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Apidaecins and Lysozyme in the Honeybee (*Apis mellifera* L.) from Environment Nonheavily Contaminated with Heavy Metals

Aktywność apidycyn i lizozymu w hemolimfie pszczoły miodnej (*Apis mellifera* L.) ze środowiska nie skażonego silnie metalami ciężkimi

The insect organism, from the time of conception to natural death, must maintain its integrity in the face of a changing and threatening environment, attacks of pathogens and parasites. From the three main integrating systems of insect body: central nervous system, endocrine system and immune system, mechanisms of immune responses have a key role in control of homeostatis in the insect hemocoel infected by microorganisms and parasites. The structure of the defense system and mechanisms of the immune responses in insects and vertebrates differ profoundly, but the final effects of the protective barriers and internal defense mechanims in these two groups of animals lead to death or elimination of pathogens from an invaded organism by the generated active effector cells and effector substances, haemolymph immune proteins (15).

The protective immunity in insects is attributable to the presence of anatomical and physiological barriers of body coverings, intestines, tracheal system, and mechanisms of internal defense both innate and inducible (13, 14). In social insects defensive behavior such as grooming, cleaning can lower the number of pathogens in the nest (16).

Investigators are generally agreed that any disturbance of an insect body integrity, stress, bacterial invasions, attacks of parasites and predators affect immune status of an insect. In response to invaded bacteria, insects synthesize antibacterial peptides and proteins, which are secreted into hemolymph. The lysozyme (11, 15, 19, 23), cecropins (2, 17) and attacins (18) are the main well known classes of inducible immune proteins of the circulating insect blood. Apidaecins (3, 4), and abaecin (5) and probably also royalisin (8) seem to be the most prominent compounds of the honeybee humoral defense against microbial invasion.

Some insect parasites (6, 7, 20) are known to depress antibacterial immunity producing extra cellular protease of the type A that interfere with the inducible cell-free antibacterial immunity of cecropin type. Also stenophagous mite, *Varroa jacobsoni* during prasitism lowers the level of lysozyme in invaded bees (11).

Information is presented on deleterious effects on bees of various agents polluting envronment from which bees collect nectar, pollen and water. The main role play insecticides, fungicides, and, to some extent, fertilizers. The role of environment polluted by heavy metals on internal defense reactions of bee organism is scant. It was found that a high level of some elements lowers significantly the content of low molecular fractions of bee brood hemolymph (10). The phagocytic activity of

hemocytes is not disturbed in bees fed honey of a high, but without acceptable limits, content of Cu, Ni, Cr, Zn, Pb and Mn (9).

In this paper we report observations on activity of internal immune defenses represented by antibacterial action of lysozyme and activity of inducible immune peptides-apidaecins in worker bees from an environment not heavily polluted by heavy metals.

MATERIALS AND METHODS

Samples of bees. The worker bees (*Apis mellifera* L.) at the age of about 30-33 days collected from one colony from an apiary in Lublin were kept in wire cages at 32°C, RH 50%, in darkness. The insects were fed candy for the experimentation time. The amount of Pb, Cd, Ni, Cu, Zn, Mn and Fe in honey fed by colony and in honey used to prepare candy was determined. Twenty-four hours before the experiments bees were divided into small groups (10 insects in each group) used for experimentation to induce hypersynthesis of lysozyme and apidaecins. Identically have been sampled bees from one apiary in Finland.

Preparation of honey samples. Samples of honey used to feed bees are derived from a colony from whch the bees are under experiments. Samples of honey after heating at 60°C were thoroughly mixed, and then three 2.0 g subsamples were transferred to 150 ml volumetric flask each to 22.5 ml of concentrated HNO_3 and 0.5 ml of 60% $HClO_4$. After mixing subsamples were heated at 150°C for 3 hrs. Cooled content was transferred into 50 ml volumetric flask and deionized water was added to the mark.

Determination of the content of heavy metals in honey. The content of Mn, Zn, Cu and Fe in honey samples was measured by the flame atomic absorption spectrophotometer (ASS). Pb, Cd and Ni were determined after Yeager et al. (26), using the flameless electrothermal spectrophotometer (ET ASS). Standards prepared covered the range from 0.0 to 0.50 $\mu g\ g^{-1}$. All determinations were performed at the atomic absorption spectrophotometer (ASS-3, Carl Zeiss).

