ANNALES
UNIVERSITATIS MARIAE CURIE-SKŁODOWSKA
LUBLIN – POLONIA
VOL. XXIX (4) SECTIO EE 2011

Department of Biological Basis of Animal Production, University of Life Sciences
Akademicka 13, 20-950 Lublin
aneta.strachecka@up.lublin.pl

ANETA STRACHECKA, JERZY PALEOLOG, GRZEGORZ BORSUK, MAGDALENA GRYZIŃSKA, KRZYSZTOF OLSZEWSKI, KRZYSZTOF GRZYWNOWICZ, KORNEL KASPEREK

Proteases on the body surface of honeybee *Apis mellifera* L. in cage and beehive

Proteazy powierzchni ciała pszczół *Apis mellifera* L. w środowisku klatki i ula

Summary. The aim of the work was to determine the type and activity of body-surface proteases of bee workers kept in a natural habitat and in a cage. 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 isolated proteins were tested as follows: protein concentration assay by the Lowry method; proteolytic activity in relation to various substrates (gelatine, haemoglobin, ovoalbumin, albumin, cytochrome C, casein) by the modified Anson method; proteolytic activity in relation to diagnostic inhibitors of proteolytic enzymes (pepstatin A, PMSF, iodoacetamide, o-phenantrolin), using the Lee & Lin method; acidic, neutral and basic protase activity by means of the modified Anson method; and electrophoretic analysis of proteins in a polyacrylamide gel for protease detection with the Laemmli method. The concentration of hydrophobic proteins on the body surface of the bees was found to be higher than that of hydrophilic proteins. Both in the hive and in the cage, proteolytic activity was observed only in relation to gelatine. The proteolytic activity of the hive bees remained at a steady level during the five weeks, whereas that of the cage bees varied. The hive workers were found to have aspartic, serine, thiolic and metallic proteases. On the other hand, the cage bees had aspartic and serine proteases on their body surfaces.

Key words: proteases, body surface, *Apis mellifera*, cage, hive

INTRODUCTION

An important element of the external protective barrier of insects is the protein layer on their body surface. Its main function is to protect the organism from pathogen invasion. It has recently been found that many body-surface proteins in *Apis mellifera* are

active as proteases and protease inhibitors [Grzywnowicz *et al*. 2009]. Proteolytic enzymes (proteases) participate in intracellular protein digestion (inorganic proteolysis) and such biological processes as: zymogen activation, the release of hormones and physiologically active proteins from their precursors, translocation through membranes, protein compound ordering and receptor activation (organic proteolysis) [Barrett 1999]. The enzymes are present in the alimentary duct, haemolymph, moult liquid, venom and cuticle of bees [Barrett 1999, Evans *et al*. 2006, Grzywnowicz *et al*. 2009. Up to date, serine, cysteine, aspartic and metallic proteases have been found in bees.

Laboratory cage tests during which bees are kept in cages are increasingly more often used in bee breeding and biology research, as well as when testing medicines and toxic substances or in advanced biomedical tests [Paleolog *et al*. 2003, Schmickl and Crailsheim 2004]. The chief principle of cage tests involves an appropriate adjustment of the breeding conditions so that they do not impinge on the health, resistance and welfare of the insects [Ustawa… 1997]. In comparison with field assessments, the use of cage tests is an easier and cheaper solution that additionally makes it possible to eliminate enormous environmental variation, such as the effect of the season [Milne 1985]. However, the problem consists in the fact that some authors have found a positive correlation between apiary and laboratory results, while others have observed no such correlation, probably due to the differences between the cage and hive habitats [Milne 1985]. The cage habitat is also stressful to bees [Paleolog *et al*. 2003].

Bearing this in mind, it seems extremely interesting to verify the hypothesis that, in comparison with the natural hive habitat, the cage habitat also negatively affects the body-surface proteolytic activity of bees, including the level of their non-specific immunity.

