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Original article

Profiles of the body-surface proteolytic system of honey bee queens, workers and drones: Ontogenetic and seasonal changes in proteases and their natural inhibitors*

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Abstract – We describe how protease and protease inhibitor activity patterns vary during ontogenesis, with season, and in relation to caste and sex in the honey bee (*Apis mellifera*). Extraction of body surfaces with water and detergent was followed by the in vitro analysis of proteolytic activity and protease inhibitor level, as well as the electrophoretic separation of extracts in polyacrylamide gels. In in vitro experiments, we compared two groups of detectable proteolytic activities: neutral and alkaline water-soluble *versus* acidic detergent-soluble. The most active proteases appeared to be acidic ones and were detected on drone pupae in spring. The most distinct and most active protease bands in electrophoretic separations were those obtained for neutral and alkaline activities on queens in all seasons. The highest levels of protease inhibitor activities in vitro were obtained from worker samples in all seasons. The enzymatic properties suggest that all catalytic types of proteases were present in the extracts, but at different activity levels, depending on pH: asparagine and cysteine proteases at pH 2.5, cysteine proteases and metalloproteases at pH 7.0, and serine proteases at pH 11.5, respectively.

honey bee / proteases / protease inhibitors / body surface proteolysis / zymography

1. INTRODUCTION

In recent years, there has been much attention on natural, transgenic, and synthetic inhibitors of proteases, both proteic and non-proteinic ones, for their potential use in inhibiting the digestive process of developing larvae and imagines of pest insects (Ryan, 1990; Jongsma and Bolter, 1997). Consequently, a wide range of investigations have been focused on identifying and characterizing different proteases in a variety of insect orders and species (Terra and Ferreira, 1994; Lopes et al., 2004). In bees, some proteinases have been investigated and the effects of protease inhibitors on survival during ontogenesis and food consumption of workers were described (Malone et al., 1998; Malone, 2004).

As social insects, honey bees (*Apis mellifera* L.) spend most of their lifetime densely crowded inside their hives or natural nesting sites. As can be expected from the selective pressures of parasites and pathogens, social insects have evolved both individual and group strategies to combat diseases and illnesses. Grooming, nest hygiene, and other behaviors can reduce the impacts of pathogenic bacteria, fungi, and parasitic mites. Insects have diverse mechanisms to combat infections by bacteria and fungi. Many insects are protected by layers of antimicrobial secretions on their body surface and by a gut-specific environment that is hostile to microorganisms and pathogens. Should microorganisms and pathogens defeat the morphological defences

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in insects (the cuticle and/or the epithelium are often already sufficient to stop invasion), they are met by efficient cellular and humoral immune defences. Honey bees, like all invertebrates, lack an adaptive immune system. Instead, they have evolved an efficient strategy for combating infections called the "innate immune response". Four lines of defence define this strategy: hygienic behaviors; physical barriers (e.g., the cuticle and the epithelial lining of the gut); cellular immune responses (e.g., phagocytosis, nodule formation, encapsulation and phenoloxidase activation); and humoral immune responses (e.g., induction of a spectrum of antimicrobial peptides upon infection) (Evans et al., 2006; Tautz, 2007; Tautz et al., 2007).

Insect immunity shows many parallels to the innate immune response of vertebrates, involving a varying set of molecular actions, like synthesis and secretion of antimicrobial peptides (many of them have domains of or are protease or protease inhibitors; Izadpanah and Gallo, 2005; www.merops.sanger.ac.uk), hemolymph coagulation, phagocytosis, melanization of pathogen surfaces, and the enzymatic degradation of pathogens (Gorman and Paskewitz, 2001; Hoffman, 2003; Hultmark, 2003). The enzymatic degradation of pathogenic bacteria, fungi, and parasitic mites also involves proteolytic enzymes, present not only in the hemolymph and in the gut but also on the body surface. So far, only a limited number proteases and/or protease inhibitors, present have been identified on the surface of the body in different organisms, including cockroaches (Cornette et al., 2002; Wünschmann et al., 2005), an amphibian (Zhao et al., 2005), and humans (Zeeuwen, 2004; Tobin, 2006). Also, differences between invertebrate and higher vertebrate proteases and immune systems appear not to be as large as one might expected (Check, 2006; The Honeybee Genome Sequencing Consortium, 2006). Novel proteins, including proteases and cuticle proteins, have been found during the recent honey bee genome investigations (The Honeybee Genome Sequencing Consortium, 2006; Kucharski et al., 2007), but for most proteases only the encoding genes are known or predicted (Zou et al., 2006). For a successful defence against invading pathogens, cooperation is necessary between cellular and humoral immune reactions in a well-balanced network. The "body-surface" proteolytic system can be expected to play an important role in this network to improve insect health. This is suggested by a previous study from our laboratory on a non-social insect: staphylinid beetles living in "dirty" environments (Grzywnowicz and Staniec, 2008).

The aim of this work is to describe in vitro activities and zymographic patterns of the major body surface proteases and their natural inhibitors during the ontogenesis of honey bees (*Apis mellifera*), taking into account the factors of season, sex, and caste.

2. MATERIALS AND METHODS

2.1. Chemicals and biological samples

Chemicals were obtained from Sigma Chemicals (St Louis, MO, USA).

Adult honey bees were collected either at the hive entrance (workers) or from inside the hive (queens and drones). Pupae, larvae, and eggs were removed from brood frames. First-, second-, and third-instar larvae were pooled as "small larvae", and fourth- and fifth-instar larvae were pooled as "big larvae" to simplify further analyses. Samples were collected in spring (May), summer (July) and autumn (October) over the course of two years from the same apiary. All samples were kept frozen until preparation of crude extracts. Since we did not obtain all categories of bees in the three sampling periods, those data are absent in the figures.

