Apidologie 32 (2001) 275–283

Original article

Isolation of a peptide fraction from honeybee royal jelly as a potential antifoulbrood factor

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(Received 18 December 2000; revised 12 February 2001; accepted 9 March 2001)

Abstract – A peptide fraction was isolated from honeybee royal jelly (RJ) using dual dialysis under acidic conditions. The N-terminal amino acid sequence of the major peptide within the fraction was V-T-C-D-L-L-S-F-K-G. This sequence corresponds to the honeybee defensin royalisin of MW 5523 Da which has been shown to exert antibacterial activity against some Gram-positive bacteria. Diffusion tests on agar plates showed that the peptide fraction had an inhibitory effect against the honeybee pathogen *Paenibacillus larvae larvae*, the primary pathogen of American foulbrood disease, as well as against other Gram-positive bacteria such as *Bacillus subtilis* and *Sarcina lutea.* Moreover, the peptide fraction was shown also to have antifungal effect against the model fungus *Botrytis cinerea.* It is the first evidence of an antibiotic effect of royalisin against a honeybee pathogen. The procedure described is very simple and does not require application of complicated separation techniques. It is based on dialysis of RJ using membranes with different pore sizes, which enable to separate the compounds having molecular weight below 2 kDa, between 2 kDa and 10 kDa, and over 10 kDa.

Apis mellifera* L. / royal jelly / isolation of antibacterial and antifungal peptides / American foulbrood disease / *Paenibacillus larvae larvae

1. INTRODUCTION

Honeybee larvae can be afflicted with several diseases which are caused by pathogenic bacteria, fungi or protozoa. The most serious and widespread bacterial disease of honeybee larvae is American foulbrood disease (AFB) caused by spores of *Paenibacillus larvae larvae* (*P. l. larvae*) (Hansen and Brødsgaard, 1999). As in all insects (Bulet et al., 1999), the honeybee defends itself against bacteria and parasites

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by production of antimicrobial peptides as components of their highly effective cellular and humoral defense systems (Casteels et al., 1989; Casteels et al., 1990; Casteels et al., 1993; Gliński and Jarosz, 1995). A special group of protective substances in the developing organism of honeybee is represented by proteins and peptides and by low-molecular-weight compounds present in royal jelly (RJ) – the larval diet of queen larva secreted by nurse honeybees. The antibacterial activity of 10-hydroxy-*trans*-2-decenoic acid, the natural component of royal jelly and worker jelly, was described (Bonvehí and Jordá, 1991). The peptide royalisin isolated from RJ was found to have antibacterial activity against some Gram-positive bacteria (Fujiwara et al., 1990). For preparation of royalisin, the authors used a complicated procedure that included combined alkaline and acidic precipitation of the water-soluble fraction of RJ, subsequent fractionation by column chromatography on a Sephadex gel, and purification by HPLC.

The proteins secreted by honeybees into RJ and other hive products have different roles in the functioning of a honeybee colony as a superorganism. A substantial part of the RJ proteins (about 90%) a protein family which plays a role in nutrition (Schmitzová et al., 1998). The low-molecular-weight proteins and peptides of RJ might play a host-defense role against honeybee pathogens.

The aim of the present work was to develop a simple method for isolation and characterization of antibacterial and antifungal peptides from RJ in natural form. In some cases, it is not possible to prepare a biologically active peptide by recombinant DNA technology because of its specific post-translation modifications; e.g., amidation. Antimicrobial activity of honeybee peptides has been previously tested predominantly against bacterial pathogens of general significance. This work studies the specific efficiency of the peptides against the honeybee pathogenic bacterium, *P. l. larvae*.

2. MATERIALS AND METHODS

2.1. Isolation of the peptide fraction from RJ

One-day old larvae were grafted into queen cells in the apiary in the Slovak Republic during June 1997. Honeybee RJ was collected 48 hours after acceptance of the larvae. The RJ was stored at –20 °C for 3 months and thawed before use at room temperature. 30 g of RJ suspension was homogenized in 90 ml phosphate buffer (50 mM NaH_2PO_4, 50 mM Na_2HPO_4, 100 mM NaCl, 20 mM EDTA, pH 7.0). The suspension was dialyzed first for three days in tubing with a MW cut-off of 2 kDa and subsequently three days in the tubing with a MW cut-off of 10 kDa (Serva Electrophoresis, Heidelberg, Germany). Dialysis was performed at 15 °C against 1 liter deionized water adjusted with acetic acid to pH 2.0. The diffusates were changed every 24 hours and concentrated by lyophilization (Lyovac GT-2, Leybold-Heraeus GmbH, Germany). The dialysis was monitored by measuring absorbancy of diffusates in the range from 200 nm to 300 nm using Beckman DU-20 Spectrophotometer (USA).

