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The mutual influence of proteins from *Varroa destructor* extracts and from honeybee haemolymph on their proteolytic activity – in vitro study

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Abstract

The influence of extracts from *Varroa destructor*, a parasitic mite of the honeybee *Apis mellifera*, on the proteinase activity of worker bee haemolymph was analysed in vitro, along with the influence of bee haemolymph on the proteolytic activity of *V. destructor* extract. The study was conducted in three different environments: pH 7.5 (high activity of bee enzymes and very low activity of parasite enzymes), pH 5 (moderate activity of enzymes from both sources) and pH 3.5 (limited activity of bee proteinases and high activity of mite proteinases). Based on electrophoretic studies, the inhibition of the activity of bee haemolymph proteinases by *V. destructor* extracts was observed at each pH. The study at pH 7.5 with commercial inhibitors of the 4 main classes of proteinases (pepstatin A, ethylenediaminetetraacetic acid (EDTA), E-64 (trans-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane), soybean trypsin inhibitor and Kunitz inhibitor) suggested that parasite extracts mainly inhibited serine proteinases and, to a lower degree, cysteine and aspartyl proteinases. At pH 3.5 and pH 5, a decrease of approximately 40% in parasite proteinase activity was also observed in the presence of bee haemolymph. The result points to the presence of aspartyl proteinase inhibitors in bee haemolymph, which may be an important defence element for bees during food intake by a mite. It was demonstrated that trypsin and trypsin inhibitors are active in the excretion/secretion products of *V. destructor*, the proteinases of which may assist the parasite in food suckling by preventing haemolymph coagulation, among other things.

Keywords

Apis mellifera, inhibitors, honeybee, proteinases, *Varroa destructor*

Introduction

The protein exoskeleton of insects is a strong barrier against most pathogens. Therefore, exoparasites that penetrate the insect cuticle, such as fungi and arthropods, use histolytic enzymes, including specific proteinases, for that purpose. Insects, in turn, fight back by producing inhibitors directed towards parasite proteinases (Bania and Polanowski 1999; Grzywnowicz *et al.* 2009; Cerenius *et al*. 2010; Vilcinskas 2010). *Varroa destructor* Anderson and Trueman 2000, belongs to the group of ectoparasitic bee arthropods and is considered one of the significant factors reducing the western bee population (Rosenkranz *et al*. 2010). This mite feeds on the haemolymph of the capped brood and imagos of *Apis mellifera* L. Hemolymph is the mite's only source of nutrients and metabolic precursor compounds.

In our previous study, we characterised the proteolytic enzymes of *V. destructor* and demonstrated their presence in its excretion/secretion products (Frączek *et al.* 2012). These enzymes may be transferred to the host haemolymph along with other secretions of the parasite salivary glands (Cicero and Sammataro 2010). It is assumed that, in addition to their contribution in disturbing bee body coatings and opening the wound used by larval and adult parasites for food collection, these secretions may facilitate the digestion of haemolymph proteins. Conversely, it is known that the body surface of the bee is equipped with its own system of proteinases and proteinase inhibitors that play a defensive role (Strachecka and

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Grzywnowicz 2008; Grzywnowicz *et al*. 2009). Thus, it is important to investigate the interactions between parasite and host at the level of the proteinases and their inhibitors generated by both. We decided to analyse this issue in vitro on a simplified model. We determined both the influence of soluble proteins in the mite extract on the proteolytic activity of bee haemolymph and, in the reverse configuration, the influence of parasite proteinases on the proteins of bee haemolymph. In this model, the possibility of such interactions had been confirmed, as the presence of proteolytic enzymes and proteins similar to proteinase inhibitors had been previously demonstrated in both the bee haemolymph and the mite secretions. The inhibitors present in *V. destructor* were preliminarily classified as serine, cysteine and aspartyl proteinase inhibitors. It was also demonstrated that, in addition to the previously described serine proteinase inhibitors (Bania *et al*. 1999; Cierpicki *et al*. 2000; Cerenius *et al*. 2010), inhibitors of aspartyl proteinases were present in bee haemolymph.

