (Crailsheim and Riessberger-Gallé, 2001). The authors found that preparations obtained from WJs had lower activity in comparison with that of RJ. The antibacterial substance responsible for the inhibition activity was not identified. It is reasonable to consider that it could be royalisin.

The important result of the present work is the finding that RJs of individual colonies differed in their content of the antibacterial factor active against *P. l. larvae*. Each analyzed RJ contained a certain amount of the antibacterial factor, although it was very low in some RJ samples, suggesting that royalisin might be a regular component of RJ. The differences in the content of the antibacterial peptide could be associated with genetic variability between colonies. This is supported especially by the finding of RJs with high and very low amounts of the antibacterial peptide in colonies of two different apiaries. The other interesting finding was that most colonies from the apiary with AFB produced RJs with higher amounts of antibacterial peptide than the colonies from the healthy apiary. It is possible that genetic factors were responsible for higher content of the antibacterial peptide in some of the RJs. However, it is also possible that the presence of *P. l. larvae* in some of these colonies could have affected the content of the royalisin in RJs. Identification of royalisin in both antibacterial bands detected by employing *B. subtilis* suggests that royalisin can exist in two distinct forms. The differences between the individual forms are unclear at present. They could be related to C-termini of the peptides. The presence of C-terminally truncated form of bee defensin in hemolymph was described (Casteels, 1998).

The lower antibacterial band containing royalisin had an identical position on the polyacrylamide gel as the weak antibacterial band detected at analysis of honeybee thorax extracts. We speculate that the thorax antibacterial peptide could correspond to bee defensin. Bee defensin is a peptide differing from royalisin in a single amino acid substitution. It was found in hemolymph of honeybees after its injection with *E. coli*. Its expression is the lowest and the most delayed in comparison with the other induced antibacterial peptides found in honeybee hemolymph (Casteels-Josson

et al., 1994). However, its activity in hemolymph persists for a long time, up to two weeks, after the infection (Casteels, 1998). The fact that the analyzed thoraxes were from honeybees collected at the apiary with AFB occurrence, and in one case from a colony with symptoms of AFB, suggests that either presence of *P. l. larvae* or possibly other honeybee pathogens in a colony could be the factor causing induction of expression of the detected antibacterial peptide.

Future research should focus on confirmation and further investigation of the influence of genetic factors and the infection of colonies with *P. l. larvae* on the secretion of royalisin by honeybees into RJs and WJs. Finally, the role of royalisin in protecting honeybee larvae against *P. l. larvae* infection should be confirmed to determine if it is one factor that contributes to the resistance of honeybee colonies against AFB.

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**Résumé – Identification d'un peptide de l'abeille domestique actif contre *Paenibacillus larvae larvae* à l'aide d'un test d'inhibition de croissance bactérienne sur gel de polyacrylamide.** L'un des mécanismes proposés pour la résistance des colonies d'abeilles domestiques (*Apis mellifera* L.) à la loque américaine (AFB) est associé aux composés antibactériens sécrétés par l'abeille dans la nourriture larvaire, qui peut être active contre l'agent pathogène bactérien de la loque améri-

caine, *Paenibacillus larvae larvae*. On estime que les acides aminés, principalement l'acide hydroxy-10-trans-décénoïque, font partie de ces substances antibactériennes. On a montré que la gelée royale (GR) renferme un peptide antibactérien, la royalisine. Dans nos recherches nous avons cherché à savoir si la royalisine est active contre *P. l. larvae* et si la GR renferme d'autres peptides ou protéines à activité antibactérienne. Nous avons récemment montré qu'une fraction isolée de la royalisine est active contre *P. l. larvae* (REF).

Le travail présent porte sur l'identification de peptides/protéines à activité antibactérienne sécrétés par l'abeille dans la GR en utilisant un test d'inhibition de croissance bactérienne sur gel de polyacrylamide. Après extraction des protéines et des peptides et séparation par électrophorèse sur gel de polyacrylamide acide, ce test permet d'identifier dans les extraits acides des échantillons les peptides ou protéines à activité antibactérienne grâce aux bandes d'inhibition qui apparaissent après que l'on a fait croître des bactéries à la surface du gel.

Les bandes d'inhibition et les bandes correspondantes des peptides (marquées B1 et B2) ont été détectées par analyse des extraits suivants sur gel de polyacrylamide : (A) extraits de tête et de thorax préparés à partir de 25 abeilles prélevées au hasard dans deux colonies d'un même rucher, l'une présentant des symptômes de loque américaine (marquée « s »), l'autre n'en présentant pas (Fig. 1, bandes H, Hs et T, Ts respectivement) ; (B) extraits d'échantillons mélangés de GR (Fig. 1, bandes J1 et J2) ; (C) extraits de GR provenant de colonies d'un rucher sain (Fig. 2, bandes L1-L3) ; (D) extraits de GR provenant de diverses colonies d'un rucher atteint de loque américaine (Fig. 2, bandes V1-V3). Différentes souches bactériennes ont été utilisées pour le test : *Bacillus subtilis* (Fig. 1b, Fig. 2b) et *P. l. larvae* (Fig. 2b et 2c).

