Profile of the body surface proteolytic system in *Apis mellifera* queens

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ABSTRACT: The proteolytic system on the body surface of the honey bee has been insufficiently researched. In this study the body surface proteolytic activity was examined in queens at various developmental stages (eggs, larvae, pupae and imagines) in different seasons (spring, summer, autumn, winter). Extracts of the body surface material with water and detergent were used for an *in vitro* analysis of the proteolytic activity and protease inhibitor level assaying, as well as for an electrophoretic separation of the extracts in polyacrylamide gels. The following methods were used: protein content testing by the Lowry method (modified by Schacterle-Pollack), protease activity testing by the Anson method and protease inhibitor activity testing by the Lee and Lin method. Our studies revealed a high protease activity in an acidic environment (pH = 2.4; the material rinsed with detergent), as well as in neutral (pH = 7) and alkaline (pH = 11.2) environments (the material rinsed with water in both cases). The highest protein concentration values were observed in the imagines from summer. The lowest activities of the proteases and protease inhibitors were determined in the eggs from summer. The highest activities of the acidic, neutral and alkaline proteases were observed in the pupae from spring. The highest number of protease activity bands in PAGE zymography was obtained for the neutral and alkaline activities in the queens for all the seasons. In the queens all the catalytic protease types were present: asparagine and cysteine proteases at pH = 2.4; cysteine proteases and metalloproteases at pH = 7 and serine proteases at pH = 11.2. These results were crucial for the analysis of immunity mechanisms on the body surface of the honey bee.

Keywords: proteolytic system; *Apis mellifera*; queens

A proteolytic system exists on the body surface of all living organisms. Such a system constituted by proteases and protease inhibitors is also present in the honey bee. The proteolytic enzymes catalyze the hydrolytic disintegration of the peptide bond and take part in such biological processes as: zymogen activation, translocation through membranes, the releasing of hormones and physiologically active proteins from their precursors, as well as in the allocation of protein compounds and the activation of receptors (Walter and Clélia, 1994; Barrett, 1999; Bode et al., 1999; Otlewski et al., 2001).

The proteolytic system as part of the immune system is a safeguard against infections and helps to maintain physiological homeostasis (North, 1982; Brownless and Williams, 1993). The insect immune system has many parallels with the innate immune response of vertebrates (Izadpanah and Gallo, 2005). The enzymatic degradation of pathogenic bacteria, fungi and parasitic mites also involves the proteo-

lytic enzymes present not only in the haemolymph, the moult liquid and the alimentary duct but also on the body surface (Walter and Clélia, 1994; Evans et al., 2006). The body surface proteolytic system can be expected to play an important role in improving the insect health (Grzywnowicz and Staniec, 2008).

The proteolytic system on the body surface of the honey bee has been explored scantily. That is why only a few papers on the surface proteolysis in bees were published (Bania and Polanowski, 1999; Pliszczyński et al., 2006a,b). Only a limited number of proteases and protease inhibitors were identified on the body surface of various organisms, e.g. cockroaches (Cornette et al., 2002; Page et al., 2005; Wünschman et al., 2005), scab mites (*Psoroptes* spp.) (Nisbet and Billingsley, 2002), amphibians (Zhao et al., 2005) and humans (Zeeuwen, 2004; Tobin, 2006). The proteolytic system of the honey bee body surface was analyzed and described in our laboratory, but these were preliminary studies (Strachecka et al., 2008; Strachecka and Grzywnowicz, 2008). The results we obtained suggested that we should continue exploring this subject. Therefore, the aim of this study is to describe proteases and protease inhibitor activities *in vitro* during the ontogenesis of honey bee (*Apis mellifera*) queens, taking into account the season factor.

MATERIAL AND METHODS

Samples

We conducted our research in 2005–2007. The materials for the experiments were eggs (100 samples), larvae (100 samples), pupae (100 samples) and imagines (80 samples) of the queens that were collected in spring (May), summer (July), autumn (October) and winter (January). The material was frozen (–8°C) immediately after taking it from a hive. Next, after being defrosted, the samples for biochemical analyses were taken threefold from each set of the biological material. After that the samples were placed on Miracloth, rinsed with distilled water and the polluted refuse was discarded (previously no proteins, proteases and protease inhibitors were detected in that solution). Afterwards, the samples were placed into test-tubes again and shaken for three minutes in distilled water (in the case of neutral and alkaline proteases) and then into a 1% detergent solution (Triton X-100, Serva; in the case of acidic proteases) in order to wash out the body surface proteins. Next, the washings were frozen in Eppendorf test-tubes at the temperature of –20°C.