Induction of antibacterial immune proteins. For immunization, worker bees were injected with a microsyringe into the dorsal sinus with about 1,200,000 or 20,000 living cells of *Escherichia coli*, 5,000 or 10,000 cells of *Xenorhabdus nematophilus* and 1,200,000 particles of latex (Sigma) in 2 µl of Saline W (25). Eighteen hour broth culture of *E. coli* and *Xenorhabdus* were used to induce the synthesis of immune hemolymph proteins. To control the exact number of injected bacteria, diluted samples were cultured in Petri dishes for 24 hrs at 28°C, then the colonies were counted and the number of bacteria was calculated.

Antimicrobial assays. Blood for examinations of antibacterial immune proteins (lysozyme, apidaecins) in the immunized insect was collected into a microsyringe from the dorsal sinus of bees before vaccination and after 2, 6, 24 and 29 hrs after induction. An aliquot of 2 µl of blood was taken from each insect, and pulled samples (10 l) were prepared.

A specific activity of lysozyme (EC 33.2.1.17; endo-β-(1-4)-acetylmuramide glycanhydrolase) in *Apis mellifera* hemolymph was quantified by a conventional agar-diffusion assay µl technique (23). Freeze-dried cells of *Micrococcus luteus* (Sigma, Chemical Co.), a general substrate for C (chicken) type of lysozymes were used as an indicator bacterium for antibacterial lysozyme activity. An assay plate composed of 10 ml of 0.066 M Sorensen buffer (pH 6.4), 7.5 mg of *M. luteus*, 300 µg of oxytetracycline sulfate to inhibit the growth of bacterial contaminants, and 100 mg of agarose (Serva, Hedelberg). Wells 3 mm in diameter in assay plates were filled with hemolymph, 10 µl per well. Diameters of the zone of lysis around the wells were measured after 24 hrs of incubation at 28°C. Eight known concentrations (500, 250, 125, 62.5, 31.25, 15.62, 7.81 and 3.9 µg/ml) of chicken egg white lysozyme were used as standards. The activity of bee hemolymph lysozyme is expressed in g per ml of hemolymph in equivalent to egg white lysozyme.

Apidaecins antibacterial activity elicited in immune *Apis hemolymph* by particulate inducers

(*E. coli*, *X. nematophilus*, latex beads) was recorded as the diameters of the *Escherichia coli* D31 inhibition zones around 3.0 mm wells into which 10 µl of immune hemoloymph was added. Each thin-layer plate of 10 mm diameter contained 8 ml of 0.7% nutrient agar with about 100,000 log phase bacterial cells of *E. coli* D31. 800 µg of streptomycin sulfate and a trace of 1-phenyl-2-thiourea was added to the agar medium to inhibit the growth of gram positive bacteria and to prevent melanization of insect blood.

As a standard there were used seven (100, 50, 25, 12.5, 6.25, 3.12 and 1.56 µg/ml) concentrations of gentamycin sulfate, and the activity of apidaecins was expressed in µg per 1 ml of hemolymph in equivalents to gentamycin concentrations. The results were elaborated by regression analysis. All bioassays were in triplicate and arranged in completely randomized sets of insects.

RESULTS

Honey from Poland and Finland fed to bee colonies and to bees intrahemocoelic injected by living cells of *E. coli* and *X. nematophilus* to induce hypersynthesis of blood lysozyme and synthesis of apidaecins differ clearly by the content of hevy metals studied (Fig. 1). The most pronounced differences are found between the level of Mn, Zn, Cu and Pb. Therefore, in the used system of experimentation the effect of pollution of the environment on the activity of native and inducible immune proteins could be examined.

Tab. 1. Innate and inducible level of lysozyme and bactericidal activity of apidaecins in blood of the worker honeybee

Sample	Time after induction (h)							
	0		6		24		29	
	L	A	L	A	L	A	L	A
Poland								
E. coli 20,000 cells	5.0	0	7.0	0.3	7.0	0.4	8.0	0.5
1,200,000 cells	4.8	0	7.2	0	7.2	0.3	8.5	0.4
X. nematophilus 5,000	4.8	0	9.0	0	8.9	0.3	9.4	0.3
10,000	5.0	0	6.8	0	9.0	0.3	9.2	0.4
Finland								
E. coli 20,000 cells	4.3	0	6.7	0	7.0	0.7	8.0	1.0
1,200,000 cells	4.8	0	6.4	0	7.3	0.7	7.9	0.9
X. nematophilus 5,000	5.1	0	8.1	0	9.0	0.5	9.5	0.7
10,000	5.0	0	8.8	0	9.2	0.9	9.8	0.9

L – lysozyme expressed in terms of EVL activity (µg/ml).
A – apidaecins expressed as gentamycin activity (µg/ml).