Materials and Methods

Biological material

The materials for study were collected at the beginning of September 2010 from 30 colonies of *A. mellifera* derived from the Carnolian honeybee naturally infected with *V. destructor*. These colonies were kept at 10 apiaries located within 20 km of Nidzica (north-eastern Poland). The transfer of honeybee combs to the laboratory in Olsztyn lasted approximately 1 h. Immediately following this transfer, mature female mites (n = 2,600) were isolated from the capped cells of honeybee combs. The extracts and excretory/secretory (E/S) products were obtained from them and then prepared according to a previously described procedure (Frączek *et al*. 2012).

Haemolymph was collected from freshly emerged healthy worker bees from the same combs after anaesthetising them briefly with cold (temp. –15°C, 15 min) (Chan *et al*. 2006). Hemolymph was centrifuged 5,000 x g for 10 minutes at 4°C. All collected materials were stored at –70°C until use.

Protein analysis. The protein concentration was determined using the method of Lowry *et al*. (1951).

Proteolytic activity determination. The proteolytic activity in both materials was examined according to the procedure of Mendiola *et al*. (1996) using 2% (w/v) haemoglobin solution as a substrate. The proteolytic activities of the following materials were measured: i) worker bee haemolymph (H), ii) mite extract (V) and iii) a mixture of both materials (worker bee haemolymph and parasite extract, HV). In the first case, the incubation mixture contained 25 µL of haemolymph (15 µg of protein); in the second case, the mixture contained 25 µL of extract from *V. destructor* (15 µg of protein); and in the third case, the 25 µL volume contained 15 µg of haemolymph proteins and 15 µg of mite extract proteins. The material was diluted with 0.9% NaCl to obtain a suitable protein content in analysed samples. A 225 µL volume of Theorell and Steinhagen buffer (Küster and Thiel 1993) pH 3.5, pH 5.0 or pH 7.5 was added to each sample and allowed to incubate for 10 minutes at 37°C. Then the reaction was initiated by the addition of 50 µL of 2% haemoglobin solution. The incubation lasted 30 minutes. The reaction was terminated by the addition of 300 µL of 10% TCA. After careful mixing and a 15 min incubation at 4°C, the samples were centrifuged for 10 min at 3,000 x g. The concentration of peptides released from haemoglobin by proteinases was determined in the supernatant (Frączek *et al*. 2012). The activity was expressed as µg of released peptides per 1mg of protein from sample.

Zymography – Zymography was performed by SDS-PAGE using 12.5% polyacrylamide gels containing 0.1% gelatine in the presence of sodium dodecyl sulphate (SDS) according to Felicioli *et al*. (2004). Haemolymph (15 µg of protein), mite extract (15 µg of protein), and a mixture of haemolymph (15 µg of protein), and mite extract (15 µg of protein) were used in the applied probe. After electrophoresis, the gels were washed with gentle shaking at room temperature in a 2% (v/v) aqueous solution of Triton X-100 for 30 min to remove the SDS and restore the full activity of the proteinases. The gels were then rinsed with Theorell and Steinhagen buffer at pH 3.5, pH 5 or pH 7.5. The gels were transferred to Petri dishes filled with the above buffers and incubated for 12 h at 37°C prior to staining with 0.1% colloidal Coomassie Brilliant Blue G-250. 12-hour gel incubation. The active fractions appeared as unstained bands on a blue background.

