Polish J. of Environ. Stud. Vol. 19, No. 4 (2010), 855-859

Short Communication

Influence of Environmental Pollution on the Protective Proteolytic Barrier of the Honey Bee *Apis mellifera mellifera*

Aneta Joanna Strachecka[1]*, Magdalena Maria Gryzińska[1], Magdalena Krauze[2]

[1]Department of Biological Basis of Animal Production,
[2]Department of Biochemistry and Toxicology,
Faculty of Animal Biology and Breeding, University of Life Sciences,
Akademicka 13, 20-950 Lublin, Poland

Received: 25 May 2009
Accepted: 4 January 2010

Abstract

Environmental pollution is of great significance to the protective barrier of the proteinous nature on the body surface of the honey bee. Crucial elements of this barrier are proteases and protease inhibitors (enzymes). They contribute to the protection of the bee against pathogens. Constant weakening of this barrier can lead to the rapid fall of its immunity, and even death.

Bees collected from two apiaries were compared (polluted and clean environments). This pollution contributed to the deterioration of the surface proteolytic barrier of respective groups of bees. Bees kept in a clean environment exhibited high protease and protease inhibitor activity levels.

Keywords: environmental pollution, honey bee, proteases, protease inhibitors, proteolytic barrier

Introduction

The proliferation of anthropogenic pollution sources (industry, including automotive; the expansion of cities; intensive agriculture) entails negative consequences for the natural environment. Industrial dust and smoke, as well as car fumes that pollute the air, soil, and water, also contaminate plants [5, 12, 23]. The increasing intensity of agricultural production also contributes to this pollution manifested by the disappearance of many insect species [6, 17]. A large part of these insects are honeybees needed for the correct functioning of natural fauna [17, 18].

If a bee colony lives in a polluted environment, the plant material used by bees and the air they breathe are also contaminated. As a consequence, part of this pollution accumulates in their bodies. This can cause a loss of balance in their homeostasis, including weakening of the immune system, which involves external defence body structures of proteinous nature. This hypothesis is supported by the fact that in apiaries there are a lot of fatalities in bee colonies [2, 8, 17, 21].

The data mentioned above clearly indicate that surface body structures are the first and fundamental line of the anti-infectious defence whose purpose is to protect and prevent the intrusion, implantation, and subsequent multiplication of a pathogen in apian tissues and organs. Considering this, onto the mentioned surface both proteases and protease inhibitors are secreted. It must be underlined that the proteolytic enzymes are present in the apian alimentary duct and in hemolymph [3]. In addition, the presence of proteases in apian moult liquid and venom was confirmed [3, 4, 10, 25].

It is also noteworthy that proteases and protease inhibitors are active in extra- and intracellular protein digestion (inorganic proteolysis) and take part in biological processes such as zymogene activation, the release of

*e-mail: aneta.strachecka@up.lublin.pl

Table 1. The biological material sample database.

Sample type	Total number of collected entities	Sampling pattern for biochemical analysis
worker eggs	2,400	3 samples x 10 entities
worker larvae	1,200	3 samples x 10 entities
worker pupae	1,200	3 samples x 10 entities
worker imagines	1,200	3 samples x 7 entities

hormones and physiologically active proteins from their precursors, translocation through membranes, protein compound ordering, and receptor activation (organic proteolysis) [3, 4, 11, 13, 25].

Thus, the objective of our research was to determine to what extent environmental pollution affected:
(1) the surface protein concentration,
(2) the activity of proteases and protease inhibitors, and
(3) the anti-entomopathogenic activity in the bee workers at definite developmental stages (eggs, larvae, pupae, and imagines).

Experimental Procedures

The research was performed at two stationary apiaries with differing levels of environmental anthropopression: one of them was situated at a trunk road (No. 17) in a an industrialized area of Lublin (polluted environment), the other one by a forest, away from major roads, in Nowiny village, Lublin district (clean environment).