2.2. Protein determination

The protein content of samples was determined using the bicinchoninic acid protein assay (Smith, 1985). Absorbancy at 562 nm of a mixture of 20 µl test solution and 1 ml working reagent (50 parts of the bicinchoninic acid solution plus 1 part of a 4% $CuSO_4$ solution) was measured after incubation for 30 min at 60 °C. In the case of diffusates obtained after dialysis of RJ in the tubing with a MW cut-off of 2 kDa, where the high content of sugars was expected (Šimúth, 2001), the Bradford method of protein determination was used (Bradford, 1976). Bovine serum albumin (Sigma, USA) was used as a reference protein.

2.3. Electrophoresis and N-terminal amino acid sequencing

The lyophilized samples obtained from dual dialysis were solubilized in 0.2 M Tris-HCl, pH 9.0. Tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) was carried out according to Schägger and Jagow (1987). Acrylamide and bisacrylamide concentrations for separating and stacking gels were 16.5% T, 3% C and 4% T , 3% C, respectively. Samples were incubated for 40 min at 40 °C in the buffer containing 4% SDS, 12% glycerol (w/v), 50 mM Tris, 1.5% dithiothreitol 0.01% Serva Blue G adjusted with HCl to pH 6.8. After electrophoresis the gels were stained with Serva Blue G (Serva, Electrophoresis GmbH, Heidelberg, Germany).

N-Terminal amino acid sequence was determined directly from the lyophilized sample as well as from the individual peptide bands after SDS-PAGE. Proteins were separated by Tricine SDS-PAGE and electroblotted onto PVDF membranes ProBlott (Applied Biosystems, USA) using the tank method of protein transfer (Mini Trans-Blot Electrophoretic Transfer Cell, Bio-Rad Laboratories, USA). 10 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) in 10% methanol was used as transfer buffer according to the procedure recommended by membrane manufacturer. Peptide bands were visualized using Coomassie Blue R 250 (Serva, Feinbiochemica, Heidelberg, Germany), excised, and sequenced by automated Edman degradation in a LF3600D Protein Sequenator (Beckman, USA).

2.4. Bacteria, media, and antibacterial diffusion assay

Spore culture of *Bacillus subtilis* and bacterial strains of *Sarcina lutea*, *Escherichia coli*, and *Seratia marcescens* were obtained from the collection of microorganisms from Biotika, Inc., Slovenská L'upča (Slovak Republic). Bacterial strains of *Paenibacillus larvae larvae* 5084, 5085, and 5086 were from ATCC (USA).

All cultures (except for spore suspension of *Bacillus subtilis*) were maintained as frozen stocks at –80 °C. Before experimental use, cultures were propagated at 35 °C in PCBY medium (0.6% bactopeptone, 0.4% casein hydrolysate, 0.15% beef extract, 0.3% yeast extract, 0.1% glucose, pH 7.9). Microbial concentrations were determined by measuring absorbancy at 595 nm.

For preparation of *P. l. larvae* spore suspension, 2% agar in the PCBY medium was used. 20 µl of *P. l. larvae* bacterial overnight culture (1×10^6 bacteria per ml) in the liquid PCBY medium was spread over the surface of 2% agar PCBY plates. The plates were incubated at 30 °C for 20 hours and the colonies of *P. l. larvae* were subsequently replaced into the sterile glass tube and suspended in physiological saline (sterile 0.9% NaCl). The bacterial suspension (vegetative form of *P. l. larvae*) was stored at 15 °C. After one month, the bacterial suspension was incubated at 80 °C for 20 min in order to kill non-spore-forming bacteria.