Le séquençage N-terminal des peptides présents sur le gel sur les positions des deux

bandes d'inhibition détectées (Fig. 2, échantillon V3) a montré que chaque bande contenait de la royalisine comme peptide majeur ainsi qu'un autre peptide inconnu comme composé mineur.

Les données indiquent que la royalisine est le peptide des bandes d'inhibition qui est actif contre les bactéries testées. L'analyse des échantillons de GR prélevés dans les diverses colonies des deux ruchers, dont l'un était atteint de loque américaine, a montré des différences dans la teneur en peptide antibactérien. Les résultats suggèrent que ces différences pourraient être associées à la variabilité génétique entre colonies d'abeilles.

***Apis mellifera* / *Paenibacillus larvae larvae* / loque américaine / gelée royale / peptide antibactérien**

**Zusammenfassung – Identifizierung eines gegen *Paenibacillus larvae larvae* aktiven Honigbienen-Peptids mittels eines Bakterienwachstum-Inhibitions-Tests auf Polyacrylamid-Gel.** Bei der Resistenz von Honigbienenvölkern gegen Amerikanische Faulbrut (AFB) werden als ein Mechanismus antibakterielle Komponenten diskutiert, die von den Honigbienen in das Larvenfutter abgegeben werden, und die gegen den bakteriellen Erreger der AFB, *Paenibacillus larvae larvae* wirken könnten. Es wird vermutet, dass Fettsäuren, insbesondere die 10-hydroxy-trans-2-Decensäure solche Substanzen darstellen. Es konnte gezeigt werden, dass Gelee Royal (RJ) auch ein antibakterielles Peptid, Royalisin, enthält. Wir wollten herausfinden, ob Royalisin gegen *P. l. larvae* aktiv ist und ob RJ weitere antibakterielle Peptide oder Proteine aufweist. Kürzlich konnten wir zeigen, dass eine isolierte Royalisin-Fraktion gegen *P. l. larvae* wirkt.

Die vorliegende Arbeit behandelt die Identifizierung antibakterieller Peptide/Proteine, die von Honigbienen in RJ sezerniert werden, wobei wir als Verfahren einen Bakterienwachstum-Inhibitions-Test auf einem

Polyacrylamid-Gel anwandten. Der Test ermöglicht die Identifizierung von antibakteriellen Peptiden/Proteinen in sauren Extrakten der Proben, nachdem die extrahierten Proteine und Peptide über eine saure Polyacrylamid-Gel-Elektrophorese aufgetrennt wurden. Sie werden auf dem Polyacrylamid-Gel durch Inhibitions-Banden identifiziert, die dann auftreten, wenn man Bakterien auf der Geloberfläche wachsen lässt. Die Peptide/Proteine, die mit den Inhibitionsbanden gekoppelt sind, werden über eine Protein-Färbung auf dem anderen Teil des Gels, auf den ein identischer Satz der Proben aufgebracht wurde, sichtbar gemacht.

Die Inhibitionsbanden und die dazugehörigen Peptid-Banden (B1 und B2) wurden mit Hilfe des Tests auf dem Polyacrylamid-Gel bei folgenden Fraktionen untersucht: (A) Kopf- und Thorax-Extrakte (Abb. 1, Spur H, Hs bzw. T, Ts) von 25 zufällig ausgewählten Honigbienen aus 2 Völkern eines Bienenstandes, eines ohne, das andere mit (s) AFB Symptomen; (B) Extrakte verschiedener RJ Proben (Abb. 1, Spur J1, J2); (C) RJ-Extrakte von einzelnen Völkern eines gesunden Bienenstandes (Abb. 2, Spuren L1-L3); (D) RJ-Extrakte von einzelnen Völkern eines Bienenstandes mit AFB (Abb. 2, Spur V1-V3). Verschiedene Bakterienstämme wurden bei dem Test eingesetzt: *Bacillus subtilis* (Abb. 1b, Abb. 2b) und *P. l. larvae* (Abb. 2, c und d).

Die N-ständige Sequenzierung der Peptide, die auf dem Gel an der Position zweier Inhibitionsbanden (Abb. 2, Probe V3) auftraten, ergaben, dass jede Bande Royalisin als Hauptpeptid und ein anderes, unbekanntes Peptid als Nebenkomponekte enthielt.

Die Daten deuten darauf hin, dass Royalisin das Peptid war, das in den Inhibitionsbanden gegen die getesteten Bakterien wirksam war. Die Analyse der RJ-Proben von einzelnen Völkern zweier Bienenstände, von denen der eine mit AFB infiziert war, zeigte Unterschiede im Gehalt an antibakteriellen Peptiden. Die Ergebnisse lassen vermuten, dass die Unterschiede auf die

genetische Variabilität zwischen den Honigbienen-Völkern zurückzuführen sein könnten.

***Apis mellifera* / Weiselfuttersaft / *Paenibacillus larvae larvae* / Amerikanische Faulbrut / antibakterielle Peptide**

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