

BODY-SURFACE PROTEASE INHIBITORS IN CAGE AND HIVE *APIS MELLIFERA* L.

Aneta Strachecka, Jerzy Paleolog, Grzegorz Borsuk,
Krzysztof Olszewski, Krzysztof Grzywnowicz, Magdalena Gryzińska

University of Life Sciences in Lublin, Poland

Abstract. The aim of the work was to determine the activity of protease inhibitors sampled from the body surface of bee workers kept in a natural hive environment and in a cage. The samples were collected for five weeks. 40 cage samples and 50 hive samples were gathered, each containing 10 bees. Hydrophilic (water-treated) and hydrophobic (Triton-rinsed) proteins were isolated from the insects. The samples containing washed-out proteins were tested as follows: the activity of aspartic and serine protease inhibitors by the Lee and Lin method; electrophoretic analysis of proteins in a polyacrylamide gel for protease inhibitor detection by means of the modified Felicioli method; and *in vivo* tests of antifungal and antibacterial activity using the double application method. The cage environment had a destabilizing effect on the natural protease inhibitor system causing radical variation in its activity, which was not the case with the hive environment. The samples were not found to be active in relation to *M. luteus* and *E. coli*. The cage bees were less resistant to microorganisms. The results of the *in vivo* microorganismal test confirmed the fact of weaker protease inhibitor activity in the washed-out body-surface samples of the cage bees that was also observed in *in vitro* biochemical analyses. The results of cage-based analyses of non-specific apian resistance should be treated with caution when used in reference to hive bees.

Keywords: *Apis mellifera*, beehive, body surface, cage, protease inhibitors

INTRODUCTION

The body-surface proteinaceous layer is an essential element of insect external resistance. Numerous body-surface proteins in *Apis mellifera* were found to have protease and protease inhibitor activity [Grzywnowicz et al. 2009]. Insects protect themselves against pathogen invasion and accidental activation of various cellular processes by synthesising protease inhibitors [Bania and Polanowski 1999]. Protease inhibitors are crucially active in homeostasis maintenance by suppressing their target enzymes [Gawlik et al. 2005]. Inhibitors sampled from insect haemolymph were divided into two main groups: low-mole-

Corresponding author – Adres do korespondencji: dr Aneta Strachecka, Department of Biological Basis of Animal Production, University of Life Sciences in Lublin, Akademicka 13, 20-950 Lublin, Poland, e-mail: aneta.strachecka@up.lublin.pl

cular inhibitors (below 10 kDa) that predominantly belonged to the Kunitz type of inhibitors and high-molecular inhibitors (approximately 45 kDa) from the serpin family [Bania and Polanowski 1999, Nirmala et al. 2001]. Apart from classifying the inhibitors according to their molecular masses, in another categorization they were divided as to their functions and capacity for inhibiting particular protease types. Bees were found to have serine, cysteine and aspartic proteases. The proteases are present in the alimentary duct, haemolymph, moult liquid, venom and cuticle of bees [Barrett 1999, Evans et al. 2006, Grzywnowicz et al. 2009].

Laboratory cage tests during which bees are kept in cages are increasingly more often used for breeding and scientific purposes, including drug and toxic substance testing and advanced biomedical research [Paleolog et al. 2003, Schmickl and Crailsheim 2004]. The principle of cage testing consists in adjusting breeding conditions so that they do not impinge on the health, resistance and welfare of the animals [DzU 97.111.724]. In comparison with field assessments, cage tests are an easier and cheaper method that additionally eliminates tremendous environmental variation, such as the effect of the season [Milne 1985]. However, the problem is that some authors found positive correlation between apiary and laboratory results, while others did not identify such correlation, which may be caused by differences between the hive and cage environments [Milne 1985]. The cage environment is additionally stressful to bees [Paleolog et al. 2003].

Therefore, it would be extremely interesting to verify the hypothesis that, in comparison with the natural habitat in the hive, the cage environment negatively affects the activity of apian body-surface protease inhibitors and thus, partly, the level of non-specific resistance of bees.