Study of the effects of commercial inhibitors on haemolymph proteinases by SDS-PAGE – The inhibition of the natural proteinases was performed before electrophoresis using the following inhibitors (all inhibitors from Sigma): 16 µM soybean trypsin inhibitor, 16 µM bovine pancreatic trypsin inhibitor (Kunitz), 30 µM E-64 (trans-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane); cysteine proteinase inhibitor) and 30 µM EDTA (ethylenediaminetetraacetic acid; metalloproteinase inhibitor) and 16 µM in 1% methanol pepstatin A (aspartyl proteinase inhibitor). The first four inhibitors were dissolved in water. A 15 µL sample of haemolymph containing 15 µg of protein was preincubated with each inhibitor for 15 min at 37°C. Electrophoresis and the enzymatic reactions were then performed as described above on gels containing 0.1% gelatine (omitting the incubation of the gel at pH 5). The per cent inhibition was determined by densitometry. The activity of the control probe (without inhibitors) was considered to be 100%.

Electrophoretic detection of the chymotrypsin and trypsin inhibitors activity (Uriel and Berges 1968) – SDS-PAGE electrophoresis was conducted as described above. Haemolymph (15 µg of protein), mite extract (15 µg of protein), and mite E/S products containing 15 µg of protein were used in the applied probe. After separation and washing in Triton X-100, the gels were transferred to Petri dishes containing 25 mL of chy-

Table I. The proteolytic activity of worker bee hemolymph and mite extract (μg of soluble peptides/mg proteins)

Material	The activity (μg of soluble peptides/mg proteins)		
	pH 7.5	pH 5.0	pH 3.5
Worker haemolymph (H)	90.01 ± 7.47* a, A**	57.69 ± 2.72 a, B	36.92 ± 2.36 a, C
Mite extract (V)	17.79 ± 1.35 b, A	21.45 ± 1.55 b, B	32.80 ± 2.06 a, C
Worker haemolymph + mite extract (HV)	23.45 ± 1.09 c, A	18,62 ± 1.15 b, B	16.17 ± 0.71 c, B

Explanations: *Mean ± SD, **Different small letters imply significant differences between means presented in column and different capital letters imply significant differences between means presented in row (P<0.05).

motrypsin from bovine pancreas (0.5 mg; C4129, Sigma) or trypsin from bovine pancreas (0.5 mg; T8003, Sigma) solution in 0.1 M phosphate buffer, pH 7.4. The gels were incubated for 15 minutes at 37°C with gentle mixing. Next, the gels were transferred to a staining mixture for 45 minutes. The fractions that contained serine proteinase inhibitors remained unstained on a dark red gel background.

Statistical analysis – Student's t test was used to determine significant differences between means; $p < 0.05$ was considered to be significant.

