THE SURFACE PROTEOLYTIC ACTIVITY IN *Apis mellifera*

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Summary

Proteolytic processes on the body surface of insects have barely been researched. In this work the body surface proteolytic activity was examined in different castes (queens, drones and workers). It was examined at various developmental stages (eggs, larvae, pupae and imagoes) in honey bees in spring, summer, autumn and winter. The following methods were used: protein content testing by the Lowry method (modified by Schacterle – Pollack) and protease activity testing by the Anson method. Our studies revealed a high activity of proteases for the acidic (pH = 2.4), neutral (pH = 7) and alkaline (pH = 11.2) pH. The highest protein concentration values were found in the summer (in living drone pupae – 0.7414 mg/ml; living mature queens – 0.4701 mg/ml; worker larvae – 0.8683 mg/ml). The lowest protein concentration values were in the spring (in mature workers – 0.0015 mg/ml; dead drone pupae – 0.0272 mg/ml). The worker, queen and drone eggs were found to have high protein concentrations in the summer. These high protein concentrations amounted to 0.6264 mg/ml, 0.4001 mg/ml, 0.5793 mg/ml, respectively. However, they had very low proteolytic activity. High proteolytic activity was observed in the drones in the spring and in the workers in the summer. The lowest proteolytic activity was observed on the body surface of the queens. The larvae and pupae were found to have higher proteolytic activity than the imagines. Dead insects had higher proteolytic activity than living organisms. The obtained results revealed the initial pattern of proteolysis on the body surface of honey bees. The obtained results also confirm the validity for doing further biochemical research on body surface proteolytic activity in these insects.

Keywords: surface proteolysis, proteases, castes, honey bee.

INTRODUCTION

The disintegration and synthesis of proteins are vital processes in living organisms. Protective barriers exist on the body surface of all living organisms in the form of the proteolytic system. This system safeguards against infections and helps to maintain the physiological homeostasis (Brownless and Williams 1993, North 1982). Such a barrier, in the shape of proteases and a protease inhibitors system is also present in honey bees.

The presence of proteases has also been confirmed in the molt liquid, the alimentary duct and hemolymph. The proteolytic enzymes (peptidases, proteases, proteinases) belong to the hydrolase group. This is the hydrolase group which catalyzes the hydrolytic disintegration of the peptide bond. It is also the hydrolase group which takes part in such biological processes as: zymogen activation, the releasing of hormones and physiologically active proteins from their precursors, translocation through membranes, allocation of protein compounds and activation of receptors (Walter and Clélia 1994). The proteolytic system of insects is connected with immunological mechanisms as well (Brownless and Williams 1993).

Proteolytic processes on the body surface of insects, particularly in bees, are barely researched. That is why only a few papers on the surface proteolysis in bees were published (Bania and Polanowski 1999, Pliszczyński et al. 2006a, Pliszczyński 2006b). The body surface proteases in cockroaches were described by Wünschmann et al. (2005) and Page et al. (2005). Preliminary studies were done on aminopeptidase activity in scab mites (*Psoroptes spp*.). Those studied showed that the highest aminopeptidase activity was associated with the soluble fraction obtained from the body surface and the digestive system of *P. cuniculi*. These aminopeptidases performed an important function in the immunological and chemical control strategies (Nisbet and Billingsley 2002).

The goal of the present research was to examine the surface proteolytic activity in *Apis mellifera* in the different castes, at the various developmental stages and at various times of the year.

MATERIAL AND METHODS

We did our research during two seasons in 2005 – 2007. Twenty-two types of the biological material, including eggs, larvae, pupae and imagines (queens, drones and

Table 1

The biological material sample's database.