In both apiaries the samples of *Apis mellifera* worker bees were randomly collected from 10 hives during two seasons in 2005-07. The material for the study was the following: worker eggs, worker larvae, worker pupae, and worker imagines (Table 1). These samples were collected in January, May, August, and October so as to allow for the four seasons of the year. The material was frozen (-8°C) immediately after being taking from a hive.

Next, after defrosting, the samples for biochemical analyses were taken threefold from each of the biological material types. After that the samples were placed on Miracloth, rinsed with distilled, water and the polluted washings were discarded. Afterward, the samples were placed again in test-tubes and shaken for three minutes in distilled water (for the neutral and alkaline proteases) and then in a 1% detergent solution (Triton X-100, Serva; for acidic proteases) in order to wash out the body surface proteins. Next, the washings were frozen in Eppendorf test-tubes at -20°C. On re-defrosting the samples they were assigned an optimal pH and tested for surface protein content using the Lowry method modified by Schacterle-Pollack [20] and then tested for acidic, neutral and alkaline protease activities according to the Anson [1] and the Lee and Lin methods [9] used in enzymology to determine the activity levels of such proteins.

In addition, the samples were assayed for antifungal/anti-mould and anti-ascomycetous activity in relation to the marker fungi: *Aspergillus fumigatus* and *Candida albicans* on a SABG medium (Sabouraud glucose agar) [19].

Results

The highest surface protein concentration values were observed in the case of the eggs, both in the clean and polluted environments (Tables 2 and 3). However, the values differed according to the season of the year and the investigated apiary. In the samples collected from the apiary in the clean environment the protein concentration values were approximately 2-4 times higher as compared with the concentration values in the samples from the polluted environment.

The highest surface protein concentration values in the different developmental forms of the bee were observed in summer in the case of both apiaries. The highest protein concentration value in workers from the clean environment was observed in the egg samples taken during the summer period (0.626 mg/ml), and the lowest – in the imagines taken during the spring (0.002 mg/ml) (Table 2). In the samples collected from the apiary situated in the polluted environment the highest protein concentration values were identified in the eggs collected during the summer period (0.326 mg/ml), and the lowest – in the larvae taken during the spring period (0,002 mg/ml) (Table 3).

The values of the activities of proteases and the natural protease inhibitors on the body surface in the workers were higher in the clean environment than in the polluted one. In general, the values of the activities of proteases and the natural protease inhibitors were the highest at pH = 2.4, 7, and 11.2 (Tables 2 and 3).

In the clean environment the highest values of the activities of acidic, neutral and alkaline proteases were identified in the larvae collected during summer, and the lowest in the spring material. The activities of the natural protease inhibitors were the highest at pH = 2.4 in the pupae and at pH = 7 in the spring imagines and at pH = 11.2 in the summer eggs; the lowest at pH = 2.4 in the spring eggs, at pH = 7 in the winter imagines and at pH = 11.2 in the autumn pupae (Table 2). In the polluted environment high acidic, neutral and alkaline protease activities were observed for the summer period; levels were low for the winter and spring materials. The activities of the natural protease inhibitors were the highest in the worker imagines from the summer at pH = 2.4 and 11.2 and at pH = 7 in those from the spring (Table 3).

From the analyses of the anti-entomopathogenic activities it resulted that the workers in the clean environment had much better anti-mould and anti-ascomycetous protection than the workers in the polluted environment (Table 4). In the clean environment, protection against those pathogens was at a 100% level during all the seasons of the year, whereas in the polluted environment the protection was weaker. This indicates that in the polluted environment there was an unbalance in the apian protective mechanisms, chiefly in those reacting against the yeast fungi.

Table 2. Seasonal apian body surface protein concentrations and proteolytic activities measured during the different developmental stages in workers from a clean environment.