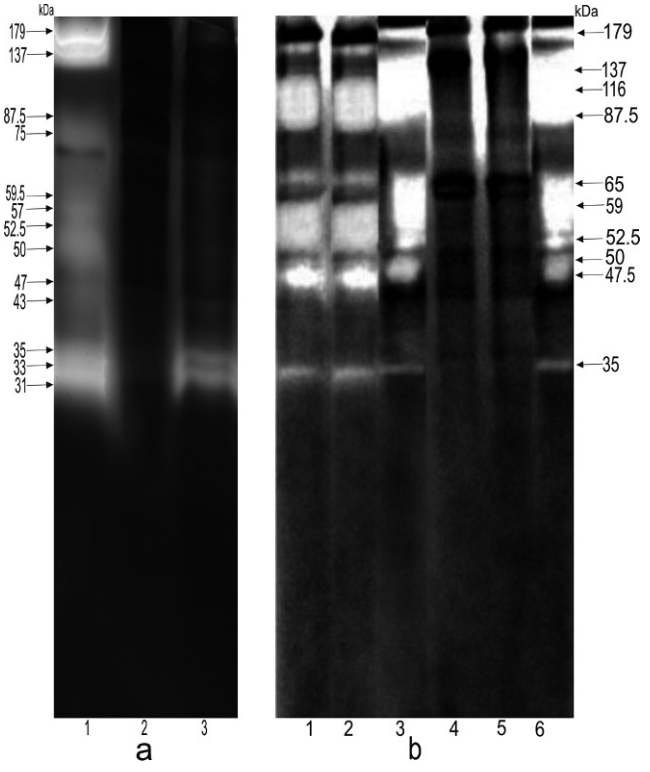

Fig. 1. Zymography PAGE after incubation at pH 7.5. Fig. 1a – lane 1, bee haemolymph; lane 2, isolated mite extract, lane 3, a mixture of bee haemolymph + mite extract. Fig. 1b – bee haemolymph exposed to commercial proteinase inhibitors. Lane 1, E-64; lane 2, EDTA; lane 3, pepstatin A; lane 4, soybean trypsin inhibitor; lane 5, bovine pancreatic trypsin (Kunitz) inhibitor; lane 6, bee haemolymph without inhibitors. Proteins were separated on a 12% gel with 1% gelatine. The slab was stained with Coomassie Brilliant Blue

Results

The proteinases of *A. m. carnica* worker bee haemolymph were characterised by high activity in an alkalane environment. This activity decreased with an increase in the acidity of the environment (Table I). The reverse situation was observed for the proteinases from *V. destructor* extract, the activity of which increased with a decrease in pH. When comparing the results from Table I, it can be observed that the overall proteolytic activity in a sample containing a mixture of haemolymph and mite extract proteins (HV) was not the sum of the individual activities of proteinases from both sources, but was instead decidedly lower than the individual activities. At pH 7.5, the activity of the HV mixture was 3.8-fold lower than

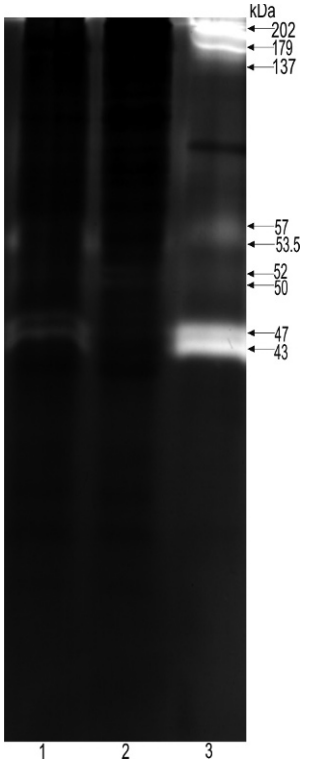

Fig. 2. Zymography PAGE of a mixture of bee haemolymph + mite extract (lane 1), isolated mite extract (lane 2) and isolated bee haemolymph (lane 3) after incubation at pH 5