MATERIAL AND METHODS

Two colonies were selected: one with yellow-hued bees and the other one with dark-coloured bees. The combs with emergent brood of the yellow-hued insects were put in an incubator for 24 hours producing approx. 2000 one-day-old workers. Four hundred of them were placed in 10 cages, each containing 40 workers. Another 1500 workers were inserted in the hive with the dark-hued bees where they were easily identifiable. The cages were kept in a conditioning chamber at 24–25°C and 60% humidity for 5 weeks. The bees were fed with treacle (1:1). During that time, dead yellow bees were collected every 7 days from the bottom board of the hive and of the cages. They were frozen in germ-free bags at –8°C 1–2 months. Each week, bees from all the ten cages were pooled together, mixed and portioned into samples – 40 after 5 weeks of experiment. A similar procedure was applied for the hive bees. 50 samples were obtained (10 samples x 10 bees x 5 weeks).

The samples were subsequently defrozen and initially rinsed in 10ml distilled water for 20 seconds to remove impurities. The resultant solution was found to contain no proteins after being tested according to the Lowry method, as modified by Schacterle and Pollack [Schacterle and Pollack 1973]. That is why the rinsings were discarded. Subsequently, the insects were put in test tubes that were then filled with 10ml distilled water and shaken/washed for

4 minutes at 3400 rpm. Filtration of each sample through Miracloth produced a solution that mostly contained hydrophilic proteins. The solution was divided into four portions, placed in four Eppendorf test tubes and refrigerated at -40°C . The procedure produced:

- 2 ml sample for protease inhibitor activity assays,
- 2 ml sample for electrophoretic analyses,
- 2 ml sample for antifungal and antibacterial activity assays,
- 2 ml back-up sample.

The remaining biological material on Miracloth was put back in test tubes. This time the procedure involved the addition of 1% detergent (Triton X-100) solution in distilled water (10 ml). The whole was shaken for 4 minutes at 3400 rpm. The same as in the case of shaking/rinsing in distilled water, four samples were prepared after Miracloth filtration, containing predominantly hydrophobic proteins. The procedure produced a total of 320 samples of the cage bees (40 samples x 2 rinsings x 4 Eppendorfs) and 400 samples of the hive bees (50 samples x 2 rinsings x 4 Eppendorfs).

Next, the samples with washed-out protease inhibitors were biochemically analysed as follows:

- determination of the levels of natural inhibitors of acidic, neutral and alkaline proteases, based on the Lee and Lin method [1995] [Lee and Lin 1995];
- electrophoretic analysis of proteins on polyacrylamide gel in order to detect aspartic and serine protease inhibitors using the modified Felicioli method [Felicioli et al. 1997],
- determination of antifungal and antibacterial activities in *in vivo* tests, using the double application method with:
 - SABG (Sabouraud, 1892) – to determine the activity in relation to *Aspergillus niger*,
 - YPD (Muthy et al., 1975) – to determine the activity in relation to *Candida albicans*,
 - LB (Bertani) – to determine the activity in relation to *Staphylococcus aureus*, *Bacillus subtilis*, *Micrococcus luteus*, *Salmonella typhimurium*, *Pseudomonas aeruginosa* and *Escherichia coli*;
- in an additional test of micro-organism survival, bacteria were passed from the surface on which the growth of *B. subtilis* had been inhibited *B. subtilis* onto a new base and observed for renewed growth or its absence;
- in the antifungal, antibacterial and survival tests, as well as in the test of micro-organism survival, each of the dishes was photographed (SONY α 100). The photographs were used to determine the area of the active anti-microorganism surface on which there was no growth with MultiScan Base software.

The statistic calculations were performed with the SAS[®] software (SAS Institute User's Guide Version 6.11. 1996). The statistical differences between the experimental factors were verified using ANOVA.

RESULTS AND DISCUSSION

The cage environment was destabilising to the system of natural protease inhibitors causing considerable oscillation in their activities (Table 1). In a dozen or so cases the re-

sult was a complete loss of inhibitor activity (especially hydrophobic protease inhibitors). This did not occur with the hive bees.

