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Comparison of body-surface proteolytic activity of live and dead honey bee Apis mellifera workers

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Summary

The aim of the study was to determine the type and activity of body-surface proteases of live and dead workers. Samples were collected for five weeks. 100 samples of live and dead worker bees were gathered, respectively, each containing 10 bees. The total number of samples was 200. Hydrophilic (water-treated) and hydrophobic (Triton-rinsed) proteins were isolated from the insects. The rinsing 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 a 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 protease 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 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 a modified Felicioli method; and in vivo tests of antifungal and antibacterial activity using the double application method.

The concentration of hydrophobic proteins on the body surface of the bees was found to be higher than that of hydrophilic proteins. The activity of proteases and their inhibitors remained at a steady level in the dead bees during the five weeks, whereas that of the live bees was variable. The dead workers were found to have aspartic, serine, thiolic and metallic proteases on the body surface; the live bees, in turn, had aspartic and serine proteases. The dead bees were less resistant to microorganisms. The methods used in the present study can be employed for assessing the condition and state of health of bee colonies, both prior to and after wintering, as well as during the beekeeping season.

Keywords: proteolysis, cuticle, honeybee, immunity, live/dead organism

Proteolytic enzymes (proteases) participate in intracellular protein digestion 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 (2). Proteases and protease inhibitors are active in the alimentary duct, haemolymph, moult liquid, venom and cuticle of bees (2, 4, 7, 16). To date, serine, cysteine, aspartic and metallic proteases have been found in bees. The protein layer on their body surface is meant to protect the organism from pathogen invasion (7, 17). The pattern of proteolysis on the body surface of honeybees is influenced by such factors as: the developmental stage, caste, environmental pollution, chemioacaricides, cage/beehive habitat and even dietary supplements containing the low-molecular epigenetic switch (11, 15, 16, 17). Proteolysis primarily takes place in living organisms during metabolic processes. These processes stop in dead organisms and saprotrophs attack the body.

Therefore, it seems of extreme interest to investigate the hypothesis that the cessation of life functions in dead workers coincides with a cessation of their body-surface proteolytic activity in contrast to live honeybees.

Material and methods

Two bee colonies were selected: one with yellow-hued bees (originating from one drone) and the other comprising dark-hued insects. Combs with emergent broods of the yellow bees were put in an incubator for 24 hours. Approximately 3000 one-day-old workers were obtained. The yellow one-day-old bees were introduced into the colony of dark-hued insects. For 5 weeks, every 7 days, 20 samples with 10 yellow bees (dead) were collected from the hive bottom and 20 samples with 10 yellow bees (alive) from the combs. The bees were refrigerated in germ-free bags at –8°C for 1-2 months. 200 samples were obtained (20 samples × 10 insects × 5 weeks × 2 physiological states = 2000 bees).

The samples were then thawed and initially rinsed in 10 ml distilled water for 20 seconds in order to remove impurities. Using the Lowry method as modified by Schacterle and Pollack (14), the resultant solution was found to contain no proteins. Therefore, the rinsings were discarded. Subsequently, the insects were put in test tubes. 10 ml of distilled water was added and the bees were shaken/rinsed for 4 min. at 3400 rpm. After filtrating each of the samples through Miracloth, a solution was obtained that contained mostly 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 anti-bacterial activities;

– 2 ml – reserve.

The biological material left on Miracloth was again placed 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 only, four samples were created, containing predominantly hydrophobic proteins. This procedure provided a total of 800 samples for the live bees (100 samples × 2 rinsings × 4 Eppendorfs) and 800 samples for the dead bees.

Next, the rinsing 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 (14);

– testing proteolytic activity in relation to the substrates (gelatine, haemoglobin, ovoalbumin, albumin, cytochrome C, casein) by the modified Anson method (1);

– determining proteolytic activity in relation to the diagnostic inhibitors of proteolytic enzymes (pepstatin A, PMSF, iodoacetamide, o-phenantroline) according to the Lee and Lin method (9);

– determining the activity of acidic, neutral and basic proteases by means of the modified Anson method (1);

– electrophoretic analysis of proteins in a polyacrylamide gel for protease detection with the Laemmli method (8);

– determination of the levels of natural inhibitors of acidic, neutral and alkaline proteases, based on the Lee and Lin method (9);

– electrophoretic analysis of proteins on polyacrylamide gel in order to detect aspartic and serine protease inhibitors using the modified Felicioli et al. method (5);

– determination of antifungal and antibacterial activities in *in vivo* tests, using the double application method with:

• SABG (12) – to determine the activity in relation to *Aspergillus niger*,

• YPD (10) – to determine the activity in relation to *Candida albicans*,

• LB (3) – to determine the activity in relation to *Staphylococcus ureus*, *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 α100). The photographs were used to determine the area of the active anti-micro-organism surface on which there was no growth with Multi-Scan Base software.