A sample type	Total number of the collected entities	The sampling pattern for the biochemical analysis
worker eggs	100	3 samples x 10 entities
worker larvae	100	3 samples x 10 entities
1-3-day old worker larvae	100	3 samples x 10 entities
4-6-day old worker larvae	100	3 samples x 10 entities
worker pupae	100	3 samples x 10 entities
mature workers	300	3 samples x 7 entities
living mature workers	300	3 samples x 7 entities
dead mature workers	300	3 samples x 7 entities
queen eggs	40	3 samples x 10 entities
queen larvae	40	3 samples x 10 entities
queen pupae	50	3 samples x 10 entities
mature queens	70	3 samples x 7 entities
living mature queens	70	3 samples x 7 entities
dead mature queens	70	3 samples x 7 entities
drone eggs	40	3 samples x 10 entities
1-3-day old drone larvae	70	3 samples x 10 entities
4-7-day old drone larvae	70	3 samples x 10 entities
living drone pupae	70	3 samples x 10 entities
dead drone pupae	40	3 samples x 10 entities
mature drones	40	3 samples x 7 entities
living mature drones	40	3 samples x 7 entities
dead mature drones	70	3 samples x 7 entities

workers) were collected in the following periods: September/October, April/May and July/August (Table 1). The material was frozen (-8°C) immediately after taking it from a hive. Next, after being defrosted, 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. Afterwards, the samples were placed again in test-tubes. They were shaken in the test-tubes for three minutes in distilled water (for neutral and alkaline proteases) and then in a 1% detergent solution (Triton X-100, Serva; for acidic proteases). This was done in order to wash out the body surface proteins. Next, the washings were frozen in Eppendorf test-tubes at a temperature of -20°C. On defrosting the samples again, they were assigned an optimal pH. They were tested for surface protein content using the Lowry method modified by Schacterle – Pollack (Schacterle and Pollack 1973). Then they were tested for acidic, neutral and alkaline protease activity according to the Anson method (Anson 1938), used in enzymology to determine the activity level of such proteins.

RESULTS AND DISCUSSION

At the beginning of the experiment, pH profiles of the samples were determined The optimal pH values were selected in which the body surface enzymes revealed the highest proteolytic activity in *Apis mellifera*. It was found that proteases displayed high activity for acidic (pH=2.4), neutral (pH=7) and alkaline (pH=11.2) pH (Fig.1).

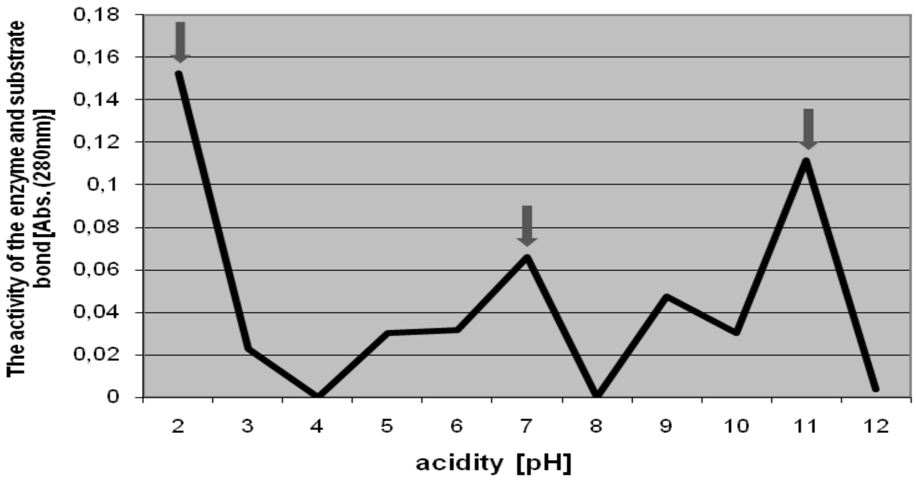

Fig.1 The proteolytic activity on the body surface of *Apis mellifera* reflects then activity of the enzyme and substrate bond.

The surface protein concentration

The body surface protein concentration (BSPC) of the drones, queens and workers were compared over the different seasons (Table 2). It was found that the highest BSPC values were noted in the summer in the drones, queens and workers. The BSPC values decreased in autumn. The proteins were almost completely „used up“ over winter. This was confirmed especially by the analysis of samples originating from the beginning of the vegetative period. The spring BSPC values were visibly higher in the drones than in the queens and workers.

This could be connected with the mating activity during that period.

Among the drones the highest BSPC values were found in the pupae in the summer (0.7414 mg/ml). In June high BSPC values were noted in the samples of eggs (0.5794 mg/ml), 4-7-days larvae (0.5556 mg/ml), 1-3-days larvae (0.5118 mg/ml) and imagines (0.4916 mg/ml) of the drones. The lowest BSPC values were observed in the dead drone pupae in the spring (0.0272 mg/ml).