Using a cup diffusion assay procedure, the antibacterial activity of lysozyme increased many times from its innate level in blood of bees from Poland (Table 1). The immunizing bacterium, *E. coli* D31 enhanced the level of lysozyme after 6 hrs since induction, and it increased steadily up to the end of experimentation. Much

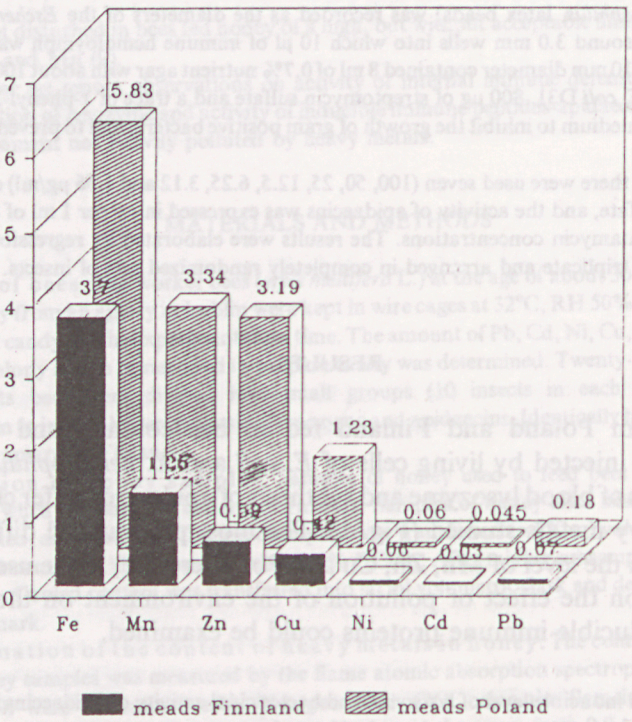

Fig. 1. The content of heavy metals in honey used to feed bee colonies under experimentation in Poland and Finland

more stronger inducer of lysozyme antibacterial activity in insect blood appears to be *Xenorhabdus*. The intensity of lysozyme response was not clearly correlated with a dose of inducing bacteria used, because the differences in antibacterial action after the used low and high doses were negligible.

Comparisons of the activity of lysozyme in hemolymph of insects from Poland and Finland could not reveal any profound differences irrespective of a type and dose of inducing bacteria used and time after induction (see Table 1).

Injection of living cells of *E. coli* or *X. nematophilus* into body cavity of the bees induced synthesis of apidaecins. The antibacterial activity could be detected as early as 6 hrs after induction wth 1,200,000 cells of *E. coli* and between 6-24 hrs after the injection of *X. nematophilus* and 20,000 living cells of *E. coli*. Irrespectively of the type of bacteria, antibacterial activiy of apidaecins increased strongly between 24 i 29 hrs after inductions. It is worth noting that in the less polluted environment (Finland) the level of apidaecins was significantly higher than that in the environment of a higher content of heavy metals.

DISCUSSION

Insecticides, fungicides, herbicides used for crop protection, when applied to crops during their blooming period or the blooming period of other plants near the treated crops may cause honey bee poisoning (22). Toxic effects also appear when bees come into contact with water, nectar of pollen contaminated with pesticides (21). The increased number of chemicals and waste products in the environment also makes most of them a potential hazard to the honeybee. They may affect bees by direct contact or they may enter the bee orally or through the respiratory system. Most commonly, like insecticides and pesticides, chemicals contaminate the interior of the hive when they are carried to it in the pollen loads or crop content (plant nectar) or the foraging worker bees. Poisoning may have immediate or delayed effect on individual bees or the bee colony as a super organism. Poisoning disrupts egg laying and normal colony functioning resulting by losses of young bees and foragers, chilling, starvation and death of brood. (1, 24).

Apart from any local toxic effects, the poison, as a stressor, may affect the whole organism, including the immune defense system of the bee. The toxicant that penetrates the cuticle, tracheae or alimentary tract is translocated by hemolymph to reactive sites, including the fat body, the main organ of hemolymph protein synthesis in insects. Lysozyme, apidaecins and abaecin are produced and released from the fat body of the bee (2, 5, 13). It is obvious that poisonings by chemicals contaminating the environment from which the foraging bees collect nectar, pollen and water are valid factors that make bees more susceptible to bacterial infections and parasitic invasions. By direct of indirect action on the bee organism, poisons may lower the effectiveness of protective barriers and/or impair defense mechanism operating in the body cavity.