The statistical calculations were carried out using SAS software (13). Statistical differences between the experimental factors were identified using one-way ANOVA.

Results and discussion

A greater amount of cuticle surface proteins was washed out with Triton (Tab. 1) than with water. The fact shows the predominance of hydrophobic proteins in worker bees. Protein concentrations of the dead workers rinsed in water were higher than those of the live bees in each week of the experiment (Tab. 1).

Proteolytic activity was observed only in relation to gelatine and ovoalbumin both in the case of the dead and the live bees. The proteolytic activity of the dead bees remained at a steady level during the five weeks. On the other hand, it varied in the live bees, with a noticeable rise at pH 2.4, 7.0 and 11.2 in the 2nd and the 3rd weeks (Tab. 1).

Aspartic and serine, although neither thiolic nor metallic, proteases were detected on the cuticles of the live bees, as proteolytic activity was identified in the case of pepstatin-A and phenylmethylsulfonyl fluoride (PMSF) but was not observed in relation to iodoacetamide or o-phenanthroline (diagnostic inhibitors of proteases). On the other hand, all these proteases were present in the dead bee samples.

More bands related with aspartic and serine proteases were observed in the zymographs of the live workers than the dead workers during the five weeks (Tab. 2). In the acidic environment, bands of medium-molecular proteins were not observed in the live bee rinsing samples during this time. The same pattern of bands at all the pH levels were observed in the electrophoretic SDS-PAGE zymographs for the live bees in the 3rd and 4th weeks of the experiment. It was these workers during the five weeks that revealed the most

Tab. 1. Protein concentration (C) and proteolytic activity (As) in the samples rinsed from the body surface of the live and dead *A. mellifera* workers 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) ($\bar{x} \pm SE$)

Physiological state	Washing	Week	C (mg/ml)	Proteolytic activity (As U × mg^{-1})		
				pH 2.4	pH 7.0	pH 11.2
Live*	Water**	1	0.091 ± 0.01	38.497 ± 0.32	52.459 ± 5.71	57.210 ± 0.18
		2	0.009 ± 0.00	321.706 ± 0.34	184.995 ± 0.50	369.663 ± 0.45
		3	0.008 ± 0.00	282.326 ± 0.20	659.563 ± 0.23	313.745 ± 0.04
		4	0.036 ± 0.00	65.448 ± 0.14	230.710 ± 0.24	79.148 ± 0.80
		5	0.002 ± 0.00	116.459 ± 0.15	572.763 ± 13.15	164.406 ± 0.25
	Triton**	1	0.293 ± 0.07	9.652 ± 0.47	1.304 ± 0.24	87.377 ± 0.20
		2	0.046 ± 0.00	65.096 ± 1.42	5.821 ± 0.08	75.631 ± 0.28
		3	0.054 ± 0.02	113.516 ± 1.22	3.338 ± 0.78	45.345 ± 2.14
		4	0.275 ± 0.01	13.271 ± 0.38	1.394 ± 0.19	11.460 ± 0.66
		5	0.125 ± 0.09	173.427 ± 0.20	55.317 ± 0.16	160.991 ± 0.48
Dead*	Water**	1	0.042 ± 0.01	68.667 ± 0.18	9.301 ± 0.19	21.992 ± 0.13
		2	0.054 ± 0.02	68.614 ± 0.06	9.253 ± 0.01	22.052 ± 0.05
		3	0.013 ± 0.00	68.987 ± 0.13	9.215 ± 0.10	22.122 ± 0.08
		4	0.008 ± 0.00	68.254 ± 0.69	9.345 ± 0.65	22.031 ± 0.07
		5	0.014 ± 0.00	68.824 ± 0.17	8.993 ± 0.29	21.998 ± 0.04
	Triton**	1	0.326 ± 0.00	72.615 ± 0.05	32.541 ± 0.02	25.227 ± 0.13
		2	0.137 ± 0.00	72.630 ± 0.16	32.594 ± 0.30	25.189 ± 0.10
		3	0.210 ± 0.07	72.789 ± 0.38	32.779 ± 0.19	24.960 ± 0.49
		4	0.338 ± 0.00	72.605 ± 0.19	32.679 ± 0.06	25.206 ± 0.13
		5	0.254 ± 0.02	72.785 ± 0.20	33.008 ± 0.34	25.175 ± 0.12