In the queens, the highest BSPC values were found in mature queens in the summer. Slightly lower BSPC values were found in the queen larvae and queen eggs at this same time. In the autumn the BSPC values decreased. BSPC values were the lowest in the pupae samples in April and May (0.0702 mg/ml).

Table 2

The seasonal honey bee body surface protein concentration measured during different developmental stages in drones, queens and workers

Samples	Protein concentration (mg/ml) in:					
	spring		summer		autumn	
	mean	± se	mean	± se	mean	± se
drone eggs	n.a.	-	0,579	0,06	n.a.	-
1-3-day old drone larvae	0,072	0,02	0,511	0,05	n.a.	-
4-7-day old drone larvae	0,064	0,005	0,555	0,009	n.a.	-
living drone pupae	0,196	0,008	0,741	0,006	n.a.	-
dead drone pupae	0,027	0,006	n.a.	-	n.a.	-
mature drones	n.a.	-	0,491	0,008	n.a.	-
living mature drones	n.a.	-	0,159	0,009	n.a.	-
dead mature drones	n.a.	-	0,240	0,009	0,325	0,009
queen eggs	n.a.	-	0,400	0,005	n.a.	-
queen larvae	n.a.	-	0,437	0,009	n.a.	-
queen pupae	0,070	0,03	0,190	0,009	n.a.	-
mature queens	n.a.	-	0,466	0,006	n.a.	-
living mature queens	n.a.	-	0,470	0,009	0,236	0,009
dead mature queens	n.a.	-	0,301	0,008	0,273	0,008
worker eggs	n.a.	-	0,626	0,009	0,462	0,08
worker larvae	n.a.	-	0,868	0,008	0,421	0,008
1-3-day old worker larvae	0,021	0,008	0,642	0,006	0,442	0,009
4-6-day old worker larvae	0,014	0,009	0,551	0,008	0,346	0,008
worker pupae	0,017	0,008	0,538	0,009	0,235	0,006
mature workers	0,002	0,003	0,520	0,009	0,028	0,008
living mature workers	n.a.	-	0,365	0,009	0,238	0,009
dead mature workers	n.a.	-	0,142	0,008	0,244	0,009

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

In the summer, the workers of all the developmental stages showed high BSPC values. These high BSPC values in summer, were the highest in the larvae (0.8683 mg/ml). In the autumn the BSPC values slightly decreased, until they were the lowest in the spring.

Additionally, the BSPC values varied between the developmental stages. Compared to the mature forms, higher BSPC values were found in the larvae and pupae. Such results could be connected with numerous mitotic processes and an increased protein biosynthesis at these stages. In the molting period the body surface of insects is not immunologically protected, therefore, they produce proteins and proteases. Generally, the BSPC values dwindled with the developmental process (larvae – pupae – imagines) in the bees. This may be the reason for the changing metabolism (Andersen et al. 1981, Merzendorfer and Zimoch 2003, Ciołek 2006).

The surface proteolytic activity

Very high proteolytic activity (PA) on the body surface was observed in the spring drones (acidic proteases). This high activity was observed in the queens also in the spring (neutral and alkaline proteases). Such high activity of proteases in those castes might be explained by their mating activity at that time. Drones have protective barriers on their body surface. These protective barriers prevent the queen from being infected during the mating flight and copulation (Bania and Polanowski 1999, Barrett 1999). Moreover, before the mating flight drones consume large quantities of food. This food is rich in nutrients (proteins, carbohydrates, mineral elements and vitamins), which might increase PA (Gliński et al. 2006).

At the beginning of the vegetative season the workers did not have the proteolytic enzyme protection on their body surface. This is related to the low temperature of the environment. The result is that the energy expenditure of the bee colony rises in order to warm up the winter cluster. This energy expenditure then leads to an accelerated exhaustion of the bees. The weakened insects are more susceptible to pathogen infection. This is the result of the destabilized immunological barrier, mainly the anatomic and physiological structures of the body surface (Pliszczyński et al. 2006a, Pliszczyński et al. 2006b, Twaróg et al. 2008). Furthermore, in winter the evacuation of excrement is stopped. Excrement is stored in the final section of the intestine (rectum) causing an enlargement of the abdomen. This consequently changes the metabolism of the insects. Only in spring and summer did the workers in our experiment start to rebuild, as it were, their protective barrier in the shape of the surface proteolytic activity.