Antibacterial diffusion test on agar plates (inhibition zone assay) was performed by spreading 8 ml of 0.5% agar PCBY medium mixed with bacterial culture of *Sarcina lutea*, *Escherichia coli*, or *Seratia marcescens* (1×10^5 cells per ml) or spore suspension of *Bacillus subtilis* or *P. l. larvae* (1×10^5 spores per ml) in plastic Petri dishes. 5 µl of the samples at protein concentration from 5.4 $\mu g \cdot ml^{-1}$ to 270 $\mu g \cdot ml^{-1}$ was applied into 2 mm diameter holes in the agar plates and incubated at 30 °C for 16–20 hours. Tetracycline (Serva, Heidelberg, Germany) at concentration 50 $\mu g \cdot ml^{-1}$ was used as a control of inhibition effect. Petri dishes with inhibition zone assays were photographed (DC120 Zoom Digital Camera, Kodak, USA) against a black background to help to visualize the inhibition zones.

2.5. Antifungal assay

The antifungal activity of the royalisin fraction was determined as described by Terras et al. (1992). A conidia suspension of the fungus *Botrytis cinerea* (Calgene, Inc., Davis, USA) was prepared from 10-day-old plates of potato dextrose agar (Difco, Detroit, USA) and diluted to a density of 10^6 spores per ml. The growth medium used for fungi was a synthetic low-ionic-strength buffer containing 2.5 mM K_2HPO_4, 50 µM $MgSO_4$, 50 µM $CaCl_2$, 5 µM $FeSO_4$, 0.1 µM $CoCl_2$, 0.1 µM $CuSO_4$, 2 µM Na_2MoO_4, 0.5 µM H_3BO_3, 0.1 µM KI, 0.5 µM $ZnSO_4$, 0.1 µM $MnSO_4$, 20 g·l^{-1} glucose, 2 g·l^{-1} asparagine, 40 g·l^{-1} methionine, 2 mg·l^{-1} *myo*-inositol, 0.2 mg·l^{-1} biotin, 1 mg·l^{-1} thiamine-HCl, 0.2 mg·l^{-1} pyridoxin-HCl. In a 96 well microtiter plate, 50 µl of protein sample at concentration 27 µg·ml^{-1} and 135 µg·ml^{-1} was mixed with 50 µl of spore suspension. For comparison of the growth inhibition effect, hydrogen peroxide (17 µg·ml^{-1}) was used. After incubation at 22 °C for 24 hours, the growth inhibition was monitored by optical microscope (Axiophot, Carl Zeiss, Jena, Germany).

3. RESULTS

3.1. Biochemical characterization of the royalisin fraction

The procedure described here is based on separation of proteins and peptides by dual dialysis of RJ at pH 2.0, which is close to the natural pH of RJ (pH 3–4). The first dialysis in tubing with a MW cut-off of 2 kDa removed non-protein, low-molecular-weight compounds (sugars, salts, metals, free amino acids, nucleotides, fatty acids, etc.) from RJ suspension. Diffusates obtained after three-day dialysis in tubing with MW cut-off of 2 kDa were lyophilized and dissolved in water to saturation. Determination of protein concentration in these solutions by Bradford method showed that there were no proteins present in the diffusates.

The second dialysis was performed after transferring the RJ suspension from the first dialysis to a new tubing with MW cut-off of 10 kDa. The obtained diffusates were collected, lyophilized, dissolved in 0.2 M Tris-HCl buffer, pH 9.0 (royalisin fraction) and used for gel electrophoresis and bioassays. Tricine-SDS-PAGE of this royalisin fraction (Fig. 1, lane b) showed the presence of

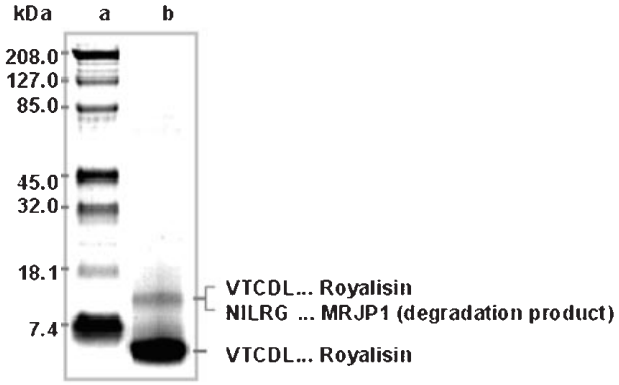

Figure 1. Tricine-SDS-PAGE of the royalisin fraction isolated by dual dialysis of RJ suspension; *lane a* – protein molecular weight standards (Bio-Rad Laboratories, USA); *lane b* – royalisin fraction (20 µg).

two peptide bands: a main peptide band with MW ca. 5 kDa, and a minor band with MW around 10 kDa.