Explanations: * – denotes statistically significant differences in values of protein concentrations and proteolytic activities between physiological states at the level of $P \leq 0.05$; ** – denotes statistically significant differences in values of protein concentrations and proteolytic activities between washing within group live workers or dead workers at the level of $P \leq 0.05$

bands of low-molecular proteins whose molecular weights ranged from 14 to 31 kDa. In the case of the dead workers, these low-molecular proteins had molecular weights of about 15, 21 and 28 kDa.

There was considerable oscillation in the system of natural protease inhibitor activities in the live workers (Tab. 3). In a dozen or so cases the result was a complete loss of inhibitor activity (hydrophobic protease inhibitors at pH 2.4 and 11.2, and hydrophilic protease inhibitors at pH 7.0). This did not occur with the dead bees and protease inhibitor activities remained at a steady level in these workers during the five weeks.

The electrophorograms (Tab. 4) of both the live and dead bees revealed broad and prominent bands of high- and low-molecular aspartic protease inhibitors during all the weeks. Basic protease inhibitor bands in the live and dead bees were few, narrow, not very prominent and predominantly associated with low-molecular proteases. During some of the weeks, the live workers were found to contain no active *in vitro* natural inhibitors, while the electrophorograms featured bands which may have resulted from a reaction with the proteases/substrates and the specificity of protease inhibitor staining.

Tab. 2. Electrophoretic SDS-PAGE zymograph showing the body-surface proteolytic activity of the live and dead workers in an acidic (pH 2.4), neutral (pH 7.0) and basic (pH 11.2) environment

Live bees															
Week	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	4	1	3	3	1	2	3	1	2	3	1	2	3
MM	0	1	0	0	2	2	0	2	1	0	2	1	0	2	1
LM	1	5	4	5	5	5	4	6	3	4	5	3	5	5	4
Dead bees															
Week	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	1	1	2	1	2	1	1	3

Explanations: HM – number of high-molecular protein bands; MM – number of medium-molecular protein bands; LM – number of low-molecular protein bands

Tab. 3. Activity of aspartic and serine protease inhibitors (As U × mg^{-1}) in the washed-out body-surface samples of the live and dead *A. mellifera* hive workers ($\bar{x}$ ± SE)

Physiological state	Washing	Week	pH 2.4	pH 7.0	pH 11.2
Live*	Water**	1	26.435 ± 0.15	19.553 ± 0.08	0.000 ± 0.00
		2	106.632 ± 0.23	99.128 ± 0.11	116.657 ± 7.70
		3	152.643 ± 0.18	0.000 ± 0.00	197.583 ± 0.02
		4	25.294 ± 0.17	0.000 ± 0.00	34.793 ± 1.10
		5	411.832 ± 0.69	0.000 ± 0.00	488.130 ± 2.39
	Triton**	1	24.585 ± 0.65	0.000 ± 0.00	31.525 ± 0.03
		2	0.000 ± 0.00	31.957 ± 0.97	32.207 ± 0.28
		3	0.000 ± 0.00	43.278 ± 0.14	0.000 ± 0.00
		4	0.000 ± 0.00	4.938 ± 0.23	0.000 ± 0.00
		5	0.000 ± 0.00	44.515 ± 0.15	0.000 ± 0.00
Dead*	Water**	1	82.556 ± 0.04	19.121 ± 0.03	16.783 ± 0.16
		2	83.195 ± 0.11	19.111 ± 0.02	16.813 ± 0.14
		3	83.125 ± 0.13	18.978 ± 0.05	16.825 ± 0.13
		4	82.777 ± 0.37	19.124 ± 0.09	16.772 ± 0.12
		5	82.686 ± 0.29	19.133 ± 0.10	16.791 ± 0.15
	Triton**	1	97.399 ± 0.03	46.421 ± 0.12	42.612 ± 0.11
		2	97.411 ± 0.21	46.551 ± 0.13	42.711 ± 0.16
		3	97.567 ± 0.31	46.415 ± 0.15	42.635 ± 0.17
		4	97.521 ± 0.21	46.537 ± 0.20	42.587 ± 0.12
		5	97.611 ± 0.32	46.459 ± 0.14	42.599 ± 0.14

Explanations: as in Tab. 1

The live bees were observed to have antifungal protection against two fungi and antibacterial protection for four bacteria (Tab. 5). The dead bees were not resistant to microorganisms. Additionally, the survival test revealed that the samples washed out of the body surface of the live bees had the capacity to destroy the *B. subtilis* strain. 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 dead bees. Reduced activity was also observed in the *in vitro* biochemical analyses.