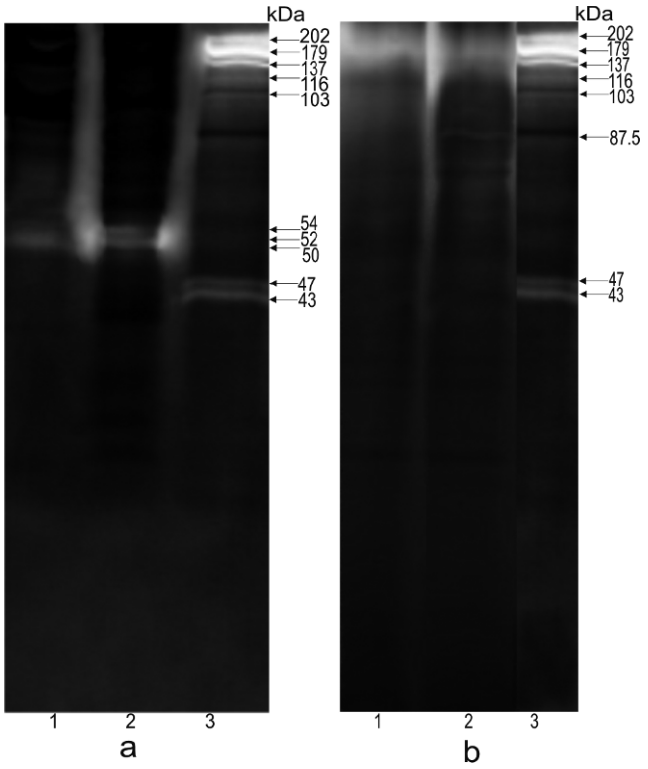

Fig. 3. Zymography PAGE after incubation at pH 3.5. Fig. 3a – lane 1, a mixture of bee haemolymph + mite extract; lane 2, isolated mite extract and lane 3, isolated bee haemolymph. Fig. 3b – bee haemolymph exposed to two commercial proteinase inhibitors. Lane 1, pepstatin A; lane 2, E-64; lane 3, bee haemolymph without inhibitors

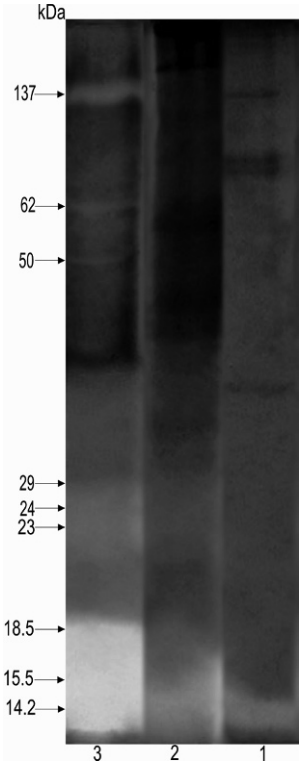

Fig. 4. Visualisation of the chymotrypsin inhibitor activity of mite E/S products (lane 1), mite extract (lane 2), and bee haemolymph (lane 3). The chymotrypsin activity was detected according to the method of Uriel and Berges (1968)

that of worker bee haemolymph and only 32% higher than the very low activity of the mite extract in this environment. The above results were confirmed by zymograms (Fig. 1, 2, 3). After the incubation of gel, at least 13 active protein fractions were observed in bee haemolymph at pH 7.5, forming wide hydrolysis zones (Fig. 1a, lane 1). In turn, the parasite extract, which was barely active at this pH, formed a poorly visible lane (Fig. 1a, lane 2).

Concurrently, a very high inhibition of proteolytic activity, originating mainly from the bees, was noted in the HV mixture (Fig. 1a, lane 1 vs lane 3). A similar decrease in the gelatinolytic activity of the protein mixture (HV) was visible after incubation of the gel at pH 5 and pH 3.5 (Fig. 2 and Fig. 3a, lane 1 vs lane 3). Based on zymograms incubated in acidic environments where the activity of the mite proteinases is higher (at pH 5 and pH 3.5), it was established that the inhibition affects not only the enzymes of bees but those of mites as well. The percent inhibition determined by densitometry was 41.4% and 45% respectively (Fig. 2 and Fig. 3a, lane 1 vs lane 2).