N-Terminal amino acid sequence analysis revealed that both main and minor bands contained sequence V-T-C-D-L-L-S-K-G identical with previously characterized honeybee defensin royalisin – the peptide with MW 5523 Da. The second, minor band also contained a degradation product of the most abundant RJ protein named as MRJP1 with N-terminal amino acid sequence N-I-L-R-G. Identical N-terminal amino acid sequences were detected also in a lyophilized royalisin fraction.

From 30 g RJ we obtained 180 mg of the lyophilized royalisin fraction. The dry matter content of the fraction estimated by drying at 105 °C until the constant weight was 38.71%. Thus, the lyophilized royalisin fraction contained 61.29% humidity. In addition, content of dry matter in royalisin fraction exposed to the air at 22 °C and relative humidity 45–50% for 24 hours reached approximately the same value as the humidity of the royalisin fraction before drying at 105 °C.

3.2. Antibacterial and antifungal properties of the royalisin fraction

The lyophilized royalisin fraction solubilized in 0.2 M Tris-HCl, pH 9.0 was used to assay its antimicrobial properties. Antibacterial effect of the royalisin fraction was tested by inhibition zone assay. It was found that this fraction inhibited growth of some Gram-positive microorganisms such as *Bacillus subtilis* and *Sarcina lutea*, as well as of the bacteria *P. l. larvae* ATCC 5084, ATCC 5085, and ATCC 5086. Figure 2 presents the diffusion tests for the antibacterial effect of the royalisin fraction at protein concentrations ranging from 5.4 $\mu g \cdot ml^{-1}$ to 180 $\mu g \cdot ml^{-1}$ against *Bacillus subtilis* (plate A) and *P. l. larvae* ATCC 5084, ATCC 5085, and ATCC 5086 (plates B, C, D). Tetracycline, a common antibiotic used in treatment of honeybee colonies, was used as a control of growth inhibition effect at concentration 50 $\mu g \cdot ml^{-1}$ (Fig. 2, position 5 for plates A, B, C and position 6 for plate D). No inhibition effect was observed for the royalisin fraction against the tested Gram-negative bacteria *Escherichia coli* and *Seratia marcescens* (data not shown).

Antifungal activity of the royalisin fraction was tested using the model fungus *Botrytis cinerea.* Figure 3 shows the antifungal activity of the fraction (Fig. 3A, 3B) in comparison with a standard solution of hydrogen peroxide (Fig. 3D) and control, i.e. growth of *Botrytis cinerea* without antifungal agent addition (Fig. 3C). These results demonstrate that the royalisin fraction is fungicidal at a protein concentration higher than 27 $\mu g \cdot ml^{-1}$.