2.2. Crude extract preparations

All specimens were gently rinsed with distilled water to free them of debris and unfixed adherent microorganisms. Then, each specimen was gently washed with cotton swabs. These were soaked with distilled water and pressed out into sample vials (Cornette et al., 2002). A second wash was made the same way, but using 1% detergent (Triton X-100) in water. This rendered about 1 mL of distilled water or 1 mL of 1% detergent wash for each specimen.

Eggs and small larvae were washed by gently stirring a few specimens in sample vials and then filtering them. Samples were collected in triplicate for each stage.

2.3. Assay of protease activities

General protease activities were determined at optimal pH by the method of Anson (1938) using hemoglobin as the substrate. Briefly, 0.2 mL of each sample was incubated for 60 min with 0.5 mL of 1% hemoglobin in appropriate buffer. The incubation buffer at pH 2.5 was 100 mM glycine-HCl; at pH 7.0 was 100 mM Tris-HCl; and at pH 11.5 was 100 mM glycine-NaOH. After the addition of 2 mL 5% TCA, undigested proteins were precipitated and centrifuged, then the peptide and amino acid content in the supernatant was spectrophotometrically determined by absorbance at 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).

2.4. Assays of protease inhibitor activities

Determination of general levels of protease inhibitor activity was carried out by the method of Lee and Lin (1999), with pepsin as a marker protease for acidic pH and trypsin as a marker protease for neutral and alkaline pHs. 0.1 mL of marker protease in appropriate buffer was preincubated with 0.1 mL of sample for 30 min. After this time, 0.5 mL of 1% hemoglobin in appropriate buffer was added and the incubation was continued for 60 min. Subsequently, proteins were TCA-precipitated and the supernatant was measured as described above. Inhibitor levels were calculated according to Lee and Lin (1999).

2.5. Detection of enzyme activity in PAGE

Electrophoreses were performed in a Laemmli (1970) system modified for non-denaturing conditions and with 1% gelatin as precast protein substrate in 10% gel. Zymographies were performed in a Miniprotean II apparatus (Bio-Rad, USA). Ten to 50 μL of each extract (~20 μg total proteins) was loaded in each lane. After electrophoresis, the gels were incubated in appropriate buffer (100 mM glycine-HCl at pH 2.5; 100 mM Tris-HCl at pH 7.0; or 100 mM glycine-NaOH at pH 11.5) for 2 hours. They were stained according to the methods of Heussen and Dowdle (1980) with 1% Coomassie Brilliant Blue R250 and de-stained with 40% methanol and 10% acetic acid. Gel analysis and densitometric scans were performed with a G:Box apparatus and software (Syngene, USA).

2.6. Determination of protein

Concentration of total proteins was determined by the method of Bradford (1976), with bovine serum albumin as the standard.

3. RESULTS

3.1. Partial characterization of major proteolytic activities on the honey bee body surface

For a partial characterization of the major proteolytic activities on the honey bee body surface, we used samples from adult workers collected in the summer, as this was the largest sample set at the start of our experiments. Figure 1 shows the proteolytic activities of water-soluble and detergent-soluble crude extracts and their variation within the pH range. The maximum peaks were near pH 2.5 for the detergent wash and around pH 7.0 and 11.5 for water washes; these pH optima were chosen for further proteolytic activity assays. There were two additional activity peaks between pH 4 and 6 and between pH 8 and 10, but these were less significant peaks and thus not chosen for further analyses.

Table I shows the proteolytic activity of crude extracts and the variation across the various substrates used. Hemoglobin turned out to be the best substrate for our analyses. Since gelatin also gave satisfactory results, we used it in PAGE zymography because it gave sharper activity bands (results not shown). Other substrates did not give satisfactory activity measurements, especially in PAGE (ovoalbumin).

Table II shows the results for the inhibition of proteolytic activity of crude extract in the

Table I. Activity of proteolytic enzymes sampled from the body surface of honey bees against some proteinaceous substrates.

Substrate	Relative activity at pH 2.5 (%)	Relative activity at pH 7.0 (%)	Relative activity at pH 11.5 (%)
Bovine serum albumin	50	35	45
Casein	0	85	80
Cytochrome c	0	70	30
Ferritin	10	25	5
Gelatin	70	85	80
Hemoglobin	100	100	100
Ovoalbumin	80	75	65

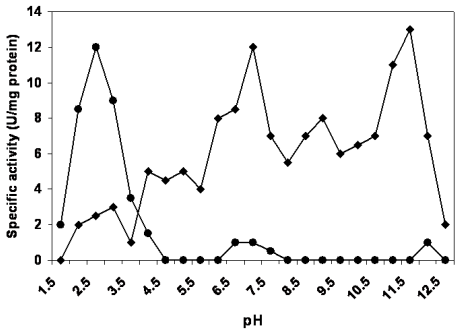


Figure 1. pH activity profile of extracts of proteolytic activities from the body surface of adult honey bee workers. Activity was determined by the method of Anson (1938) using hemoglobin as substrate. Each 0.2 mL sample aliquot was incubated with 0.5 mL of 1% hemoglobin in appropriate buffer for 60 min. The incubation buffer at pH from 1.5 to 2.5 was 100 mM Glycine-HCl; at pH from 2.0 to 5.5 was 100 mM Mc Ilvaine; at pH from 5.0 to 7.0 was 100 mM Tris-acetate; at pH from 7.0 to 9.0 was 100 mM Tris-HCl; and at pH from 8.5 to 13.0 was 100 mM glycine-NaOH. Results are shown for water-soluble proteases (♦) and for detergent-soluble proteases (●).

presence of so-called diagnostic protease inhibitors. All catalytic types of protease were present in the extracts, but their activity levels differed. At pH 2.5, we found the highest activity for asparagine (inhibited by pepstatin A and DAN) and cysteine proteases (inhibited by iodoacetamide and pCMB); at pH 7.0, cysteine proteases and metalloproteases (inhibition by *o*-phenantroline) were prominent; and at pH 11.5 serine proteases (inhibition by PMSF and STI) had the highest activity.