MATERIAL AND METHODS

Two bee colonies were selected: one with yellow-hued bees and the other with dark-hued insects. Combs with emergent brood of the yellow bees were put into an incubator for 24 hours. Approximately 2000 one-day-old workers were obtained. Four hundred of them were placed in 10 cages, 40 insects in each, whereas 1500 yellow bees were introduced into a hive containing the dark bees in which the former were easily recognisable. The cages were stored in a conditioning chamber at 24–25°C and 60% humidity for 5 weeks. During that period, every 7 days the yellow bees were collected from the hive bottom and dead bees were gathered from the cage bottoms. The bees were frozen in germ-free bags at -8°C for 1–2 months. Each week, bees from all the ten cages were accumulated, mixed, and then divided into samples, altogether 40 after 5 weeks. The same procedure was applied in the case of the bees collected from the hive. 50 samples were obtained (10 samples × 10 insects × 5 weeks).

The samples were successively defrozen and initially rinsed in 10 ml distilled water for 20 seconds in order to remove impurities. The resultant solution was found to contain no proteins using the Lowry method as modified by Schacterle and Pollack [1973]. Therefore, the rinsings were discarded. Subsequently, the insects were put in test tubes, 10 ml distilled water were added and the bees were shaken/rinsed for 4 min. at 3400 rpm. After filtrating each of the samples through Miracloth, a solution was ob-

tained that mostly contained hydrophilic proteins. The solution was then divided into four aliquots, poured into four Eppendorf tubes and frozen in a refrigerator at -40°C. The procedure produced:

– 2 ml – a sample to determine protease and protease inhibitor activities,
– 2 ml – a sample for electrophoretic assays,
– 2 ml – a sample to determine anti-fungal and antibacterial activities,
– 2 ml – reserve.

The biological material left on Miracloth was again put in test tubes. This time, a 1% detergent solution (Triton X-100) was poured into distilled water (10 ml). The whole was shaken for 4 minutes at 3400 rpm and filtrated through Miracloth. As in the case of shaking/rinsing in distilled water, four samples were created, containing mostly hydrophobic proteins. This procedure provided altogether 320 samples of the cage bees (40 samples × 2 rinsings × 4 Eppendorfs) and 400 samples of the hive bees (50 samples × 2 rinsings × 4 Eppendorfs).

Next, the samples containing washed-out proteins were analysed biochemically as follows:

– quantitative total protein concentration assay using the Lowry method, as modified by Schacterle and Pollack [1973];

– testing proteolytic activity in relation to the substrates (gelatine, haemoglobin, ovoalbumin, albumin, cytochrome C, casein) by the modified Anson method [1938];

– determining proteolytic activity in relation to the diagnostic inhibitors of proteolytic enzymes (pepstatin A, PMSF, iodoacetamide, o-phenantroline) according tot the Lee and Lin method [1995];

– determining the activity of acidic, neutral and basic proteases by means of the modified Anson method [1938];

– electrophoretic analysis of proteins in a polyacrylamide gel for protease detection with the Laemmli method [1970].

The statistical calculations were carried out using the SAS software (SAS Institute User's Guide Version 6.11., 1996). The verification of statistical differences between the experimental factors was performed using ANOVA.

RESULTS

A significantly greater amount of cuticle surface proteins was washed out with Triton (Fig. 1) than with water. The fact shows the predominance of hydrophobic proteins in bee workers. In the third week, the protein concentration in both the hive and the cage bees was higher with both the solvents.

Proteolytic activity was observed only in relation to gelatine in the case of both the hive and the cage bees. The proteolytic activity of the hive bees remained at a steady level during the five weeks. On the other hand, it varied in the cage bees, with a noticeable rise at pH 7.0 and 11.2 from the 3rd to the 5th week (Tab. 1).

Table 1. Proteolytic activity As in relation to gelatine in the samples rinsed from the body surface of *A. mellifera* workers kept in the hive and the cages in an acidic (pH 2.4), neutral (pH 7.0) and basic (pH 11.2) environment during the consecutive weeks of the experiment (age-related)

Tabela 1. Aktywność proteolityczna As wobec żelatyny w próbkach wypłukanych z powierzchni ciała robotnic *A. mellifera* utrzymywanych w ulu i w klatkach w środowiskach kwaśnym (pH 2,4), obojętnym (pH 7,0) i zasadowym (pH 11,2) w kolejnych tygodniach doświadczenia (wieku pszczół)