It was demonstrated that infections of body cavity with *E. coli* induce a cecropin antibacterial activity (2) and increase the actual lysozyme titre in insect hemolymph (23). In the honeybee, the synthesis of immune proteins have been studied previously (3, 4, 5) but in no case were questions asked if contamination of environment, especially with heavy metals, may affect this process. In the light of the present investigations it seems probable that when the concentration of heavy metals in honey fed to bees is within a normal range both innate and inducible level of hemolymph lysozyme is not affected. The differences in the level of honey contamination by the metals between Poland and Finland have no visible influence on the action of this bactericidal enzyme. Like in other species of *Hymenoptera*, the low natural level of the honeybee lysozyme elevates much less than in lepidopterans, both after infection and after experimental induction with abiotic foreign bodies (12). This relatively low innate titre of lysozyme in *Apis mellifera* and only a moderate increase of the blood lysozyme after induction could partly be explained by social life of the honeybee. In this social insect, there operate additional defensive mechanisms

on the level of a whole colony such as cleaning behaviour, antibacterial action of propolis and royal jelly, antibiotic systems on honey, nectar and pollen that by lowering the number of pathogens in the hive environment protect brood and bees against bacterial infections (16).

In antibacterial defense of insect body cavity against bacterial invaders, lysozyme cooperates with cecropins-like polipeptides. By enzymatic way it destroys the murein sacculus of bacterial cells left after the cecropin action (2). Apidaecins (cecropin-like peptides) are the major immune components induced in honeybees upon bacterial infection. Examinations of induction kinetics revealed a peak concentration of this peptide in bee hemolymph (360 μg/ml) at 36 hrs post induction, with a delayed (8 hrs) onset (5). As Jacobs states (5), the peptide level at a certain time post injection depends on physical conditions of bees, such as age and stress. The persistent presence of bacteria in hemolymph of induced bee seems to keept the apidaecin transcription in the on state for days.

The results summarized in Table 1 demonstrate the tendency to depress inducible immune response in the bee in the environment heavier polluted comparing to that of a low level of contaminating heavy metals. Evidently indicates that the fact of much higher titre of apidaecins in hemolymph of insects from Finland after provoking the insect immune system by intrahemocoelic injection of bacteria, to produce immune proteins than that in Poland. The level of apidaecins in bee blood after *Xenorhabdus* induction has been at least two times higher than after *E. coli* induction. It seems, therefore, that *X. nematophilus* under the experimental conditions appeared to be better immunogen than *E. coli* D31.

Finally, some questions require further investigation: (I) the induction of abaecin, another antibacterial factor of immune bee hemolymph, in relation to stress conditions of bee organism; (II) the synergistic action of this peptide with one or more other major antibacterial factors of bee hemolymph-lysozyme, apidaecins; (III) the impact of individual heavy metals on innate and inducible immune proteins of the bee.

CONCLUSIONS

1. In the worker honeybee from colonies fed to honey contaminated with heavy metals, but in concentrations below the acceptable limits, native and induced level of hemolymph lysozyme seems to be unaffected by contamination.

2. The higher level of inducible immune proteins in the worker bee hemolymph in the environment less polluted by heavy metals, comparing to that in more contaminated environment, points to adverse effects of contaminators on synthesis of apidaecins in the fat body of the bee (1).

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STRESZCZENIE

Określono aktywność przeciwbakteryjną typu lizozymu i apidycyn (cecropin-like activity) w hemolimfie pszczół robotnic pochodzących z rodzin usytuowanych na terenach o niskiej (Finlandia) i wyższej (Polska) zawartości Pb, Cd, Ni, Cu, Zn, Mn i Fe w miodzie. W żadnym przypadku poziom zanieczyszczenia miodu nie przekraczał wartości uznanych za prawidłowe. Aktywność lizozymu oznaczono metodą agarowo-dyfuzyjną w stosunku do *Micrococcus luteus*, apidycyn w stosunku do *Escherichia coli* D31. Jako induktory odporności zastosowano *E. coli* D31 i *Xenorhabdus nematophilus*.

Aktywność bakteriobójcza lizozymu natywnego i lizozymu po indukcji w hemolimfie pszczół z pasiek usytuowanych w Polsce i w Finlandii nie różniła się. Niższa aktywność apidycyn we krwi pszczół z terenów silniej zanięczyszczonych może wskazywać na ujemny wpływ skażenia na syntezę indukowalnych białek odpornościowych w ciele tłuszczowym pszczoły miodnej.