3.2. Proteolytic activities and protease inhibitor levels

Figures 2, 3, and 4 show protease activity levels detected at the chosen pH optima with hemoglobin as the substrate. In the spring samples (Fig. 2), we could see that the highest levels of proteolytic activity was present in drone samples, and they were prominent for acidic pH and highest on pupae. Protease activity levels in queen pupae samples were also high, but these were found mainly at alkaline pH. In workers samples, proteolytic activities were very low at all pHs. In summer samples (Fig. 3), we once again saw that the highest levels of proteolytic activity were present in drone samples, but in this season they were of neutral and alkaline pH, and they were found mainly on imagines. Relatively high protease activity levels appeared on workers, especially on adults and at neutral pH. At neutral pH, we also found a high protease activity level on pupae of queens. In autumn samples (Fig. 4), high protease activity levels were found on adult queens and workers, and at neutral pH. The lowest level of proteolytic activity was present in samples of adult drones at all pHs. Generally, low levels of proteolytic activities were found in the stages of development.

Figures 5, 6, and 7 show protease inhibitor levels for the chosen conditions. In spring samples (Fig. 5), we saw that the highest level of protease inhibitor activity was present in workers samples, practically at all pHs, and on most

Table II. Effects of diagnostic protease inhibitors on proteolytic enzymes sampled from the body surface of honey bees. The enzymes were preincubated in appropriate buffer containing different inhibitors for 60 minutes at 4 °C. After preincubation, the activity was assayed by standard procedures.

Inhibitor	Concentration of inhibitor (mM)	Relative activity at pH 2.5 (%)	Relative activity at pH 7.0 (%)	Relative activity at pH 11.5 (%)
None	-	100	100	100
EDTA	5.0	95	120	110
o-Phenantroline	2.0	94	85	90
Phenylmethylsulfonyl fluoride (PMSF)	2.0	120	100	70
Soybean trypsin inhibitor (STI)	1.0	100	95	82
p-Chloromercuribenzoate (*p*-CMB)	1.0	50	80	90
Iodoacetamide	2.0	77	92	98
Pepstatin A	1.0	70	90	100
Diazoacetyl norleucine methyl ester (DAN)	2.0	68	100	100

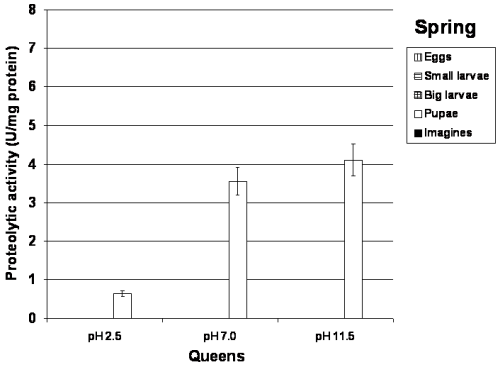
stages. In summer samples (Fig. 6), we once again noted the highest level of protease inhibitor activity in worker samples, practically at all pHs, and on most stages. Similarly, high levels of protease inhibitor activity were found in drone samples, at all pHs, and on most stages. The levels of protease inhibitor activities were generally lowest in queen samples, just as the protease activity levels. Only in autumn samples (Fig. 7) did we see the highest protease inhibitor activity levels on adult queens, and at all pHs. High levels of protease inhibitor activity were also present in workers samples, but mainly at acidic pH. In drone samples, for which we only had adults in this season, protease inhibitor activities were detected at all pHs.

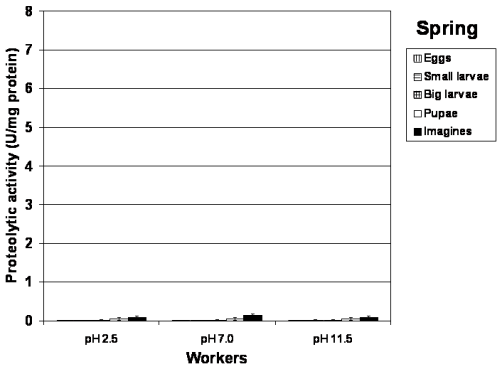
3.3. Proteolytic activities PAGE pattern variations

Table III shows the results of PAGE zymographies on gelatin gels for protein extracts from the body surface of queen, worker, and drone honey bees sampled at different developmental stages and seasons. For spring samples, we found that zymographies of queen samples gave sharp, fast-migrating protease bands in neutral and alkaline pH. Zymographies of workers samples were irregular; in acid pH, we obtained weak, slow-migrating protease bands, but in neutral pH, there were weak, medium-migrating protease bands, and in alkaline pH, we obtained blurred but strong medium-migrating protease bands and sharp, fast-migrating protease bands. This stands in contrasts with the very low proteolytic activities found in the in vitro experiments and we think that this maybe due to the separation from protease inhibitors during electrophoresis. In drone samples, for instance, we found sharp, fast-migrating protease bands in acid pH, and in neutral and alkaline pHs we found blurred protease bands across the whole range.

In summer samples, we saw, once again, that the zymographies of queen samples gave sharp, medium-migrating and fast-migrating protease bands in neutral and alkaline pH, especially for larvae and in neutral pH. Zymographies of workers samples were irregular in summer as well; in acid pH, protease bands were blurred and slow-migrating; in neutral pH, they were blurred but strong- and medium-migrating, as well as sharp and fast-migrating (despite a strong band for small larvae); and in alkaline pH, we found weak, medium-migrating protease bands. In drone samples in all pHs, we obtained wide and rather weak protease bands in all migrating ranges.