Seasons	Worker developmental forms	Protein concentration (mg/mL)	Protease activity			Protease inhibitor activity		
			pH = 2.4	pH = 7	pH = 11.2	pH = 2.4	pH = 7	pH = 11.2
spring	eggs	0.523± 0.002	0±0.005	0±0.008	0±0.004	1.234±0.002	1.675±0.003	3.432±0.002
	larvae	0.021± 0.009	0±0.003	0±0.002	0.122±0.002	12.035±0.004	3.68±0.004	4.022±0.005
	pupae	0.017±0.005	0±0.004	0±0.004	0.134±0.005	**14.076**±0.007	5.311±0.002	4.121±0.007
	imagines	0.002±0.009	0±0.007	0.023±0.003	0.011±0.002	8.121±0.005	**17.603**±0.004	7.691±0.006
summer	eggs	**0.626**±0.011	0.019±0.001	0±0.003	0±0.001	8.409±0.003	2.338±0.002	**9.691**±0.006
	larvae	0.598±0.009	**0.463**±0.006	**2.559**±0.005	**1.672**±0.003	8.486±0.004	3.168±0.003	5.071±0.002
	pupae	0.538±0.008	1.33±0.008	1.387±0.009	1.403±0.005	8.234±0.002	4.636±0.005	4.023±0.005
	imagines	0.52±0.007	0.023±0.004	0.564±0.004	1.324±0.006	9.325±0.004	11.411±0.001	8.446±0.007
autumn	eggs	0.462±0.010	0.199±0.009	0±0.002	0±0.001	8.089±0.003	0.001±0.002	0.836±0.004
	larvae	0.421±0.006	0.108±0.007	0.345±0.006	0.023±0.004	8.221±0.001	13.341±0.001	0.056±0.003
	pupae	0.235±0.008	0.089±0.008	0.342±0.003	0.022±0.005	8.123±0.003	2.611±0.004	0.012±0.001
	imagines	0.211±0.004	0.034±0.007	0.338±0.005	0.02±0.004	7.478±0.002	1.435±0.003	4.456±0.002
winter	eggs	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	larvae	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	pupae	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	imagines	0.112±0.003	0.001±0.002	0.021±0.003	0.005±0.002	3.211±0.001	0.998±0.003	1.112±0.004

The non-analyzed samples (n.a.) were due to the unavailability of specific forms at a given time.

Table 3. Seasonal apian body surface protein concentrations and proteolytic activities measured during the different developmental stages in workers from a polluted environment.

Seasons	Worker developmental stages	Protein concentration (mg/mL)	Protease activity			Protease inhibitor activity		
			pH = 2.4	pH = 7	pH = 11,2	pH = 2.4	pH = 7	pH = 11.2
spring	eggs	0.324±0.004	0±0.003	0±0.003	0±0.002	0.867±0.004	0.996±0.006	0.999±0.008
	larvae	0.002±0.007	0±0.002	0±0.005	0.092±0.004	2.141±0.006	1.111±0.005	0.856±0.009
	pupae	0.007±0.002	0±0.004	0±0.001	0.098±0.007	2.187±0.005	2.122±0.009	0.841±0.006
	imagines	0.034±0.004	0±0.001	0.003±0.002	0.001±0.003	1.678±0.007	**5.675**±0.004	1.213±0.005
summer	eggs	**0.326**±0.001	0.011±0.003	0±0.002	0±0.002	2.789±0.003	0.967±0.007	2.156±0.007
	larvae	0.245±0.005	0.125±0.005	**1.259**±0.003	**0.871**±0.009	2.986±0.004	0.881±0.003	2.341±0.004
	pupae	0.232±0.003	**0.873**±0.008	0.987±0.004	0.833±0.005	2.999±0.008	0.865±0.008	2.123±0.006
	imagines	0.212±0.005	0.008±0.009	0.265±0.006	0.765±0.004	**3.115**±0.005	4.256±0.006	**3.326**±0.005
autumn	eggs	0.211±0.004	0.101±0.005	0±0.003	0±0.002	2.561±0.005	0±0.004	0.113±0.002
	larvae	0.198±0.007	0.098±0.004	0.175±0.005	0.009±0.004	2.321±0.003	5.256±0.005	0±0.004
	pupae	0.135±0.008	0.049±0.003	0.152±0.007	0.001±0.003	2.121±0.009	0.991±0.009	0±0.001
	imagines	0.078±0.005	0.014±0.008	0.124±0.003	0±0.002	2.111±0.008	0.878±0.007	1.276±0.005
winter	eggs	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	larvae	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	pupae	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	imagines	0.045±0.003	0±0.002	0.008±0.001	0±0.001	0.998±0.006	0.018±0.007	0.992±0.003

The non-analyzed samples (n.a.) were due to the unavailability of specific forms at a given time.