Habitat Środowisko	Płukanie w Rinsed in	Tygodnie Weeks	pH 2.4	pH 7.0	pH 11.2
			$\bar{x}$ ±se	$\bar{x}$ ±se	$\bar{x}$ ±se
Beehive Ul	water wodzie	1	67.667 ±0.18	9.309 ±0.19	21.922 ±0.13
		2	68.614 ±0.06	9.153 ±0.01	22.052 ±0.05
		3	67.987 ±0.13	9.005 ±0.10	22.122 ±0.08
		4	67.254 ±0.69	8.545 ±0.65	22.031 ±0.07
		5	67.824 ±0.17	7.593 ±0.29	21.968 ±0.04
	triton tritonie	1	72.605 ±0.05	32.541 ±0.02	25.427 ±0.13
		2	71.630 ±0.16	32.594 ±0.30	25.089 ±0.10
		3	72.889 ±0.38	32.779 ±0.19	24.860 ±0.49
		4	72.205 ±0.19	32.679 ±0.06	25.206 ±0.13
		5	72.785 ±0.20	33.048 ±0.34	25.075 ±0.12
Cage Klatka	water wodzie	1	253.453 ±0.05	5.491 ±0.01	24.949 ±0.01
		2	104.720 ±0.03	24.645 ±0.02	18.745 ±0.04
		3	6.994 ±0.09	39.693 ±0.14	12.454 ±0.14
		4	39.959 ±2.01	163.583 ±0.53	64.532 ±0.21
		5	45.064 ±0.05	419.463 ±0.41	158.243 ±0.12
	triton tritonie	1	26.842 ±0.02	62.099 ±0.02	34.760 ±0.02
		2	28.712 ±0.07	0.000 ±0.00	12.845 ±0.03
		3	44.291 ±0.42	8.983 ±0.08	9.565 ±0.15
		4	71.593 ±0.06	0.000 ±0.00	15.699 ±0.12
		5	9.500 ±0.42	0.000 ±0.00	1.098 ±0.14

Aspartic, serine, thiolic and metallic proteases were found in the hive samples (activity in relation to pepstatin A, phenylmethylsulfone fluoride (PMSF), iodoacetamide and o-phenantroline; Tab. 2). The cage samples were found to contain aspartic and serine proteases (activity in relation to pepstatin A and phenylmethylsulfone fluoride – PMSF).

Summing up, the body-surface samples of *A. mellifera* workers kept in the hive were found to contain a greater variety of proteases with higher activities. On the other hand, the proteolytic activity in the cage samples underwent greater fluctuations as compared with the hive samples.

Few bands related with aspartic and serine proteases were observed in the zymographs of the hive samples, which may be ascribed to low *in vitro* protease concentration and stable proteolytic activity during the five weeks (Tab. 3). On the other hand, more bands were observed in the zymographs of the cage samples, probably corresponding with higher total protein concentration (Fig. 1).

Table 2. Mean proteolytic activity (for the 5 weeks) in relation to the diagnostic inhibitors washed out of the body surface of the bees kept in the hive and the cages
Tabela 2. Średnia (za okres 5 tygodni) aktywność proteolityczna wobec inhibitorów diagnostycznych wypłukanych z powierzchni ciała pszczół, które były utrzymywane w ulu i w klatkach

Inhibitor	pH	Hive – Ul		Cage – Klatka	
		$\bar{x}$	se	$\bar{x}$	se
Pepstatin A Pepstatyna A	2.4	34.671	0.01	21.628	0.32
	7.0	15.762	0.01	12.677	0.34
	11.2	93.566	0.59	15.762	0.20
PMSF	2.4	318.726	0.83	72.719	0.14
	7.0	187.717	1.02	32.878	0.38
	11.2	120.673	1.44	57.878	0.20
Iodoacetamide Jodoacetamid	2.4	81.626	0.07	0	0.08
	7.0	71.232	0.09	0	0.06
	11.2	32.625	0.11	0	0
o-phenantroline o-fenantrolina	2.4	30.626	0.16	0	0
	7.0	39.617	0.34	0	0
	11.2	67.173	0.47	0	0

Explanation: $\bar{x}$ – The arithmetic means for proteolytic activity in relation to the diagnostic inhibitors have been calculated from the values for the consecutive 5 five weeks; se – standard error. The intensively shaded values are significantly ($P < 0.05$) higher than those highlighted, separately presented in each line for ease of comparison