Determination of protein content

On defrosting the samples again they were tested for the surface protein concentration using the Lowry method modified by Schacterle and Pollack (1973), with bovine serum albumin as the standard.

Protease activity assaying

The samples were tested for the activity of acidic, neutral and alkaline proteases according to the Anson method (Anson, 1938). In most cases the proteolytic activity was assayed on gelatin in three buffers: in 100mM glycine – HCl at pH 2.4, in 100mM Tris – HCl at pH 7, and in 100mM glycine – NaOH at pH 11.2. Next, 0.2 ml of each sample was incubated with 0.5 ml of 1% gelatin in an appropriate buffer for 60 min at 37°C. The reactions were ended by adding 2 ml of cold 5% trichloroacetic acid; the undigested proteins were precipitated and centrifuged and the supernatant was analysed spectrophotometrically at the absorbance of 280 nm. One unit of enzyme activity was defined as the amount of enzyme producing a 0.001 increase in absorbance per minute (according to Anson).

Assaying protease inhibitor activity

The samples were tested for protease inhibitor activity according to the Lee and Lin method (Lee and Lin, 1995), which is used in enzymology to determine the activity level of such proteins and present catalytic types of proteases. In the determination of general levels of protease inhibitor activities pepsin was used as a protease marker at acidic pH and trypsin as a protease marker at neutral and alkaline pH. 0.1 ml of pepsin or trypsin was preincubated with 0.1 ml of a given sample for 30 min. After this time 0.5 ml of 1% gelatin in an appropriate buffer were added and the incubation was continued for 60 min. The reactions were ended by adding 1 ml of TCA and the supernatants were measured as described above. The inhibitor levels were calculated according to Lee and Lin (1995).

Table 1. The seasonal body surface protein concentration, protease activities and protease inhibitor activities in queens at different developmental stages

Season	Samples	Protein concentration (mg/ml)	pH = 2.4		pH = 7		pH = 11.2	
			protease activities (U/mg)	protease inhibitor activities (U/mg)	protease activities (U/mg)	protease inhibitor activities (U/mg)	protease activities (U/mg)	protease inhibitor activities (U/mg)
Spring	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	0.071	0.651	0.4331	3.512	0.634	4.121	0.021
	imagines	0.169	0.084	0.923	0.612	1.598	0.023	1.836
Summer	eggs	0.405	0.012	0.005	0.003	0.007	0.005	0.002
	larvae	0.443	0.024	1.023	0.008	0.009	0.007	0.005
	pupae	0.211	0.017	0.231	0.154	0.121	0.434	0.023
	imagines	0.474	0.015	1.268	0.006	1.701	0.004	0.007
Autumn	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	0.255	0.123	0.675	2.645	1.923	0.067	3.876
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	0.167	0.096	0.532	1.983	1.534	0.089	2.587

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

Determination of proteolytic activity in PAGE

The proteolytic activity in the queens was also assayed through PAGE electrophoresis with 1% gelatin as the initial protein substrate in a 10% gel. The electrophoreses were performed in the Laemmli (1970) system modified for non-denaturing conditions. The zymographies were performed in a Miniprotean II apparatus (Bio-Rad, USA). A sample (100 µl) from each of the different developmental stages of the queens was loaded in each lane. The gels were then incubated in appropriate buffers (0.1M glycine – HCl at pH 2.4, 0.1M Tris – HCl at pH 7, 0.1M glycine – NaOH at pH 11.4) at 37°C for 2 h at the acidic pH and 1 h at the neutral and alkaline pH. The gels were stained according to the methods of Heussen and Dowdle (1980) with 0.5% Coomassie Brilliant Blue R-250 and de-stained with 40% methanol and 10% acetic acid. The gels were then analysed and densitometric scans were performed with a G:Box apparatus and software (Syngene, USA).