Table 4. Seasonal apian body-surface anti-enthomopathogen activities measured in the different developmental forms of the workers from the clean and polluted environments.

Seasons	Worker developmental stages	Clean environment		Polluted environment	
		Anti-mould activity	Anti-ascomycetous activity	Anti-mould activity	Anti-ascomycetous activity
spring	eggs	present	present	absent	absent
	larvae	present	present	absent	absent
	pupae	present	present	absent	absent
	imagines	present	present	present	present
summer	eggs	present	present	present	present
	larvae	present	present	present	present
	pupae	present	present	present	present
	imagines	present	present	present	present
autumn	eggs	present	present	present	absent
	larvae	present	present	present	absent
	pupae	present	present	present	absent
	imagines	present	present	present	absent
winter	eggs	n.a.	n.a.	n.a.	n.a.
	larvae	n.a.	n.a.	n.a.	n.a.
	pupae	n.a.	n.a.	n.a.	n.a.
	imagines	present	present	absent	absent

The non-analyzed samples (n.a.) were due to the unavailability of specific forms at a given time.

Discussion

It is well known that the organism of a worker bee acts as a "filter" that receives from the honey matter part of the toxic elements that are dangerous to its health (from 20% to 36%, depending on the type of the element). In this situation activity of the proteolytic system on the body surface in bees, which improves their protection against pathogens [22], should be impaired by this toxic effect. It must be stressed that this barrier has not been sufficiently examined (Tables 2, 3 and 4).

Our observations (Tables 2 and 3) clearly indicate that in the clean and polluted environments, at the beginning of the vegetative season the workers did not have or had very low protease protection on their body surfaces. This happens mostly because the wintering and early spring bees are physiologically different from workers in the summer and autumn [6, 7, 16, 24]. Moreover, at this time bees are more susceptible to pathogen infection due to the destabilized immunological barrier of the body surface [14, 15, 22]. Significantly, at the turn of the spring and summer, workers started to rebuild their protective barriers in the form of surface proteolytic activity. So in opposite situations, as the year progressed, the surface protease activity in the workers began to diminish.

Thus, when comparing the respective developmental phases of the bees originating from the clean and polluted environments in different seasons (Tables 2, 3 and 4), we observed that there are significant variations in the values of the proteolytic system activity between them. Significantly, lower values of the activity were recorded for the bees collected in the polluted environment. Similarly, differences in the values of the proteolytic system activity in the workers (e.g. the spring pupae) from the clean and polluted environments result in different patterns of entomopathogenic activity.

Conclusion

In the clean environment, higher protein concentration, protease activity, and natural protease inhibitor activity values could be observed on the body surface of the workers as compared with the bees from the polluted environment. The surface proteolytic system status is reflected in the anti-enthomopathogenic activities.

References

1. ANSON M. The estimation of pepsin, tripsin, papain and cathepsin with hemoglobin. J.Gen.Physiol. **22**, 79, **1938**.
2. BANIA J., POLANOWSKI A. Bioinsecticides and insect defense mechanisms. Postępy Biochemii **45**, (2), 143, **1999**.
3. BARRETT A. Peptidases: a view of classification and nomenclature. MCBU, **1999**.