To determine the types of bee haemolymph proteinases inhibited by parasite proteins, electrophoresis of bee haemolymph was conducted after its preincubation with commercial inhibitors directed towards proteinases belonging to 4 basic classes. An image obtained after gel incubation at pH 7.5 (Fig. 1b) showed that the proteolytic activity of haemolymph was almost entirely blocked by serine proteinase inhibitors, including a type of Kunitz inhibitor and the soybean trypsin inhibitor. The haemolymph proteolytic activity at pH 7.5 was also decreased by approximately 20% following incubation with E-64 and EDTA, whereas pepstatin A did not induce a change in activity. In turn, it was demonstrated that the 202–137 kDa, 47 kDa and 43 kDa fractions of bee haemolymph that were active at pH 3.5, all of which were inhibited by the incubation of haemolymph with mite extract (Fig. 3a, lane 1 vs 3), were blocked by pepstatin A and by E-64, which are inhibitors of aspartyl and cysteine proteinases, respectively (Fig. 3b, lanes 1 and 2 vs lane 3). The 202–137 kDa fractions were inhibited by them only partly by 39% (pepstatin A) and 47% (E-64). The presence of serine proteinase inhibitors in the extract and E/S products of *V. destructor* and in bee haemolymph was confirmed by the electrophoretic migration of the proteins from both bee and mite that inhibited chymotrypsin and trypsin activity. Chymotrypsin inhibitors from E/S products and mite extract had low molecular masses, approximately 14–15 kDa (Fig. 4, lanes 1 and 2). Bee haemolymph possessed a larger range of inhibitors acting on chymotrypsin. In addition to the low molecular mass chymotrypsin inhibitors at 14–18.5 kDa and 23–29 kDa, inhibitors of high molecular mass (50 kDa, 62 kDa and 137 kDa) were also observed (Fig. 4, lane 3).

In the case of trypsin, the image was similar, but the inhibition of its activity was weaker. We were not able to obtain zymograms of a satisfactory quality; thus, these images were not included.

Discussion

The serine proteinases of insects contribute to the regulation of a few innate defensive responses connected to blood coagulation, synthesis of antimicrobial peptides and surface melanisation (Muta and Iwanga 1998; Zou *et al*. 2006; Vilcinskas 2010). The presence of inhibitors of thrombin, the main serine proteinase responsible for mammal haemostasis, has been observed in numerous blood-sucking arthropods (Liao *et al*. 2009). We suspect that serine proteinase inhibitors from *V. destructor* extracts and E/S products found in these studies may also limit bee haemolymph coagulations, thus facilitating haemolymph uptake by a mite. Richards *et al*. (2011) suggested that the salivary secretions of the mite act by suppressing haemocyte-mediated wound healing and plugging responses in the host. In the honeybee genome, as many as 44 genes encoding serine proteinases are present, most of which are probably secreted enzymes (Zou *et al*. 2006). Some serine proteinases act in the alimentary tract, while other proteinases are secreted into the haemolymph from the fat body and haemocytes and are potentially responsible for immunological reactions. Changes in the expression of genes encoding several serine proteinases following microbial challenge in larvae and adult honeybees in response to infection support this hypothesis (Zou *et al*. 2006). The presence of at least 5 genes encoding serine proteinase inhibitors belonging to the serpin family is also observed in the genome of *A. mellifera* (Zou *et al*. 2006). These proteins are high molecular mass inhibitors of approximately 40-50 kDa. In addition to these serpin family inhibitors, bee haemolymph also contains low molecular mass serine proteinase inhibitors with masses of approximately 10 kDa that mainly belong to the family of Kunitz type inhibitors. Cathepsin/chymotrypsin inhibitors AMCI 1-3 of the Kunitz type have been isolated and characterised from the haemolymph of *A. mellifera* larvae (Bania *et al*. 1999; Cierpicki *et al*. 2000). In our study, in addition to the inhibitors discussed above, we observed the presence of chymotrypsin inhibitors with a mass of approximately 62 kDa, which corresponds to the molecular mass of chymotrypsin inhibitor from human blood serum, and an inhibitor with a very high molecular mass of 137 kDa (Fig. 4). The highest activity of chymotrypsin inhibitors was, however, observed in low molecular mass proteins. These inhibitors are a very significant element of insects' protection against entomopathogens such as bacteria and fungi (Strachecka and Grzywnowicz 2008; Vilcinskas 2010). In the case of the infestation of bee colonies with *V. destructor*, proteinase inhibitors may play a similar role because this mite is a vector of fungal, bacterial and viral infections (Genersch 2010). Due to the low activity of serine proteinases in a parasite, inhibitors of that class of proteinases are not able to strongly inhibit its proteolytic activity because mites mainly contain aspartyl proteinases (Frączek *et al.* 2009, 2012). However, proteins inhibiting the proteolytic activity of mites E/S are present in bee haemolymph. It was demonstrated in our densitometric measurements that the activity of the two main proteinases of the mite was decreased by approximately 40% in the presence of *A. mellifera* haemolymph at pH 3.5, which is optimal for the activity of *V. destructor* proteinases (Fig. 3a). This is consistent with Tewerson and Jany (1982) result, who observed the intact, antigenic host proteins in mite eggs. They suspected the presence in absorbed by parasite from bee haemolymph the factors inhibiting their own proteolytic enzymes.