The electrophorograms of both the cage and hive bees revealed a similar distribution of high- and low-molecular aspartic protease inhibitors which were numerous, broad and prominent during all the weeks. The bands denoting neutral proteases of the cage bees were few. They were broad and prominent and contained high-molecular proteins. The bands were missing in the hive samples. Basic protease inhibitor bands in the hive and cage bees were few, narrow, not very prominent and predominantly low-molecular. During some of the weeks the cage samples were found to contain no active *in vitro* natural inhibitors, while the electrophorograms featured bands, which may be due to a reaction with the proteases/substrates and the specificity of protease inhibitor staining.

Table 1. The activity of aspartic and serine protease inhibitors (As U · mg⁻¹) in the washed-out body-surface samples of *A. mellifera* workers from a hive and from cages
Tabela 1. Aktywność inhibitorów proteaz asparaginowych i serynowych (As U · mg⁻¹) w próbkach wypłukanych z powierzchni ciała robotnic *A. mellifera* z ula i z klatek

Environment Środowisko	Rinsed with Płukanie	Weeks Tygodnie	Inhibitors – Inhibitory					
			pH 2.4		pH 7.0		pH 11.2	
			\bar{x}	± se	\bar{x}	± se	\bar{x}	± se
Hive Ul	water woda	1	82.676	0.04	19.828	0.04	16.456	0.16
		2	83.095	0.10	19.007	0.02	16.983	0.16
		3	83.265	0.13	18.958	0.03	17.199	0.12
		4	82.477	0.57	19.389	0.08	17.325	0.03
		5	82.486	0.29	19.132	0.10	16.669	0.18
	triton	1	97.405	0.03	46.470	0.11	42.625	0.07
		2	97.374	0.21	46.881	0.13	43.029	0.06
		3	97.867	0.41	47.015	0.05	42.535	0.34
		4	97.321	0.21	46.634	0.20	42.317	0.19
		5	97.826	0.32	47.059	0.11	42.290	0.16
Cage Klatka	water woda	1	196.615	0.08	191.488	0.01	0.000	0.00
		2	102.527	0.11	71.733	0.03	0.000	0.00
		3	13.323	0.83	14.758	0.84	7.698	0.49
		4	73.493	0.13	7.097	0.10	70.384	0.24
		5	122.370	0.16	0	0	126.497	0.11
	triton	1	19.252	0.03	0	0	25.446	0.06
		2	18.697	0.03	0	0	24.469	0.06
		3	17.330	0.19	0	0	23.164	0.70
		4	0	0	33.159	2.44	13.454	0.24
		5	0	0	4.123	0.04	0	0

No sample was found to be active in relation to *M. luteus* or *E. coli*.

The hive bees were observed to have antifungal protection against two fungi and antibacterial protection from four bacteria (Table 2). The cage bees were less resistant to microorganisms. Their body-surface samples inhibited only the growth of *B. subtilis* and *S. aureus*.

Additionally, the survival test revealed that the samples washed out of the body surface of the hive bees had the capacity to destroy the *B. subtilis* strain, whereas the cage bee samples only inhibited the growth of the bacteria.

Table 2. The antifungal and antibacterial activities in the washed-out body-surface samples of *A. mellifera* workers from a hive and from cages, calculated as the area (mm²) without pathogen development.

Tabela 2. Aktywność przeciwgrzybowa i przeciwbakteryjna próbek z powierzchni ciała robotnic *A. mellifera* z ula i z klatek, mierzona jako pole powierzchni (mm²), na której rozwój patogenu nie wystąpił

Environment Środowisko	Area, mm ² – Pole powierzchni, mm ²					
	with antifungal activity – aktywności przeciwgrzybowej			with antibacterial activity aktywności antybakteryjnej		
	<i>Candida albicans</i>	<i>Aspergillus niger</i>	<i>Bacillus subtilis</i>	<i>Staphylococcus aureus</i>	<i>Salmonella typhimurium</i>	<i>Pseudomonas aeruginosa</i>
Hive Ul	159.62	10.43	188.28	118.91	263.87	214.79
Cage Klatka	0	0	171.62	222.13	0	0

The results of the *in vivo* microorganismal test confirmed the fact of weaker proteolytic system activity in the washed-out body-surface samples of the cage bees. Reduced activity was also observed in *in vitro* biochemical analyses.