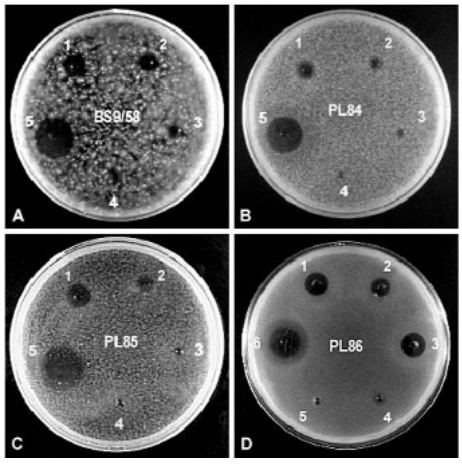

Figure 2. Antibacterial diffusion test on the agar plates of the royalisin fraction isolated from RJ. Test microorganisms: (**A**) *Bacillus subtilis*; (**B**) *P. l. larvae* ATCC 5084; (**C**) *P. l. larvae* ATCC 5085; (**D**) *P. l. larvae* ATCC 5086. Protein concentration of the royalisin fraction for the plates A, B, C: position (1) 108 $\mu g \cdot ml^{-1}$; position (2) 54 $\mu g \cdot ml^{-1}$; position (3) 27 $\mu g \cdot ml^{-1}$; position (4) 5.4 $\mu g \cdot ml^{-1}$; position(5) tetracycline 50 $\mu g \cdot ml^{-1}$; for the plate D: position (1) 108 $\mu g \cdot ml^{-1}$; position (2) 54 $\mu g \cdot ml^{-1}$; position (3) 270 $\mu g \cdot ml^{-1}$; position (4) 5.4 $\mu g \cdot ml^{-1}$; position (5) 0.2 M Tris-HCl (pH 9.0); position (6) tetracycline 50 $\mu g \cdot ml^{-1}$.

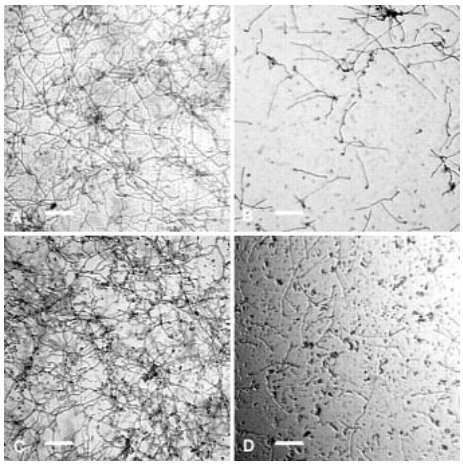

Figure 3. Inhibition effect of the royalisin fraction on the growth of fungus *Botrytis cinerea*. (**A**) royalisin fraction (27 μg·ml^{-1}); (**B**) royalisin fraction (135 μg·ml^{-1}); (**C**) control (growth of *Botrytis cinerea* without addition of antifungal agent); (**D**) hydrogen peroxide (17 μg·ml^{-1}). The bar corresponds to approximately 25 μm.

4. DISCUSSION

The presented procedure is suitable for preparation of royalisin in its natural form and in reasonable quantities to study its antibiotic activity. Until now, complicated procedures have been used to isolate honeybee proteins and peptides from RJ (Fujiwara et al., 1990; Hanes and Šimúth, 1992; Schmitzová et al., 1998). In many cases, degradation of proteins and/or loss of their biological activity could be expected. Due to protein-protein interactions and/or protein-fatty acid interactions, insoluble aggregates were formed (Šimúth, 2001) and thus, the yield of isolated proteins and peptides was low. To overcome these problems we have developed a simple method for isolation of antimicrobial peptides from RJ. The method is based on dialysis at pH close to the natural pH of RJ in the absence of any denaturing agents. For elimination of proteolytic activity present in RJ (Chen and Chen, 1995) and for release of cationic peptides from the bulk proteins of RJ, dialysis in acidic conditions at pH 2.0 was used.

The Tricine-SDS-PAGE patterns and subsequent N-terminal amino acid sequencing of the peptide fraction of RJ showed that the main band (Fig. 1, lane b) had identical N-terminal amino acid sequence as royalisin (Fujiwara et al., 1990). The second, minor band with MW around 10 kDa in addition to royalsin, also contained a degradation fragment with N-terminal amino acid sequence corresponding to MRJP1, the major protein of RJ (Hanes and Šimúth, 1992; Ohashi et al., 1997; Schmitzová et al., 1998). The low-molecular-weight degradation products of MRJP1 were detected also during non-invasive fractionation of RJ by ultracentrifugation at 6 °C (Šimúth, 2001). We hypothesized that royalisin in the minor band was present in a dimer form.

Royalisin isolated from RJ by Fujiwara et al. (1990) was found to have antibacterial effect against some Gram-positive bacteria, however, the authors did not describe its inhibition effect against honeybee pathogens of bacterial and fungal origin. The royalisin obtained herein by dual dialysis was found to inhibit the growth of some Gram-positive microorganisms, e.g. *Bacillus subtilis*, as well as *P. l. larvae* ATCC 5084, ATCC 5085, and ATCC 5086 (Fig. 2). These results are the first evidence that royalisin may have antibacterial activity against honeybee larval pathogen *P. l. larvae* – the ethiologic agent of AFB. Neither MRJP1 alone nor its degradation products obtained after 24 hours incubation at 37 °C in 50 mM Tris-HCl, pH 7.0 and 50 mM NaCl did not reveal antimicrobial effect (Šimúth, unpublished results).