The authors observed higher activity of proteases and protease inhibitors in the live bees as compared with the dead ones. Moreover, the proteolytic system activity of the dead bees remained at a relatively stable level during the consecutive weeks of the experiment, while that of the live bees varied with time. The microbiological tests also revealed a narrower range of antifungal and antibacterial activity in the dead workers.

When enzyme activity stops, life stops and the organism dies. All metabolic processes are controlled by proteolytic enzymes. In the dead honeybees enzyme activity stopped at a stable level (Tab. 1 and 3). After some days or weeks, dead organisms are decomposed by pathogens and saprotrophs. At that time, proteolysis in these bodies is induced by enzymes secreted by the saprotrophs (6). The results published in the present study were obtained for workers that had been collected directly after their death from the hive bottom before their bodies had been decomposed. A cessation in the activity of the proteolytic system at a stable level also causes the apian non-specific body-surface immune system to stop and lose activity, which was confirmed by the results of the microbiological tests (Tab. 5).

Tab. 4. Electrophoretic SDS-PAGE zymograph showing the body-surface protease inhibitor activities of the live and dead workers in an acidic (pH 2.4), neutral (pH 7.0) and basic (pH 11.2) environment

Live bees															
Week	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	1	0	2	1	4	2	1	4	2	1	4	1	1
MM	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LM	1	0	0	2	0	0	2	0	0	2	1	0	3	1	0
Dead bees															
Week	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	2	1	0	2	1	1	2	1	1	2	1	0	3	1	2
MM	0	0	0	2	0	0	1	0	0	2	0	0	0	0	0
LM	5	0	2	3	0	1	5	0	2	4	0	2	5	0	0

Explanations: as in Tab. 2

Tab. 5. The antifungal and antibacterial activities in the washed-out body-surface samples of the live and dead *A. mellifera* workers, calculated as the area (mm²) without pathogen development

Physiological state	Area, mm²					
	with antifungal activity		with antibacterial activity			
	Candida albicans	*Aspergillus niger*	*Bacillus subtilis*	*Staphylococcus aureus*	*Salmonella Typhimurium*	*Pseudomonas aeruginosa*
Live	159.62	10.43	188.28	118.91	263.87	214.79
Dead	0	0	0	0	0	0

What was surprising was the variation in the proteolytic system of the dead insects (aspartic, serine, thiolic and metallic proteases), whereas the living hive bees only had aspartic and serine proteases on their body surfaces. Thiolic and metallic proteases are induced in workers by the stress resulting from untoward environmental conditions (i.e. acaricides, environmental pollution etc.) (15, 17). Bees may also undergo certain metabolic changes towards the end of their lifetime which activate those enzymes.

Activation/deactivation of the proteolytic system depends on the environment in which bees exist. The activity of the system in the dead hive bees stopped at a stable steady level. On the other hand, as indicated in a previous study by the authors, the activity changed on the body surface of dead cage bees during five weeks of the experiment (17, 18). The cage environment is stressful to bees and can affect body-surface proteolytic system activity of workers. Additionally, it is propitious to pathogen development. Most probably, part of the enzymes may have originated from pathogens. Hence, the conclusion is that living bees should be collected for biochemical analyses, preferably from the hive (not the cage), to be refrigerated forthwith at –24°C, pending the analyses.

The immune system of live bees is assisted by the effect of the pollen, honey, propolis and royal jelly. Additionally, a pollen-rich diet stimulates protein production (6). Oscillation in protease and protease inhibitor activities also results from changing weather conditions (7, 19).

Conclusion

Enzyme activity results for dead and live bees should be viewed and compared with caution. Bees intended for biochemical analyses should be collected live and immediately refrigerated at the lowest possible temperature to preserve actual enzyme activity and avoid possible environmental and/or pathogenic interference.

The methods used in the present study can be employed for assessing the condition and state of health of bee colonies, both prior to and after wintering, as well as during the beekeeping season. In a majority of cases such assessments are performed by means of visual monitoring. The paper presents additional instruments that can be taken advantage of both by scientists and professional beekeepers. The metalloprotease activity assay can be used as a marker and bee welfare bioindicator on its own.

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