In autumn samples for queens, we saw blurred slow- and medium-migrating protease





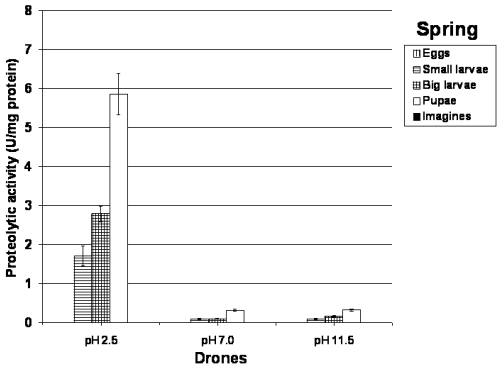
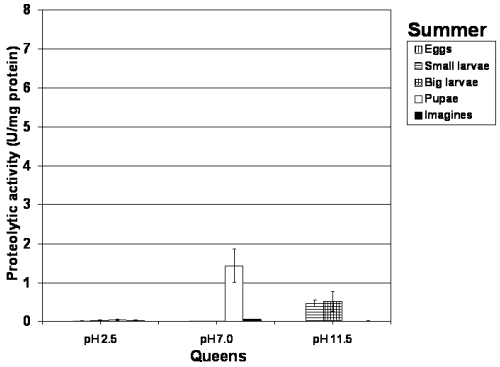
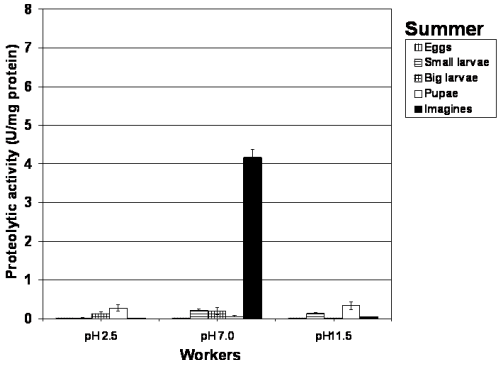


Figure 2. Levels of protease activities from body surface preparations of different development stages (see legend) of honey bee queens, workers and drones sampled in spring. Activity was determined by the method of Anson (1938) using hemoglobin as substrate. Aliquots of 0.2 mL of sample were incubated with 0.5 mL of 1% hemoglobin in appropriate buffer for 60 min. The incubation buffer at pH 2.5 was 100 mM glycine-HCl; at pH 7.0 was 100 mM Tris-HCl; and at pH 11.5 was 100 mM glycine-NaOH. Standard errors of the means are shown.





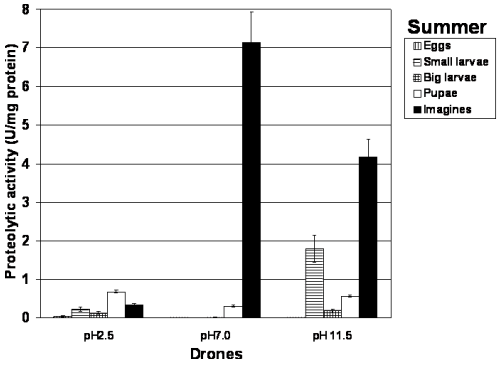


Figure 3. Levels of protease activities from body surface preparations of different development stages (see legend) of honey bee queens, workers and drones sampled in summer. Activity was determined by the method of Anson (1938) as in Figure 2. Standard errors of the means are shown.

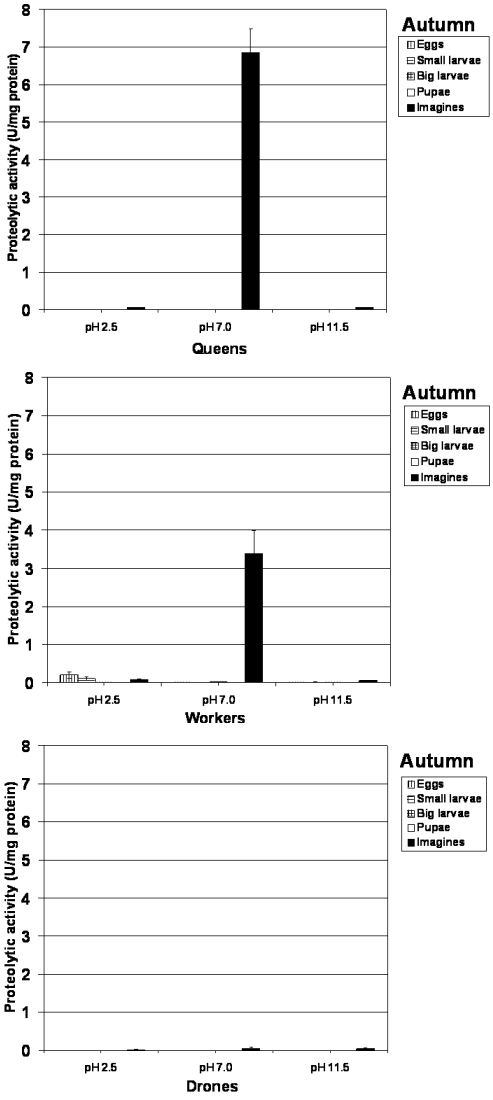


Figure 4. Levels of protease activities from body surface preparations of different development stages (see legend) of honey bee queens, workers and drones sampled in autumn. Activity was determined by the method of Anson (1938) as in Figure 2. Standard errors of the means are shown.