The spectrum of biological activity of royalisin was broadened by discovering its antifungal activity against *Botrytis cinerea* (Fig. 3A, 3B). It is possible to suggest that royalisin exhibits both antibacterial and antifungal properties. This finding corresponds with the data on defense of insects against

pathogens that were essentially based on synthesis of cationic peptides/polypeptides exhibiting a broad spectrum of antimicrobial and antifungal activity (Bulet et al., 1999; Otvos, 2000).

The antimicrobial properties of royalisin support the indirect experimental evidence on antifoulbrood factor(s) presented in larval diet. It was found that water suspension of RJ killed vegetative cells of *P. l. larvae* within 5 min. This suspension had no effect on survival of the *P. l. larvae* spores (Hornitzky et al., 1996). Brødsgaard et al. (1998) found that 24–28 hour old larvae were susceptible to infection with *P. l. larvae.* Older larvae became more resistant to the infection and no significant dose-mortality relationship existed when the larvae were older than 48 hours.

Hygienic colonies resistant to *P. l. larvae* were resistant also to chalkbrood, caused by fungus *Ascoshaera apis* (Taber, 1988). For resistance to chalkbrood, physiological resistance is required in addition to hygienic behavior (Spivak and Gilliam, 1993), which implies the multifactorial rather than simple, single-factor resistance mechanism.

Most honeybee diseases are treated with antibiotics and fungicides. However, application of these compounds can bring about contamination of honeybee products and could lead to increased resistance of pathogens. Miyagi et al. (2000) confirmed the presence of oxytetracycline-resistant AFB pathogens in the bee hives. It is only question of time that antibiotics having molecular-biological mechanism of action different from that of tetracyclines will induce resistant strains of *P. l. larvae*. One approach to the solution of these serious problems of beekeeping is to determine the properties of antimicrobial proteins and peptides of honeybee and then to start a coordinated applied genome research of honeybee defensive mechanisms for use in breeding resistant colonies against pathogens.

ACKNOWLEDGMENTS

This work was supported by the European Commission programme: INCO-COPERNICUS (Project number IC15-CT96-0905) and by the American-Slovak Sciences and Technology Program (Project number 93012).

Résumé – Isolement d'une fraction peptidique de la gelée royale comme facteur potentiel anti-loque. Les protéines sécrétées par l'abeille domestique (*Apis mellifera* L.) dans la gelée royale (GR) et d'autres produits de la ruche ont différents rôles dans le fonctionnement d'une colonie d'abeilles comme super-organisme. Pour étudier les mécanismes de résistance physiologique de l'abeille aux maladies bactériennes et fongiques, nous avons entamé une recherche systématique des protéines et des peptides présents dans la GR. Une partie importante des protéines de la GR (environ 90 %) est représentée par un groupe de cinq protéines dénommées protéines majeures de la GR qui, d'après la forte homologie séquentielle (identique à 72 %), peuvent être classées dans une même famille de protéines and jouer un rôle dans la nutrition des larves. D'autre part, les protéines de la GR de faible poids moléculaire et ses peptides pourraient jouer un rôle dans la défense de l'abeille contre les agents pathogènes.
L'activité antimicrobienne des peptides de l'abeille a été testée principalement contre des bactéries pathogènes d'importance générale et très peu d'efforts ont été dirigés vers la recherche de leur efficacité contre des bactéries ou des champignons pathogènes pour l'abeille. Le but de notre travail a été de mettre au point une méthode simple pour isoler et caractériser les peptides de la GR à action antimicrobienne et antifongique et d'étudier leur action contre les agents pathogènes responsables des maladies infectieuses de la colonie d'abeilles.
Une fraction peptidique de la GR a été isolée à l'aide d'une double dialyse en conditions acides. La séquence N-terminale d'acides aminés du principal peptide de la