Antifungal and anti-ascomycetous activity assaying

The samples were assayed for antifungal: antimould and anti-ascomycetous activities in relation to the marker fungi *Aspergillus fumigatus* and *Candida albicans* on a SABG medium (Sabouraud glucose agar). Having mixed all the substrates of the SABG medium, it was autoclaved. After this medium was cooled down, to 200 ml of SABG we added 200 ml of the fungal solution: *Aspergillus fumigatus* or *Candida albicans,* and 20 ml of this mixture were poured onto each Petri dish. Little holes were cut out in the solidified medium. The rinsed queen samples were lyophilized and 400 µl of distilled water were added to them. The little holes were filled with 200 µl of the preparation. The Petri dishes were kept in an incubator. After three days a photographic documentation was performed and the results were checked.

RESULTS

We analyzed only mature queens from all the seasons, because some samples were missing due to the unavailability of specific forms at a given time (Table 1).

The highest protein concentration value was observed in the imagines from summer and the lowest in the pupae from spring (Table 1). High protein concentrations in the case of all the developmental stages of the queens were noted for the summer seasons.

The lowest protease activities were found for the summer seasons. The lowest protease activities and protease inhibitor activities were observed in the queen eggs from summer and these values amounted to 0.003–0.012 U/mg for proteases and to 0.002–0.007 U/mg for protease inhibitors. The highest activity values of acidic, neutral and alkaline proteases were observed in the pupae from spring and they amounted to 0.651 U/mg at pH = 2.4; 3.512 U/mg at pH = 7 and 4.121 U/mg at pH = 11.2. The highest values for acidic protease inhibitors were noted in the imagines from summer (1.268 U/mg), for neutral protease inhibitors in the imagines from autumn (1.923 U/mg) and for alkaline protease inhibitors in the imagines from autumn (3.876 U/mg). We observed the highest neutral and alkaline protease inhibitor activities in the queen imagines from autumn.

All the catalytic types of proteases were present in the queen samples but their activity levels differed. We found the highest activity in asparagine (inhibited by pepstatin A) and cysteine proteases (inhibited by iodoacetamide) at pH = 2.4; cysteine proteases and metalloproteases (inhibition by *o*-phenetroline) at pH = 7 were prominent and serine

Table 2. The activity of proteolytic enzymes sampled from the body surface of *Apis mellifera* queens in relation to some diagnostic protease inhibitors

Diagnostic protease inhibitors	Relative activity (%)		
	at pH 2.4	at pH 7	at pH 11.2
Pepstatin A	100	0	0
Iodoacetamide	100	70	0
O-phenantroline	0	80	70
PMSF	0	0	100

Table 3. Rm in gelatin PAGE zymography of the body surface preparations sampled from queens at different developmental stages and seasons (A and B)

Rm	Spring												Summer											
	pH 2.4				pH 7				pH 11.2				pH 2.4				pH 7				pH 11.2			
	eggs	larvae	pupae	imagines	eggs	larvae	pupae	imagines	eggs	larvae	pupae	imagines	eggs	larvae	pupae	imagines	eggs	larvae	pupae	imagines	eggs	larvae	pupae	imagines
0.1			▬										▬	▬	▬	▬	▬				▬			▬
0.3								▬								▬				▬				
0.6	n.a.	n.a.		▬	n.a.	n.a.		▬	n.a.	n.a.		▬					▬	▬		▬		▬		
0.7											▬													
0.8			▬				▬																▬	
0.9																		▬	▬	▬		▬		

Rm	Autumn												Winter											
	eggs	larvae	pupae	imagines	eggs	larvae	pupae	imagines	eggs	larvae	pupae	imagines	eggs	larvae	pupae	imagines	eggs	larvae	pupae	imagines	eggs	larvae	pupae	imagines
0.1				▬				▬				▬				▬				▬				▬
0.3																								
0.6	n.a.	n.a.	n.a.		n.a.	n.a.	n.a.	▬	n.a.	n.a.	n.a.		n.a.	n.a.	n.a.		n.a.	n.a.	n.a.		n.a.	n.a.	n.a.	
0.7												▬												▬
0.8																								
0.9																								

n.a = non-analysed samples

proteases (inhibition by PMSF) had the highest activities at pH = 11.2 (Table 2).