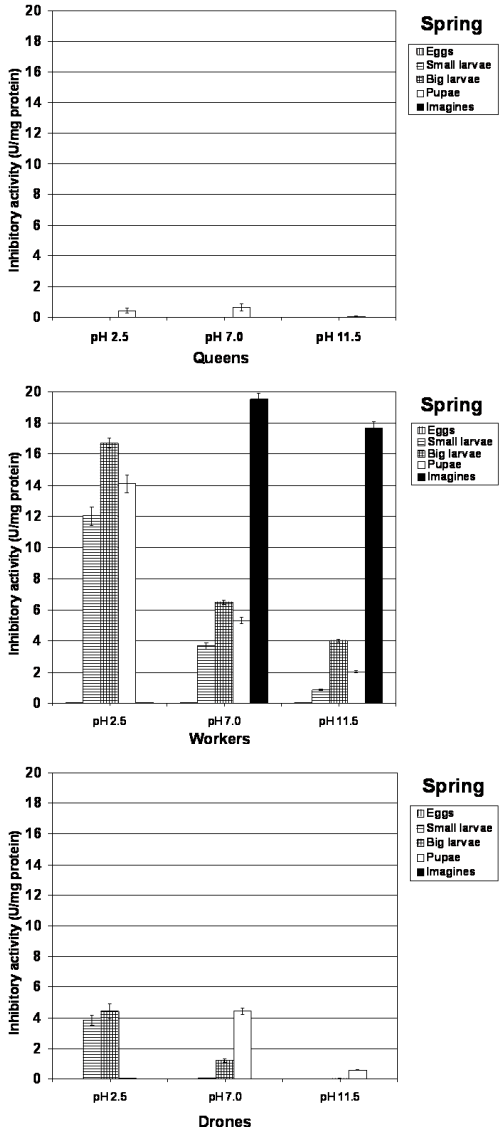
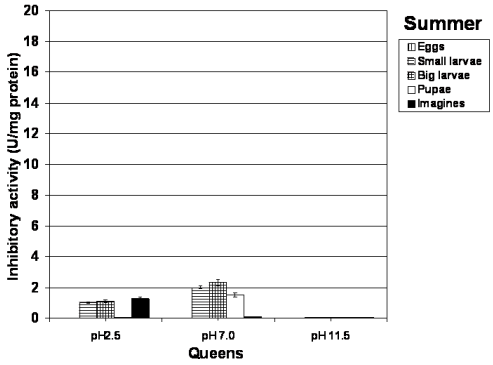
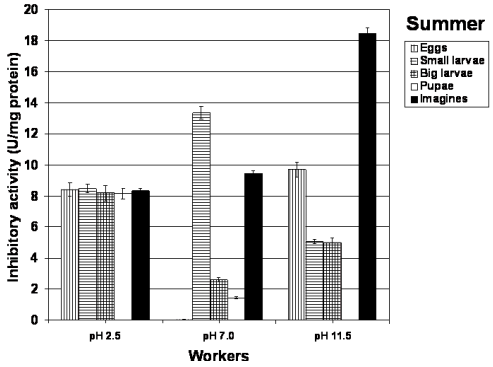


Figure 5. Levels of protease inhibitor activities from body surface preparations of different development stages (see legend) of honey bee queens, workers and drones sampled in spring. Determination of general levels of protease inhibitor activities was determined by the method of Lee and Lin (1999), with pepsin as marker protease for acidic pH and trypsin as marker protease for neutral and alkaline pH. Standard errors of the means are shown (from multifactorial analysis of variance $P < 0.001$).

bands in neutral and alkaline pH. From workers samples, we obtained weak, slow-migrating protease bands in acid pH, and weak, medium- and fast-migrating protease bands in neutral and alkaline pHs. In drone samples, we obtained blurred protease bands





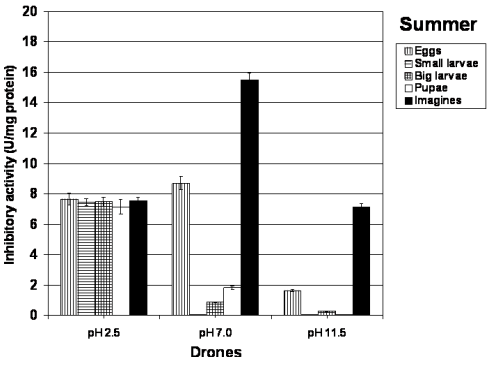
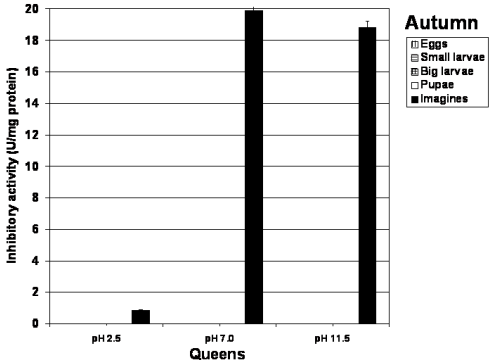
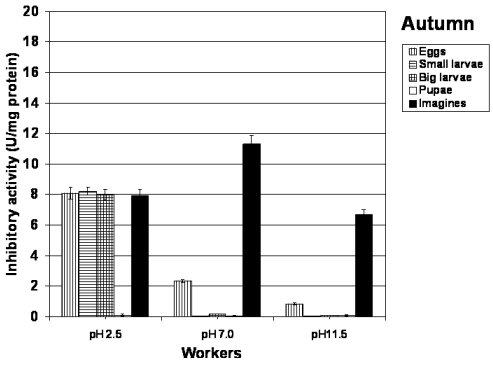


Figure 6. Levels of protease inhibitor activities from body surface preparations of different development stages (see legend) of honey bee queens, workers and drones sampled in summer. Determination of general levels of protease inhibitor activities was determined by the method of Lee and Lin (1999) as in Figure 5. Standard errors of the means are shown.





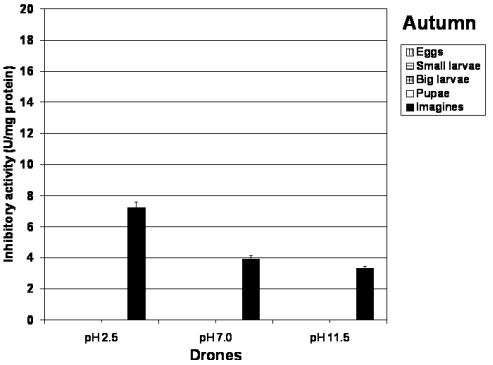


Figure 7. Levels of protease inhibitor activities from body surface preparations of different development stages (see legend) of honey bee queens, workers and drones sampled in autumn. Determination of general levels of protease inhibitor activities was determined by the method of Lee and Lin (1999) as in Figure 5. Standard errors of the means are shown.

for all pHs and in all migrating ranges, much like those in summer samples.