It was demonstrated in this study that the reverse interactions are also possible, i.e., the inhibition of bee haemolymph proteinases by mite proteins. In extracts from *V. destructor*, inhibitors that were highly active towards proteinases from host haemolymph were observed at all analysed pH values. The comparison of worker bee haemolymph zymograms incubated at pH 7.5 demonstrated that zones indicating the inhibition of proteolysis by commercial inhibitors and proteins extracted from mites suggested the presence of metalloproteinases, cysteine proteinases, a dominant level of serine proteinases and a low level of aspartyl proteinases in bee haemolymph. These proteinases were inhibited by EDTA, E-64, bovine and soybean trypsin inhibitors of the Kunitz type and pepstatin, respectively (Fig. 1a vs Fig. 1b). Therefore, it may be hypothesised that inhibitors acting on the classes of proteinases listed above are present among the soluble proteins of *V. destructor*.

Anticoagulation factors, including thrombin inhibitors belonging to the Kunitz family, have been observed in the salivary glands, saliva, intestine and haemolymph of ticks (Martiz-Olivier *et al*. 2007, Liao *et al*. 2009). In addition to the thrombin inhibitor, a cysteine proteinase (legumain) is also present in the mid-intestine of *Haemaphysalis longicornis*. This enzyme takes part in host blood digestion (Alim *et al*. 2008). Previous studies confirmed the possibility of the introduction of an inhibitor from the tick *Boophilus microplus* into the host bloodstream (Willadsen and Riding 1980). A similar situation may be observed in the case of *V. destructor*. We cannot exclude the possibility that the proteinase inhibitors present in the mite's E/S products may be transferred to bee haemolymph in the process of suckling. We detected a serine proteinase inhibitor with a low molecular weight ca. 17 kDa, in the E/S products of the mite, and protein of the same weight was recently detected by Richards *et al*. (2011) in pure salivary secretions from *V. destructor*. We can suppose that a similar as in vitro activity on the proteolytic enzymes of bee haemolymph induced by mite proteinases and proteinase inhibitors may be observed in vivo. In turn, the observed inhibition of the proteolytic activity of

mite extract induced by bee haemolymph in vitro suggests that its proteinase inhibitors may act protectively by limiting the activity of *V. destructor* proteinases to some degree (Frączek *et al*. 2012).

The obtained results fit well with the theory of the co-evolutional adjustment of parasite proteinases and host proteinase inhibitors presented by Vilcinkas (2010). This theory highlights the improvement and formation of mechanisms that allow the achievement of a specific equilibrium and a limitation of the costs of organism coexistence to the highest possible degree in both sides of the parasite-host system. For a mite, it is profitable to use a wide range of proteinase inhibitors. These proteins are important in nutrition and in the mite's strategy to subvert host defences. The system of proteinases and their inhibitors in a parasite may be an important factor in the maintenance of the non-healing wound in the bee cuticle formed by mite females, which is used for the constant collection of haemolymph by *V. destructor* adults and nymphs (Cicero and Sammataro 2010). Concurrently, the fact that *V. destructor* mainly possesses aspartyl proteinases limits the possibility of their complete blockade by the proteinase inhibitors of bee haemolymph, its main food source. This system does not offer full protection, however, because proteins that decrease the activity of mite proteinases are present in bee haemolymph.

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