The authors observed a markedly higher activity of protease inhibitors in the hive bees as compared with the cage ones. Moreover, inhibitor activity in the hive bees remained at a relatively stable level during the consecutive weeks of the experiment, while that of the cage bees varied with time. The cage bees had difficulty maintaining constant activity / balance / homeostasis of their body-surface protease inhibitor protection. Microbiological tests also revealed a narrower range of antifungal and antibacterial activity in the cage samples.

The cage environment is more conducive to pathogen proliferation than the hive habitat [Gliński et al. 2006]. With incomplete colony representation and limited flight possibilities, cage bees are more vulnerable to stress [Paleolog and Wilczyńska 1998] which may in turn bring about changes in hormone and ectohormone secretion [Lipiński 2002]. The immune system of hive bees is enhanced thanks to pollen, honey, propolis and royal jelly consumption [Gliński et al. 2006] and pollen-rich diet stimulates the production of proteins, enzymes, protein inhibitors and enzyme inhibitors [Woyke 2008]. The mobility of bees in a cage is limited, with the concomitant pollen deficit.

The hive and cage environments may differently affect the expression of many characteristics [Milne 1985]. This is consistent with Olszewski and Paleolog's [2005] conclusions to the effect that laboratory cage test results should be treated with caution in comparisons with the hive environment. The present research confirms that, considering the negative effect of the cage environment on the body-surface proteolytic system, the results of cage tests of apian non-specific resistance should be used tentatively in reference to bees kept in hives.

CONCLUSIONS

The cage environment had a destabilizing effect on the natural protease inhibitor system causing radical variation in its activity, which was not the case with the hive environment. The cage bees were less resistant to microorganisms.

REFERENCES

- Bania J., Polanowski A., 1999. Bioinsecticides and insect defense mechanisms. *Postępy Biochem.* 45 (2), 143–150.
- Barrett A.J., 1999. Peptidases: a view of classification and nomenclature. *MCBU* 5, 1–12.
- Evans J.D., Aronstein K., Chen Y.P., Hetru C., Imler J.L., Jiang H., Kanost M., Thompson G.J., Zou Z., Hultmark D., 2006. Immune pathways and defence mechanisms in honey bee *Apis mellifera*. *Insect Mol. Biol.* 15 (5), 645–656.
- Felicioli R., Garzelli B., Vaccari L., Melfi D., Balestreri E., 1997. Activity staining of protein inhibitors of proteases on gelatin-containing polyacrylamide gel electrophoresis. *Anal. Biochem.* 244 (1), 176–179.
- Gawlik K., Poręba W., Gutowicz J., 2005. Cystatins, thyrpines and inhibitors homologous to propeptides of cysteine proteases. *Postępy Biochem.* 51 (3), 318–327.
- Gliński Z., Kostro K., Luft-Deptuła D., 2006. Choroby pszczół [Apian diseases]. PWRiL, Warszawa, 37–43 [in Polish].
- Grzywnowicz K., Ciołek A., (Strachecka), Tabor A., Jaszek M., 2009. Profiles of body-surface proteolytic system of honey bee queens, workers and drones: Ontogenetic and seasonal changes in proteases and their natural inhibitors. *Apidologie* 40 (1), 4–19.
- Lee T., Lin Y., 1995. Trypsin inhibitor and trypsin – like protease activity in air – or submergence – grown rice (*Oryza sativa* L.) coleoptiles. *Plant Sci.* 106, 43–54.
- Lipiński Z., 2002. Istota oraz mechanizm porzucania gniazd przez roje pszczół miodnych (rójka, ucieczka, migracja i zjawiska im towarzyszące) [The essence and mechanizm of nest abandonment by honeybee swarms (mating flight, escape, migration and related phenomena)]. *Blenam, Olsztyn*, 5–322 [in Polish].
- Milne Ch.P., 1985. The need for using laboratory tests in breeding honeybees for improved honey production. *J. Apic. Res.* 24 (4), 237–242.
- Nirmala X., Kodrik D., Zurovec M., Sehnal F., 2001. Insect silk contains both a Kunitz-type and a unique Kazal-type proteinase inhibitor. *Eur. J. Biochem.* 268, 2064–2073.
- Olszewski J., Paleolog J., 2005. Foraging and hoarding efficiency in Buckfast purebreds and Norwegian black bee (*A. m. mellifera*) hybrids. Part 1. Annual honey yield versus results of field flying cage and laboratory tests. *J. Apic. Sci.* 49 (1), 17–25.
- Paleolog J., Borsuk G., Olszewski K., 2003. Some factors influencing the results of the cage tests of a life span and food intake in *Apis mellifera* workers. *Ann. Univ. Mariae Curie-Skłodowska*, 21 (N2), 95–104.
- Paleolog J., Wilczyńska R., 1998. The influence of queen pheromones, environment factors, genotypes and their interactions on length of life of caged workers in laboratory tests in bees. *Pszczeln. Zesz. Nauk.* 42 (2), 65–66.