Table 3 shows the results of PAGE zymographies on the gelatin gel for the protein extracts from the body surface of queens sampled at different developmental stages and seasons. The zymographies of the queen samples were sharp. For the spring samples, we found medium-migrating protease bands at the acidic pH, fast-migrating protease bands at the neutral and alkaline pH. In the case of summer samples the zymographies were sharp with medium-migrating protease bands for the eggs at all pH values, the fastest-migrating protease bands for the larvae at the neutral and alkaline pH, fast-migrating protease bands for the pupa and imago samples at the acidic, neutral and alkaline pH. In autumn and winter, we studied only the queen imagines because the other stages were missing due to the

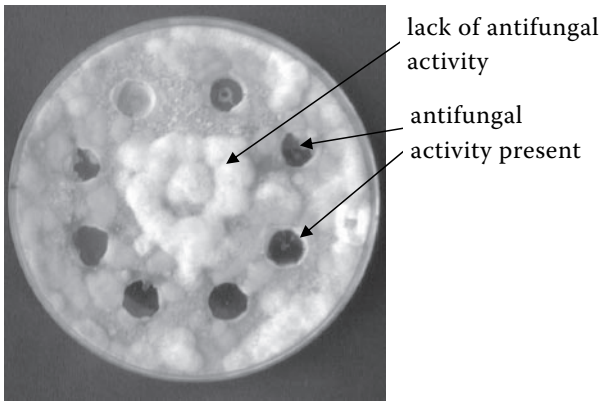

Figure 1. A specimen Petri dish showing the antifungal activity in *Apis mellifera* in relation to the marker fungus *Aspergillus fumigates*

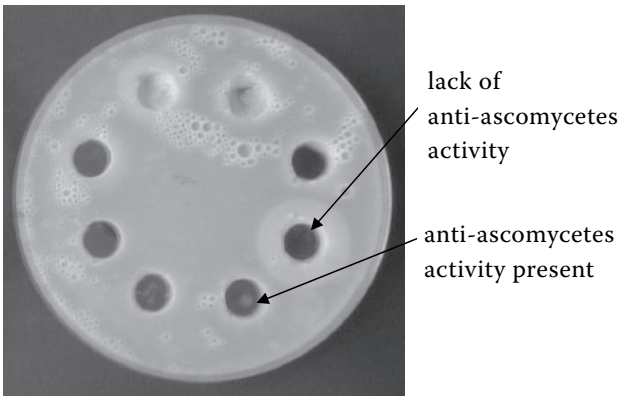

Figure 2. A specimen Petri dish showing the anti-ascomycetous activity in *Apis mellifera* in relation to the marker fungus *Candida albican*

Table 4. The seasonal body surface antifungal (anti-mould and anti-ascomycetous) activities in queens at different developmental stages

Season	Stage	Antifungal activity	Anti-ascomycetous activity
Spring	eggs	n.a.	n.a.
	larvae	n.a.	n.a.
	pupae	yes	no
	imagines	yes	no
Summer	eggs	yes	no
	larvae	yes	no
	pupae	yes	no
	imagines	yes	yes
Autumn	eggs	n.a.	n.a.
	larvae	n.a.	n.a.
	pupae	n.a.	n.a.
	imagines	yes	yes
Winter	eggs	n.a.	n.a.
	larvae	n.a.	n.a.
	pupae	n.a.	n.a.
	imagines	yes	yes

The non-analysed samples (n.a.) are due to the unavailability of specific forms at a given time
yes – activity present, no – lack of activity

unavailability of specific forms at a given time. We saw blurred slow-migrating protease bands at the acidic pH and medium-migrating protease bands at the neutral and alkaline pH.

The results for the anti-mould and anti-ascomycetous activities (Table 4, Figures 1 and 2) show a better protection from the mould and yeast. The anti-ascomycetous activity was present only in the mature queens from all the seasons of the year.