The number of proteolytic enzyme bands differed between males and females. The largest number of bands (one to six) was found for drones and the smallest number (one to three) for queens. Workers had a medium number of proteolytic bands (one to four).

4. DISCUSSION

In this work, we demonstrated by in vitro analyses and PAGE zymography how the profiles of water- and detergent-soluble proteases, and those of their natural inhibitors, from the body surface of hone bee queens, workers, and drones vary during ontogeny and time of year. In the in vitro experiments, we compared two groups of detectable proteolytic activities — neutral and alkaline water-soluble and acidic detergent-soluble. The most active proteases appeared in the acidic range on drone pupae in spring (Fig. 2), in the neutral and alkaline ranges on adult drones in the summer (Fig. 3), and at neutral pH on adult queens in the fall (Fig. 4) and on queen pupae in the spring (Fig. 2). The lowest protease activity was found on workers in the spring (Fig. 2) and on drones in autumn (Fig. 4). In general, protease activities on pupae and adults were higher than those on other stages. These observations may be related with fluctuations in immune system functions during the year, during development, and in relation to hygienic conditions (eggs and larvae are well protected by nurse bees). There were also clear difference between the sexes and caste, possibly reflecting other physiological differences (Hrassnigg and Crailsheim, 2005).

It is important to note that the proteolytic activities found in vitro differed in some aspects from their zymography on precast gelatin gels. The sharpest and most active protease bands in PAGE zymography were obtained for neutral and alkaline activities on queens (Tab. III). The most blurred bands of proteases were those obtained for neutral and alkaline activities on drones. For workers, the zymography protease bands were both sharp and blurred in all seasons. An interesting result was the inverse relationship between levels of in vitro protease activities and intensity of protease bands in PAGE zymography. In some cases, for example, we obtained from the same samples low activity in vitro and intense activity band(s) on the gel. We think that the reason for this discrepancy does not only lie in the different substrates and in different protease substrate specificity (Dahlmann et al., 1978; Felicioli et al., 2004), but also the separation of proteases and their in vivo protease inhibitors during electrophoresis. This question can be resolved when isolating different proteases from the total body surface extracts.

Another interesting observation was the agreement between low protease activity levels with high levels of protease inhibitor activity in vitro on workers in all seasons. Comparable high protease inhibitor levels in vitro were observed on drones in summer (Fig. 6) and on adult queens in autumn (Fig. 7). Because of the lack of success in protease inhibitor PAGE zymography, we could not to make direct comparisons between PAGE profiles of protease activities and protease inhibitor activities.

The proteolytic system of the honey bee body surface that we investigated is a very interesting arrangement between enzymes and their natural inhibitors, and it may be involved in some processes occurring on the cuticle surface. A first suggestion would be its participation in the immune system of insects, as described for a variety of organisms including cockroaches (Cornette et al., 2002; Wünschmann et al., 2005), an amphibian (Zhao et al., 2005), and humans (Zeeuwen, 2004; Tobin, 2006). There is also the possibility that proteases participate in cuticle-associated immunity, e.g. by enzymatic degradation of pathogens (Gorman and Paskewitz, 2001), but the majority of their functions is still unknown (Evans et al., 2006; Zou et al., 2006). The participation of protease inhibitors in cuticle-associated immunity is quite well investigated investigated (Kanost, 1999; Selitrennikoff, 2001; Zasloff, 2002; Bulet et al., 2004). A second and possibly parallel role in insect body-surface immunity may be the control of microbial biofilms, like those in ants (Currie et al., 1999; Currie, 2001).

Table III. Precast gelatin PAGE zymography of body surface preparations honey bee queens workers and drones sampled at different developmental stages and seasons. (A) Rm of proteolytic activity bands and (B) intensity of proteolytic bands by densitometric evaluation. Proteins were separated in 10% polyacrylamide gels containing 0.1% gelatin and incubated, after development, in appropriate buffers (100 mM Glycine-HCl at pH 2.5; 100 mM Tris-HCl at pH 7.0; and 100 mM glycine-NaOH at pH 12.5), at 37 °C for two hours. The slabs were stained with Coomassie Brilliant Blue R 250 and de-stained. Twenty μg of crude protein extracts were loaded per lane. na, not analyzed.

A. Rm

Sex, caste and optimum pH of activity		Spring					Summer					Autumn				
		Eggs	Small larvae	Big larvae	Pupae	Imagines	Eggs	Small larvae	Big larvae	Pupae	Imagines	Eggs	Small larvae	Big larvae	Pupae	Imagines
Queens	pH 2.5	na	na	na	-	na	na	0.09	0.09	0.09	0.09	na	na	na	na	0.09
	pH 7.0	na	na	na	-	na	na	-	-	-	0.09	na	na	na	na	0.09
					-			0.55	0.55	-	0.55					0.55
					0.82			-	-	-	-					-
					-			0.90	0.90	0.90	0.90					0.90
	pH11.5	na	na	na	-	na	na	-	-	-	0.09	na	na	na	na	0.09
					-			0.55	-	-	-					0.55
					0.82			0.82	0.82	0.82	-					-
Workers	pH 2.5	-	-	-	-	-	0.09	0.09	0.09	0.09	0.09	-	0.09	0.09	0.09	0.09
		-	0.19	0.19	-	-	-	0.19	0.19	0.19	0.19	-	-		-	-
	pH 7.0	-	-	-	-	-	0.09	-	-	-	-	0.09	0.09	0.09	0.09	0.09
			0.40	-	-	-	-	-	-	-	-	-	-	-	-	-
			-	-	-	-	0.55	0.55	0.55	-	0.55	-	-	-	0.55	0.55
			-	-	-	-	-	0.64	0.64	0.64	0.64	-	-	-	-	-
			-	-	-	0.73	-	-	-	-	-	-	-	-	-	-
			-	-	-	-	-	-	0.82	0.82	0.82	-	-	-	-	-
			-	-	-	-	-	0.90	-	-	-	0.90	0.90	0.90	0.90	0.90
	pH11.5	-	-	-	-	-	0.09	-	-	-	0.09	-	-	-	0.09	0.09
		-	0.55	0.55	0.55	0.55	-	0.55	0.55	-	0.55	-	-	-	-	0.55
		0.64	0.64	0.64	-	0.64	-	0.64	-	0.64	-	-	-	-	-	-
		0.73	0.73	0.73	-	0.73	-	0.73	-	-	-	-	-	-	-	-
		-	0.82	0.82	0.82	0.82	-	0.90	0.90	0.90	-	0.90	0.90	0.90	0.90	0.90

Table III. Continued.