During their six-week lifetime, workers perform increasingly complicated activities. These activities range from cleaning old cells, feeding larvae, picking up and storing pollen, cleaning the other bees, and acting as guards, to collecting pollen. During those activities workers require protease protection from an enthomopathogen attack (Bania and Polanowski 1999). Workers which collect pollen and nectar have adapted to the toxic components of the food produced by plants. This forms one of the fundamental theories of coevolution. The honey bee has responded to the untoward circumstances by the detoxification of the substances. It has also responded by forming appropriate protective barriers in the shape of surface proteolysis (Harborne 1997).

The larvae and pupae were found to have higher PA than the imagoes. This may be because of the numerous mitotic processes and an increased protein biosynthesis at the time. It may also be

Table 3

Seasonal proteolytic activity in queens, drones and workers.

samples	spring						summer						autumn					
	pH=2,4	±se	pH=7	±se	pH=11,2	±se	pH=2,4	±se	pH=7	±se	pH=11,2	±se	pH=2,4	±se	pH=7	±se	pH=11,2	±se
drone eggs	n.a.	-	n.a.	-	n.a.	-	0,044	0,008	0	0,001	0	0,001	n.a.	-	n.a.	-	n.a.	-
1-3-day old drone larvae	1,716	0,009	0	0,001	0,157	0,006	0,231	0,009	0	0,002	0	0	n.a.	-	n.a.	-	n.a.	-
4-7-day old drone larvae	2,797	0,009	0,302	0,009	0	0,002	0,133	0,009	0	0,001	0	0,003	n.a.	-	n.a.	-	n.a.	-
living drone pupae	4,873	0,008	0,107	0,008	0,128	0,009	0,681	0,009	0	0	0	0,002	n.a.	-	n.a.	-	n.a.	-
dead drone pupae	7,204	0,009	0,245	0,009	0,346	0,009	n.a.	-	n.a.	-	n.a.	-	n.a.	-	n.a.	-	n.a.	-
mature drones	n.a.	-	n.a.	-	n.a.	-	0,329	0,006	0,938	0,008	1,248	0,009	n.a.	-	n.a.	-	n.a.	-
living mature drones	n.a.	-	n.a.	-	n.a.	-	0,022	0,008	0	-	0	0,001	n.a.	-	n.a.	-	n.a.	-
dead mature drones	n.a.	-	n.a.	-	n.a.	-	0	0,001	0	-	0	0,001	0	0,001	0	0,002	0	0,001
queen eggs	n.a.	-	n.a.	-	n.a.	-	0,014	0,006	0	0	0	0,002	n.a.	-	n.a.	-	n.a.	-
queen larvae	n.a.	-	n.a.	-	n.a.	-	0,019	0,009	0	0,001	0,047	0,006	n.a.	-	n.a.	-	n.a.	-
queen pupae	0,6493	0,008	3,546	0,009	4,068	0,009	0	0,001	0,144	0,008	0	0,003	n.a.	-	n.a.	-	n.a.	-
mature queens	n.a.	-	n.a.	-	n.a.	-	0	0,001	0	0	0	0,001	n.a.	-	n.a.	-	n.a.	-
living mature queens	n.a.	-	n.a.	-	n.a.	-	n.a.	-	n.a.	-	n.a.	-	0	0,001	0,385	0,009	0	0,002
dead mature queens	n.a.	-	n.a.	-	n.a.	-	n.a.	-	n.a.	-	n.a.	-	0	0,002	0	0,001	0	0,001
worker eggs	n.a.	-	n.a.	-	n.a.	-	0,019	0,008	0	0	0	0	0,199	0,006	0	0,002	0	0,002
worker larvae	n.a.	-	n.a.	-	n.a.	-	0,463	0,009	2,559	0,009	1,672	0,009	0,109	0,009	0	0,002	0	0,001
1-3-day old worker larvae	0	0,001	0	0,002	0,122	0,006	0,598	0,009	1,7751	0,009	0,875	0,009	n.a.	-	n.a.	-	n.a.	-
4-6-day old worker larvae	0	0,002	0	0	0	0,001	1,48	0,009	2,6588	0,009	2,178	0,008	n.a.	-	n.a.	-	n.a.	-
worker pupae	0	0	0	0,001	0	0	1,33	0,006	1,3873	0,007	1,403	0,006	n.a.	-	n.a.	-	n.a.	-
mature workers	0	0,002	0	0,001	0	0,002	0	0,001	0,4171	0,005	0	0,002	0,034	0,009	0,338	0,009	0	0
living mature workers	n.a.	-	n.a.	-	n.a.	-	0	0	0,1561	0,009	0,04	0,009	0,076	0,005	0,256	0,008	0	0,001
dead mature workers	n.a.	-	n.a.	-	n.a.	-	0	0,001	0,2651	0,009	0	0,001	0	0,003	0,158	0,006	0	0,003