Objaśnienia: $\bar{x}$ – średnie arytmetyczne aktywności proteolitycznej wobec inhibitorów diagnostycznych liczone na podstawie wartości z 5 kolejnych tygodni; se – błąd standardowy. Wartości mocno zacieniowane są istotnie ($P < 0,05$) wyższe od tych oznaczonych jako jasne zaciemnienie, dla porównań w każdym wierszu oddzielnie

Table 3. Electrophoretic SDS-PAGE zymograph for the proteolytic activity of the hive and cage samples (body-surface rinsings) in an acidic (pH 2.4), neutral (pH 7.0) and basic (pH 11.2) environment
Tabela 3. Zymograf elektroforezy SDS-PAGE aktywności proteaz w środowisku kwaśnym (pH 2,4), obojętnym (pH 7,0) i zasadowym (pH 11,2) dla próbek wypłukanych z powierzchni ciała pszczół z ula i klatki

Week Tydzień	Cage – Klatka														
	1			2			3			4			5		
pH	2.4	7.0	11.2	2.4	7.0	11.2	2.4	7.0	11.2	2.4	7.0	11.2	2.4	7.0	11.2
HM	1	1	2	3	1	1	2	2	2	1	1	2	1	0	0
MM	1	0	0	3	0	1	0	0	1	0	1	0	0	0	0
LM	2	1	2	6	1	4	4	2	1	4	1	1	3	4	2
	Hive – Ul														
Week Tydzień	1			2			3			4			5		
pH	2.4	7.0	11.2	2.4	7.0	11.2	2.4	7.0	11.2	2.4	7.0	11.2	2.4	7.0	11.2
HM	0	1	0	4	2	1	0	1	1	1	1	2	0	1	1
MM	0	0	0	1	1	0	0	2	0	1	2	0	0	0	1
LM	3	1	1	1	1	1	3	0	1	2	1	2	1	1	3

HM – band number of high-molecular protein – ilość prążków białek wysokocząsteczkowych
MM – band number of medium-molecular protein – ilość prążków białek średniocząsteczkowych
LM – band number of low-molecular protein – ilość prążków białek niskocząsteczkowych

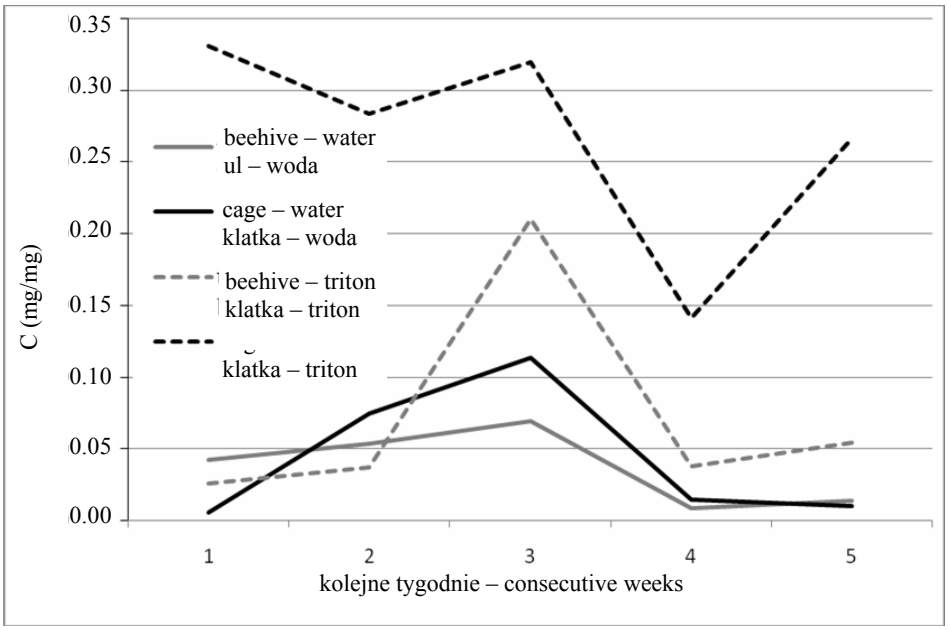

Fig. 1. Total protein concentration (C) in the samples washed out of the body surface of *A. mellifera* workers kept in the hive and the cages
Ryc. 1. Stężenie białka ogólnego (C) w próbkach wypłukanych z powierzchni ciała robotnic *A. mellifera* utrzymywanych w ulu i w klatkach

DISCUSSION

The authors observed a slightly higher activity of the proteolytic system of the hive bees than of the cage ones. Moreover, the proteolytic activity of 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 seemed to have difficulty maintaining a steady activity / balance / homoeostasis of their non-specific proteolytic resistance system.