Sex, caste and optimum pH of activity		Spring					Summer					Autumn				
		Eggs	Small larvae	Big larvae	Pupae	Imagines	Eggs	Small larvae	Big larvae	Pupae	Imagines	Eggs	Small larvae	Big larvae	Pupae	Imagines
Drones	pH 2.5	na	-	-	0.09	na	-	0.09	-	-	-	na	na	na	na	0.09
			-	0.19	-		-	-	-	-	0.19					-
			-	-	-		-	0.55	0.55	0.55	0.55					-
			0.64	0.64	0.64		-	-	-	-	-					-
			0.73	0.73	0.73		-	-	-	-	-					0.73
			-	-	-		0.82	0.82	-	-	0.82					-
			-	-	-		-	-	-	-	-					0.90
	pH 7.0	na	0.09	0.09	0.09	na	0.09	0.09	0.09	0.09	-	na	na	na	na	0.09
			0.19	0.19	0.19			-	-	-	0.19					0.19
			0.55	0.55	0.55			-	-	-	-					0.55
			-	0.64	-			0.64	-	-	0.64					-
			0.73	0.73	0.73			-	0.73	0.73	0.73					0.73
			0.90	0.90	0.90			0.90	0.90	0.90	0.90					-
	pH11.5	na	0.09	-	0.09	na	-	0.09	0.09	-	-	na	na	na	na	0.09
			-	-	0.19		-	-	-	-	-					-
			-	-	-		-	-	-	-	-					0.36
			-	-	0.55		-	-	-	-	-					-
			-	0.64	0.64		-	-	-	-	-					0.55
			-	-	-		0.73	-	-	0.73	0.73					-
				0.82	0.82		-	-	-	-	-					-
				-	0.90		0.90	0.90	0.90	0.90	0.90					-

Table III. Continued.

B. Intensity

Caste and optimum pH of activity		Spring					Summer					Autumn				
		Eggs	Small larvae	Big larvae	Pupae	Imagines	Eggs	Small larvae	Big larvae	Pupae	Imagines	Eggs	Small larvae	Big larvae	Pupae	Imagines
Queens	pH 2.5	na	na	na	-	na	na	5	4	5	4	na	na	na	na	6
	pH 7.0	na	na	na	-	na	na	-	-	-	8	na	na	na	na	5
					-			72	70	-	15					11
					12			-	-	-	-					-
					-			65	63	58	22					7
	pH11.5	na	na	na	-	na	na	-	-	-	9	na	na	na	na	4
					-			19	-	-	-					11
					13			41	20	45	-					-
Workers	pH 2.5	-	-	-	-	-	3	8	9	9	11	-	21	22	15	12
		-	12	7	-	-	-	11	11	10	9	-	-		-	-
	pH 7.0	-	-	-	-	-	2	-	-	-	-	5	4	4	6	5
			14	-	-	-	-	-	-	-	-	-	-	-	-	-
			-	-	-	-	4	32	26	-	18	-	-	-	3	3
			-	-	-	-	-	7	9	11	16	-	-	-	-	-
			-	-	-	5	-	-	-	-	-	-	-	-	-	-
			-	-	-	-	-	-	19	18	20	-	-	-	-	-
			-	-	-	-	-	15	-	-	-	13	12	12	11	10
	pH11.5	-	-	-	-	-	3	-	-	-	6	-	-	-	9	8
		-	15	14	15	18	-	18	17	-	19	-	-	-	-	11
		11	10	9	-	11	-	17	-	0.64	-	-	-	-	-	-
		9	8	9	-	10	-	8	-	-	-	-	-	-	-	-
		-	18	21	20	18	-	8	9	9	-	8	9	9	8	10

Table III. Continued.

Caste and optimum pH of activity		Spring					Summer					Autumn				
		Eggs	Small larvae	Big larvae	Pupae	Imagines	Eggs	Small larvae	Big larvae	Pupae	Imagines	Eggs	Small larvae	Big larvae	Pupae	Imagines
Drones	pH 2.5	na	-	-	16	na	-	8	-	-	-	na	na	na	na	8
			-	15	-		-	-	-	-	21					-
			-	-	-		-	7	19	13	24					-
			16	14	17		-	-	-	-	-					-
			15	14	15		-	-	-	-	-					11
			-	-	-		6	8	-	-	12					-
			-	-	-		-	-	-	-	-					18
	pH 7.0	na	16	14	13	na	7	8	8	9	-	na	na	na	na	7
			17	15	15			-	-	-	6					17
			15	11	14			-	-	-	-					18
			-	12	-			5	-	-	6					-
			12	15	11			-	7	8	8					21
			13	21	19			21	20	11	5					-
	pH11.5	na	6	-	18	na	-	8	8	-	-	na	na	na	na	6
			-	-	23		-	-	-	-	-					-
			-	-	-		-	-	-	-	-					7
			-	-	15		-	-	-	-	-					-
			-	23	24		-	-	-	-	-					6
			-	-	-		11	-	-	12	11					-
				20	18		-	-	-	-	-					-
				-	19		17	19	19	18	17					-

Such speculations would be supported by finding the presence of specific metalloproteases (only *o*-phenantroline-inhibited) and their inhibitors, which are typical for such relations (Imamura et al., 2001; Zhang et al., 2003; Harrison et al., 2005; Rohlfs, 2005). We are also considering that the body-surface proteolytic system may be involved in pheromone metabolism (Cornette et al., 2002) indicated by the high proteolytic activities in queens. We are currently starting experiments to isolate specific proteins, as well as to find and identify genes encoding body surface proteases and their inhibitors. This should help to find and better define potential functions of individual body surface proteases and their inhibitors.