fraction obtenue est V-T-C-D-L-L-S-F-K-G. Ceci correspond à la royalisine défensine d'un poids moléculaire de 5523 Da, substance qui a montré une activité antibactérienne contre les bactéries Gram+. La procédure décrite ici est très simple et ne nécessite pas l'utilisation de techniques de séparation sophistiquées. Elle est basée sur la dialyse de la GR à l'aide de membranes de différentes porosités, ce qui permet de séparer les composés qui ont un poids moléculaire < 2 kDA, ceux qui sont entre 2 kDA et 10 kDa et ceux qui sont au-dessus de 10 kDa. Les tests de diffusion sur plaques de gélose (Fig. 2) montrent que la fraction peptidique préparée a une action inhibitrice vis-à-vis de *Paenibacillus larvae larvae*, principal agent de la loque américaine et aussi vis-à-vis des bactéries Gram+ telles que *Bacillus subtilis* et *Sarcina lutea*. On a montré en outre que la fraction peptidique avait une action antifongique (Fig. 3) contre le champignon modèle *Botrytis cinerea*.

gelée royale / isolement / peptide / antibactérien / antifongique / loque américaine / *Paenibacillus larvae larvae*

Zusammenfassung – Isolierung einer Peptidfraktion aus Königinnenfuttersaft als möglicher Faktor gegen Faulbrut (*Apis mellifera*). Proteine, die von den Honigbienen dem Königinnenfuttersaft (RJ) und anderen Bienenprodukte beigefügt werden, dienen auf verschiedenene Weise der optimalen Entwicklung des Bienenvolkes als Superorganismus. Um den physiologischen Abwehrmechanismus gegen bakterielle und Pilzkrankheiten zu untersuchen, begannen wir mit einer systematischen Untersuchung der Proteine und Peptide im Königinnenfuttersaft. Eine Hauptgruppe (etwa 90 %) der RJ Proteine bildet die 5 sogenannten Hauptproteine des Königinnenfuttersafts. Auf der Basis von hoher Homologie in den Sequenzen (72 % identisch) können sie in häufig vorkommende Proteinfamilien eingeordnet werden. Sie spielen bei der Ernährung der Larven eine Rolle. Andererseits könnten die Proteine mit kleinem Molekulargewicht und die Peptide eine Rolle bei der Abwehr von Pathogenen der Honigbienen spielen.

Antimikrobielle Wirkung von Peptiden der Honigbienen wurde bisher vor allem bei allgemeinen bakteriellen Pathogenen getestet und es gab wenig Arbeiten über ihre Wirkung bei für Honigbienen spezielle Bakterien- oder Pilzinfektionen. Mit dieser Arbeit wollten wir eine einfache Methode entwickeln, um die bakteriziden und fungiziden Peptide des RJ zu isolieren, zu charakterisieren und ihre Wirkung auf Pathogene, die Krankheiten im Bienenvolk verursachen, zu testen.

Eine Peptidfraktion wurde vom RJ über eine zweifache Dialyse unter sauren Bedingungen isoliert. Die N-terminalen Aminosäure Sequenz der meisten so erhaltenen Peptide bestand aus V-T-C-D-L-L-S-F-K-G , das dem Defensin Royalisin mit dem Molekulargewicht 5523 DA entspricht, das eine bakterizide Wirkung gegen gram-positive Bakterien hat. Die Isolierung, die hier beschrieben wird, ist sehr einfach und bedarf keiner komplizierten Trennungstechnik. Sie beruht auf der Dialyse von RJ über Membranen mit unterschiedlichen Porengrößen. Dadurch lassen sich die Verbindungen mit einem Molekulargewicht von weniger als 2 kDA, die mit 2 kDa bis 10 kDa und die mit über 10 kDa trennen. Diffusionstests auf Agarplatten (Abb. 2) zeigten, dass die aufbereitete Peptidfraktion eine Inhibitionswirkung auf das Bienenpathogen *Paenibacillus larvae larvae*, dem Hauptpathogen der Amerikanischen Faulbrut, aufwies. Außerdem wirkte es gegen andere grampositive Bakterien wie *Bacillus subtilis* und *Sarcina lutea*. Des weiteren konnte nachgewiesen werden, dass die Peptidfraktion auch eine fungizide Wirkung gegen den Testpilz *Botrytis cinerea* besitzt (Abb. 3).

Honigbiene (*Apis mellifera* L.) / Königinnenfuttersaft / Isolation von bakteriziden und fungiziden Peptiden / Amerikanische Faulbrut / *Paenibacillus larvae larvae*

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