- Schachterle G., Pollack R., 1973. Simplified method for quantitative assay of small amounts of protein in biological material. *Anal. Biochem.* 51, 654 – 655.
- Schmickl T., Crailsheim K., 2004. Inner nest homeostasis in a changing environment with special emphasis on honey bee brood nursing and pollen supply. *Apidologie* 35, 249–263.
- DzU 97.111.724. Ustawa z dnia 21 sierpnia 1997 r. o ochronie zwierząt [The Act on Animal Protection of 21 August 1997] [in Polish].
- Woyke J., 2008. *Biologia pszczół. Hodowla pszczół* [Apian biology. Apiculture]. PWRiL, Poznań, 77–119 [in Polish].

INHIBITORY PROTEAZ NA POWIERZCHNI CIAŁA PSZCZÓŁ (*APIS MELLIFERA* L.) W KLATCE I W ULU

Streszczenie. Określono aktywność inhibitorów proteaz wyizolowanych z powierzchni ciała robotnic utrzymywanych w naturalnym środowisku ula oraz w klatce. Próby pobierano przez pięć tygodni, pozyskując 40 prób z klatek i 50 prób z ula, w każdej po 10 pszczół. Z owadów wyizolowano białka hydrofilne (przy użyciu wody) oraz hydrofobowe (przy użyciu tritonu). Próbkę z wyplukanymi białkami poddano następującym oznaczeniom: aktywność inhibitorów proteaz asparaginowych i serynowych wg metody Lee i Lina; analiza elektroforetyczna białek w żelu poliakrylamidowym do wykrywania inhibitorów proteaz wg zmodyfikowanej metody Felicioliego; aktywność przeciwgrzybowa i antybakteryjna w testach *in vivo* metodą płytek dwuwarstwowych. Środowisko klatki działało destabilizująco na system naturalnych inhibitorów proteaz wywołując duże wahania ich aktywności, co nie zdarzyło się w ulu. W próbkach nie zaobserwowano aktywności wobec *M. luteus* i *E. coli*. Pszczoły w klatce miały słabszą oporność przeciwko mikroorganizmom. Wyniki testu z mikroorganizmami *in vivo* potwierdziły słabszą aktywność inhibitorów proteaz w próbkach wyplukanych z powierzchni ciała pszczół w klatkach, wykazaną również w analizach biochemicznych *in vitro*. Uzyskane w klatkach wyniki badań oporności nieswoistej pszczół należy ostrożnie odnosić do pszczół w ulu.

Słowa kluczowe: *Apis mellifera*, inhibitory proteaz, klatka, powierzchnia ciała, ul

Accepted for print – Zaakceptowano do druku 10.11.2011