4. BODE W., FERNANDEZ-CATALAN C., NAGASE H., MASKOS K. Endoproteinase – protein inhibitor interaction. AMPIS **107**, 3, **1999**.
5. CURZYDŁO J. Contamination of *Trifolium pratense* (red clover) with car fume lead along the Cracow region roads. Kraj. Konf., IUNG, Puławy, **1978**.
6. GROMISZ Z. Protection of bees against contamination. PWRiL,Warsaw, Poland, pp. 1-235, **1990**.
7. HARBORNE J. Ecological biochemistry. PWN, Warsaw, Poland, pp. 1-287, **1997**.
8. HOFFEL I. Schwermetallen in Bienen und Bienenprodukten. Apidologie **16**, 196, **1985**.
9. LEE T., LIN Y. Trypsin inhibitor and trypsin – like protease activity in air – or submergence – grown rice (*Oryza sativa* L.) coleoptiles. Plant Science **106**, 43, **1995**.
10. LIMA P., BROCHETTO-BRAGA M., CHAUD-NETTO J. Proteolytic activity of Africanized honeybee (*Apis mellifera*: hymenoptera, apidae) venom. J.Venom.Anim.Toxins **6**, (1), **2000**.
11. LOCHT A., LAMBA D., BAUER M., HUBER R., FRIEDRICH T., KROGER B., HOFFKEN W., BODE W. Two heads are better than one: crystal structure of insect derived double domain Kazal inhibitor rhodniin in complex with thrombin. Eur. Mol. Biol. Organ. J. **14**, 5149, **1995**.
12. MIGULA P., KAFEL A., KĘDZIORSKI A., MARCZAK G., NAKONIECZNY M. Heavy metals in bee nutrients, products and tissues collected in industrialised areas. XXVI-II Nauk. Konf. Pszczel., Puławy, **1991**.
13. OTLEWSKI J., JASKÓLSKI M., BUCZEK O., CIERPIŃSKI T., CZAPIŃSKA H., KROWARSCH D., SMALAS A.O., STACHOWIAK D., SZPINETA A., DADLEZ M. Structure – function relationship of serine protease – protein inhibitor interaction. Acta Biochimica Polonica **48**, (2), 419, **2001**.
14. PLISZCZYŃSKI M., CHEŁMIŃSKI M., BIZOŃ K. Hemocytic immune parameters of the wintering workers of the honey bee *Apis mellifera* L. (Apidae). Ann. Univ. Mariae Curie-Skłodowska **20**, 157, **2006**.
15. PLISZCZYŃSKI M., LUFT-DEPTUŁA D., BIZOŃ K. Monitoring of immunity in wintering workers of the honey bee, *Apis mellifera* L. (Apidae), by a protection test. Ann. Univ. Mariae Curie-Skłodowska **21**, 173, **2006**.
16. PRABUCKI J. Apiculture. Albatros Szczecin, Poland, pp. 1-901, **1998**.
17. ROMAN A. Bioaccumulation levels of some trace elements in the organisms of worker bees and drones. Med. Wet. **62**, (12), 1439, **2006**.
18. ROMAN A. Bees and bee products as environmental pollution bioindicators in areas affected by the copper (LGOM) and concrete and lime industries (Opole). Zesz. Nauk. AR, Wrocław **323**, 175, **1997**.
19. SABOURAUD R. A Contribution to the Study of Human Trichophytosis. A Clinical, Microscopic and Bacteriological Study of the Variety of Trichophytosis in Man. Ann. Dermatol. Syphil. **3**, 1061, **1892**.
20. SCHACTERLE G., POLLACK R. Simplified method for quantitative assay of small amounts of protein in biological material. Anal. Biochem. **51**, 654, **1973**.
21. SPODNIEWSKA A. Lead and cadmium content in bees from apiaries of Warmia and Mazury province. Med. Wet. **63**, (6), 736, **2007**.
22. STRACHECKA A., GRZYWNOWICZ K. Activity of protease inhibitors on the body surface of the honeybee. Med. Wet. **64**, 1256, **2008**.
23. STRUSIŃSKI A. Environmental pollution caused by car fume lead. Kraj. Konf., IUNG, Puławy, **1978**.
24. TWARÓG D., STRACHECKA A., PALEOLOG J., KASPEREK K., MISIURA E., CHOROSZYŃSKA D. Winter ambient temperature effect on surface protein concentration values in bees. Mat. Konf., Puławy, **2008**.
25. WALTER R., CLELIA F. Insect digestive enzymes: properties, compartmentalization and function. Comp. Biochem. Physiol. **109B**, 1, **1994**.