Le système protéolytique de la surface corporelle des reines, des ouvrières et des mâles d'abeilles (*Apis mellifera*) : variations du profil des protéases et de leurs inhibiteurs naturels au cours de l'ontogenèse et au cours des saisons.

***Apis mellifera* / protéase / inhibiteur de protéase / zymographie / protéolyse / surface corporelle**

Zusammenfassung – Das proteolytische System der Körperoberfläche von Königinnen, Arbeiterinnen und Drohnen der Honigbiene: Ontogenetische und jahreszeitliche Schwankungen im Profil der Proteasen und ihrer natürlichen Inhibitoren. Bei verschiedenen Organismen, einschliesslich Schaben, Amphibien und Menschen, wurde bereits eine beschränkte Anzahl an Proteasen und/oder Proteaseinhibitoren auf der Körperoberfläche identifiziert. Dabei sind die Unterschiede innerhalb der Invertebraten und im Vergleich mit Vertebraten nur relativ gering. Untersuchungen basierend auf dem Genom der Honigbienen weisen bereits auf eine bestimmte Anzahl neuer Proteine, einschliesslich Kutikulaproteinen, hin, die auch Proteasen umfassen könnten. Ziel dieser Studie war die Beschreibung der Muster, der in vitro Aktivität und der Zymogramme der Proteasen der Körperoberfläche und ihrer natürlichen Inhibitoren im Verlauf der Ontogenese der Honigbiene (*Apis mellifera*). Dabei wurden auch Kasten- und Saisonunterschiede in Betracht gezogen.
Adulte Honigbienen wurden entweder am Stockeingang abgefangen (Arbeiterinnen) oder aus dem Volk entommen (Königinnen und Drohnen). Puppen, Larven und Eier wurden aus Brutwaben gewonnen. Larven der ersten drei Larvenstadien wurden als „kleine Larven" und die des vierten und fünften Larvenstadiums als „grosse Larven" gepoolt. Alle Proben wurden mit destilliertem Wasser abgewaschen, um Verunreinigungen und nicht festhaftende Mikroorganismen zu entfernen. Anschliessend wurde mittels feuchter Baumwollstäbchen die Körperoberfläche abgewaschen. Aus den Baumwollstäbchen wurden dann die biologischen Proben ausgepresst. Für eine zweite Waschung wurde destilliertes Wasser mit 1 % Detergenz verwendet. Allgemeine Proteasenaktivitäten wurden am optimalen pH mittels Hämoglobin als Substrat bestimmt (Tab. I) In den Extrakten waren alle katalytischen Typen an Proteasen vertreten. Bei pH 2,5 hatten die Asparaginproteasen (inhibiert durch Pepstatin und DAN) und Cysteinproteasen (inhibiert durch Iodoacetamid und pCMB) ihre höchsten Werte; bei pH 7,0 erreichten die Cysteinproteasen und Metalloproteasen (inhibiert durch *o*-Phenantrolin) und bei pH 11,5 die Serinproteasen (inhibiert durch PMSF und STI) die höchsten Aktivitätswerte.
Die Bestimmung der allgemeinen Niveaus der Aktivitäten der Proteaseninhibitoren wurde mit Pepsin als Proteasemarker bei saurem pH und mit Trypsin als Markerprotease für neutrale und basische pH-Werte durchgeführt. Elektrophoresen in 10 % Gelen erfolgten bei nichtdenaturierenden Bedingungen, mit 1 % Gelatine als vorgegebenem Proteinsubstrat. In in vitro-Versuchen verglichen wir unter identischen Bedingungen zwei Gruppen proteolytischer Aktivitäten: die neutralen und basischen wasserlöslichen und die sauren, detergenzlöslichen. Die aktivsten Proteasen waren saure Proteasen auf Drohnenpuppen, die im Frühling gesammelt worden waren (Abb. 2), neutrale und basische Proteasen auf adulten Drohnen im Sommer (Abb. 3), neutrale auf adulten Königinnen im Herbst, sowie alle Proteasentypen auf Königinnenpuppen im Frühjahr (Abb. 4 und Abb. 2). Die geringsten Proteaseaktivitäten fanden wir auf Arbeiterinnen im Frühjar (Abb. 2) und auf Drohnen im Herbst (Abb. 4). In allen Fällen waren die Aktivitäten auf Puppen und Imagines höher als auf den anderen Stadien. Im zymographischen Vergleich in Gelen mit Gelatine zeigten sich einige Unterschiede. Die schärfsten Banden mit der höchsten Aktivität fanden wir für neutrale und basische Werte auf Königinnen in allen Jahreszeiten. Die verschwommensten Banden waren für neutrale und basische Werte auf Drohnen in allen Jahreszeiten zu verzeichnen. Bei Arbeiterinnenproben erhielten wir für alle Jahreszeiten sowohl klare als auch verschwommen Banden. Letzteres steht in Übereinstimmung mit den niedrigen Proteaseaktivitäten, die wir für Arbeiterinnen in den in vitro-Versuchen gefunden hatten. Vergleichbar hohe Werte für Proteaseinhibitoren fanden wir bei Drohnen im Sommer (Abb. 6) und bei adulten Königinnen im Herbst (Abb. 7).

Honigbiene / Proteasen / Proteaseninhibitoren / Körperoberfläche-Proteolyse / Zymographie

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