The non-analyzed samples (n.a.) are due to the unavailability of specific forms at a given time food. Pollen is richer in amino acids than nectar which is mainly consumed by imagines (Billingsley 1999, Strachecka and Grzywnowicz 2008). With the ageing process the surface protease activity in all these insects began to diminish.

What deserves particular attention is the higher alkaline and neutral protease activity in the spring queen pupae than in the drone and worker pupae in the same period. It is suspected that this fact is connected with the fast metabolism and the crucial position held by the queen in the bee colony. A queen does not leave the hive for its whole life. It flies out of the nest only during mating flights. During the mating flight(s) it collects enough semen to be used for its whole lifetime. After such a flight it starts laying eggs in the brood comb cells which are looked after by the workers (Bania and Polanowski 1999). Our studies show that the queen imago is not exposed to an excessive contact with pathogens. It retains on its body surface an intact weak protective barrier. This protective barrier is in the form of proteases which are more active in the imagines of the workers and drones.

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POWIERZCHNIOWA AKTYWNOŚĆ PROTEOLITYCZNA *Apis mellifera*

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Streszczenie

Procesy proteolizy na powierzchni ciała owadów są słabo poznane. W tej pracy porównywano aktywności proteaz w kastach (matki, trutnie, robotnice), w różnych okresach rozwoju (jaja, larwy, poczwarki i postacie dorosłe) i w różnych porach roku (wiosna, lato, jesień, zima) u pszczoły miodnej. Zastosowano następujące metody: oznaczanie zawartości białek metodą Lowry'ego (zmodyfikowaną przez Schacterle – Pollack'a) i oznaczanie aktywności proteaz metodą Anson'a. Badania wykazały wysoką aktywność proteaz w pH kwaśnym (pH=2,4), obojętnym (pH=7) i zasadowym (pH=11,2). Najwyższe wartości stężenia białek zaobserwowano w lecie (u żywych poczwarek trutni – 0,7414 mg/ml; żywych dorosłych matek – 0,4701 mg/ml; larw robotnic – 0,8683 mg/ml) a najniższe na wiosnę (u dorosłych robotnic – 0,0015 mg/ml; martwych poczwarek trutni – 0,0272 mg/ml). Na jajach robotnic, matek i trutni zaobserwowano wysokie wartości stężenia białek w okresie lata i wynosiły one odpowiednio: 0,6264 mg/ml, 0,4001 mg/ml, 0,5793 mg/ml. Niemniej jednak miały one bardzo niską aktywność proteolityczną. Wysokie aktywności proteolityczne zaobserwowano u trutni w okresie wiosny i u robotnic w okresie lata. Najniższe aktywności proteolityczne występowały na powierzchni ciała matek. Larwy i poczwarki wykazywały wyższe aktywności proteolityczne niż postacie dorosłe. Martwe osobniki miały wyższą aktywność proteolityczną w porównaniu z żywymi postaciami. Uzyskane wyniki przedstawiają początkowy obraz proteolizy na powierzchni ciała pszczół i utwierdzają w słuszności prowadzenia dalszych badań biochemicznych nad proteolizą u tych owadów.

Słowa kluczowe: proteoliza powierzchniowa, proteazy, kasty, pszczoła miodna.