DISCUSSION

In this study we demonstrated by *in vitro* analyses and PAGE zymographies how the profiles of proteases and those of their natural inhibitors from the body surface of the honey bee queens varied according to the ontogenetic stage and the season of the year. In the *in vitro* experiments we compared two groups of detectable proteolytic activities: in the acidic detergent solution and in the neutral and alkaline water solutions. We observed very low acidic, neutral and alkaline protease activities and protease inhibitor activities in the majority of the developmental stages collected in summer.

The first suggestion would be that this fact is connected with the fast metabolism and the crucial position held by the queen in a bee colony. Queens do not leave the hive for all their lifetimes. They fly out of the nest only during mating flights. During the mating flights they collect enough semen to be used for their whole lifetime. After such flights they start laying eggs in the brood comb cells which are looked after by the workers (Bania and Polanowski, 1999; Grăff et al., 2007). Queens do not have to react by forming appropriate protective barriers in the form of surface proteolysis in the vegetation season because drones have such barriers (Strachecka and Grzywnowicz, 2008). Moreover, at the insemination time their organisms are not entered by infective pathogens: bacteria, fungi and parasitic mites (Gliński et al., 2006). A second and possibly parallel role in insect body surface immunity may be connected with pheromone metabolism (Cornette et al., 2002). Queens are oriented to lay eggs in summer. They emit a substance which stops the development of worker ovaries and as a result, the task specialization is strongly pronounced (Gliński et al., 2006).

The lowest protease activities and protease inhibitor activities were identified in queen eggs. This developmental stage is not exposed to an excessive contact with pathogens. Additionally, eggs, larvae and pupae have constant worker protection. During ontogenesis queen larvae get the royal jelly which is rich in nutrients (Prabucki, 1998). We suppose that such treatment of queens is reflected in the type of the protective barrier in the form of proteases and protease inhibitors. The proteolytic system activity decreased at the turn of winter and spring. This situation may have been caused by the changing metabolism (Andersen et al., 1981; Merzendorfer and Zimoch, 2003) and related to the low temperature of the environment.

Our studies showed that the queens had different proteases on their body surfaces. We found high activity for asparagine and cysteine proteases, metalloproteases and serine proteases. These enzymes catalyze the hydrolytic cleavage of the peptide bonds and play a regulatory role in the majority of the fundamental processes ranging from protein synthesis to protein degradation. For example, serine proteases are considered to be direct elements of defence against microbial proteinases and important factors in the control of the proteolytic cascades and in the control of multiple pathways

associated with coagulation and melanization after wounding and infection (Kanost, 1999; Polanowski et al., 2003; Evans et al., 2006; Zou et al., 2006; Grăff et al., 2007). In turn, cysteine proteases participate in various biological processes. They are involved in protein breakdown in lysosomes, antigen presentation, the proteolytic processing of proenzymes and prohormones, cell proliferation and apoptosis (Grzonka et al., 2001; Rzychon et al., 2004). Whereas metalloproteases contribute to the wound healing processes, cell migration and proliferation; they also participate in the inflammatory response, apoptosis and angiogenesis (Wawrzycka et al., 2007). Asparagine proteases play crucial roles in peptide degradation, digestion and in interactions with the pests which the honey bee encounters (Walter and Clélia, 1994; Takayuki and Reniera, 2008). Each of the proteases performs different functions but together they can form group strategies to combat diseases and infections on the body surface of queens (Evans et al., 2006; Tautz et al., 2007).

We studied natural protease inhibitors on the body surface of honey bee queens. We identified asparagine and serine protease inhibitors. In insect haemolymph these inhibitors impede activated proteases in order to maintain homeostasis and prevent an unregulated activation of immune responses such as melanization or the Toll-mediated antimicrobial protein synthesis (Evans et al., 2006). The functions of the natural inhibitors on the body surface of queens are not known but we suggest that they may control the microbial biofilms, like those in ants (Currie et al., 1999; Currie, 2001). The protective barrier formed mainly by the protease inhibitors can be correlated with the anti-mould and anti-ascomycetous activities in the queens (Bania and Polanowski, 1999). The fact that the mature queens are protected in this way can result from their social status and special treatment by the worker caste (cleaning, feeding etc.) (Prabucki, 1998).

The obtained results reveal the initial model of proteolysis on the body surface of honey bee queens and confirm the validity of further biochemical research on the body surface proteolytic activity in these insects.

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