Gliński *et al*. [2006] think that the cage environment is more propitious to pathogen development than the hive habitat. Additionally, cage bees are exposed to more stress due to a missing queen, incompleteness of the colony and flight limitation [Paleolog *et al*. 2003]. Stress, in turn, can affect hormone and ectohormone secretion [Wilde and Prabucki 2008]. The immune system of bees in a hive is assisted by the effect of the pollen, honey, propolis and royal jelly. Additionally, a pollen-rich diet stimulates protein production [Wilde and Prabucki 2008]. The mobility of bees in a cage is limited, with the concomitant pollen deficit.

Milne [1985] found that the hive and cage environments can differently affect the expression of many traits. This corresponds with the conclusion [Paleolog *et al*. 2003] that the results obtained in laboratory cage tests should be treated with caution in reference to the hive environment. The present research shows 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.

REFERENCES

Anson M., 1938. The estimation of pepsin, tripsin, papain and cathepsin with hemoglobin. J. Gen. Physiol. 22, 79–84.

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.

Gliński Z., Kostro K., Luft-Deptuła D., 2006. Choroby pszczół. PWRiL Warszawa, 37–43.

Grzywnowicz K., Ciołek A., 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. Apidolgie 40(1), 4–19.

Laemmli U., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

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.

Milne Ch. P., 1985. The need for using laboratory tests in breeding honeybees for improved honey production. J. Apic. Res. 24 (4), 237–242.

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. Annales UMCS, sec. EE, Zootechnica, 21, 2, 95–104.

SAS Institute, 1996. SAS/STAT User's Guide release 6.11, Cary, NC, Statistical Analysis System Institute.

Schacterle 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.

Ustawa z dnia 21 sierpnia 1997 r. o ochronie zwierząt. Dz.U. 97.111.724.

Wilde J., Prabucki J., 2008. Hodowla pszczół. PWRiL, Poznań, 1–496.

Streszczenie. Celem pracy było określenie rodzaju oraz aktywności proteaz wyizolowanych z powierzchni ciała robotnic utrzymywanych w naturalnym środowisku ula oraz w klatce. Próby pobierano przez pięć tygodni, otrzymując 40 prób z klatek i 50 prób z ula, w każdej po 10 sztuk pszczół. Z owadów wyizolowano białka hydrofilne (przy użyciu wody) oraz hydrofobowe (przy użyciu tritonu). Próbki z wypłukanymi białkami poddano następującym oznaczeniom: stężenie białka metodą Lowry'ego, aktywność proteolityczna wobec różnych substratów (żelatyny, hemoglobiny, owoalbuminy, albuminy, cytochromu C, kazeiny) wg zmodyfikowanej metody Ansona, aktywność proteolityczna wobec inhibitorów diagnostycznych enzymów proteolitycznych (pepstatyny A, PMSF, jodoacetamidu, o-fenantroliny) wg metody Lee i Lina, aktywności proteaz kwaśnych, obojętnych i zasadowych wg zmodyfikowanej metody Ansona, analiza elektroforetyczna białek w żelu poliakrylamidowym do wykrywania proteaz wg metody Laemmli'ego. Na powierzchni ciała pszczół zaobserwowano wyższe stężenia białek hydrofobowych niż hydrofilowych. Aktywność proteolityczną tak w ulu, jak i w klatce, wykazano tylko wobec żelatyny. U pszczół z ula aktywność proteaz w ciągu pięciu tygodni utrzymywała się na stałym poziomie, natomiast w klatkach podlegała wahaniom. U robotnic z ula stwierdzono obecność proteaz asparaginowych, serynowych, tiolowych i metalozależnych, a u pszczół w klatkach – proteaz asparaginowych i serynowych.

Słowa kluczowe: proteazy, powierzchnia ciała, *Apis mellifera*, klatka, ul