THE ACTIVITY OF NINETEEN HYDROLASES IN EXTRACTS FROM *Varroa destructor* AND IN HEMOLYMPH OF *Apis mellifera carnica* WORKER BEES

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Summary

The activity of 19 hydrolases in extracts of *Varroa destructor* mite and hemolymph of *Apis mellifera carnica* honey bee (a host) were studied. Within parasite's esterases high activity of alkaline and acid phosphatase, low esterase (C4) and esterase (C8), and no activity of lipase (C14) was noticed. Among its peptidases the activity of leucine arylamidase was high, and a low activity of valine, cysteine arylamidase and trypsin was detected. However, no activity of chymotrypsin was observed. Except for α-galactosidase, α-mannosidase, a high activity was observed in the rest of the α- and ß-glycosidases. The activity for all 19 enzymes was found in the hemolymph of worker bees. It should be noticed that the relative activity of most of the enzymes was on almost the same level in the host as in the parasite. However, the activities of glycosidases; α-fucosidase, ß-glucuronidase and α-galactosidase were higher in the parasite than in the honey bee hemolymph.

Keywords: *Apis mellifera carnica*, *Varroa destructor*, enzyme, hydrolase.

INTRODUCTION

Varroatosis is one of the most serious diseases of honey bees (*Apis mellifera*). It is caused by ectoparasitic bee mites of the genus *Varroa*. This genus consists of four species; *Varroa jacobsoni*, *V. destructor*, *V. underwoodi* and *V. rindereri*. These species differ in morphology, genetics and host specificity (Anderson and Trueman 2000, Akimov et al. 2004, Munoz et al. 2008). *V. destructor* is the widely spread mite that parasitizes on honey bees (*Apis mellifera*). Korean haplotype is the most widely spread species in Europe based on mitochondrial DNA studies. The most serious problem for beekeepers, including those in Poland, is the dynamic growth of the parasite and its pathogenic action. Emerging bees from infected pupae are smaller in size, lighter and very often have undeveloped wings and shortened abdomens. Their average length of life is shortened (De Jong et al. 1982; Schneider and Drescher 1987, Marcangeli et al. 1992). Moreover, decreased immunity is also caused by the mites. This decreased immunity often results to infections by viruses, bacteria and fungus, because *V. destructor* is also a vector for lots of bee pathogens (Bowen-Walker et al. 1999).

Parasites feeding on the hemolymph of honey bee capped brood cause disturbances in the protein metabolism of the host. This destroys the neurohormonal balance and resistance mechanisms of honey bees (Gliński and Jarosz 1984, 1988, Weinberg and Madel 1985).

The study results deal with the control of varoatosis. A lot of papers are published on the subject of the drug-resistance of the parasite (Gerson et al. 1991,Sammataro et al. 2005, Chmielewski et al. 2007, Pohorecka and Bober 2007, Lipiński and Szubstarski 2007). The biology of the development of *V. destructor* is being studied, including semiochemical interactions between the parasite and the honey bee (Salvy et al. 2001, Taylor et al. 2008, Nazzi et al. 2009). There is also a low number of data concerning the host-parasite relationship on the molecular level, particularly biochemical papers directed at the study of the parasite. Studies were done regarding catabolism of bee hemolymph proteins in *V. destructor*, activity of esterases, proteolytic enzymes, glutathione transferases of parasite, and presence of allergens (Tewarson 1981, Tewarson and Engels 1982, Tewarson and Jany 1982, Gerson et al. 1991, Dandeu et al. 1991, Fernandez-Caldas et al. 2007). In addition to the mechanical damage of integuments, the secretion enzymes of the parasite play a role in the invasion of the host. Chitinase and proteases play an important role in the degradation of the host's cuticle (Mira 2000, Colin et al. 2001).

High chitinolytic activity and lack of activity of endoproteases in the adult *V. destructor* were demonstrated by using the API ZYM assay (Colin et al. 2001). This assay enables the simultaneous measurement of 19 hydrolytic enzymes that belong to three subclasses. These three subclasses are: esterases, proteases and glycosidases. The API ZYM assay was successfully used for the comparative study of hydrolases of ectoparasitic and free-living mites. This assay was also successfully used for studies concerning the metabolic effects in the infection of *A. m. carnica* larvae by entomopathogenic nematode. The assay was used as well in the measurement of changes of hydrolases activities in ontogenesis of *A. m. carnica* drone (Nisbet and Billingsley 2000, Żółtowska et al. 2003, 2007).

Confirmation or verification of the Colin studies (Colin et al. 2001), is the main aim of this paper. According to us, the Colin et al. studies were carried out on too low number (20) of parasite individuals. Moreover, we did a comparison of hydrolytic enzym activities from extracts of adult *V. destructor* females, with activities of hemolymph enzymes of worker bees (*A. mellifera*). The same API ZYM assay was used. The assimilation of enzymes with proteins, which are being taken from host hemolymph, was also taken into consideration (Tewarson and Engels 1982).

MATERIAL AND METHODS

Adult *V. destructor* females were collected from naturally infected *A. mellifera carnica* colonies. Parasites were isolated at the end of July, 2008 from honeycomb cells selected from fifty-colonies. They were from an *A. m. carnica* apiary situated ca. 20 km west of the city of Olsztyn. Simultaneously, foragers (n=200) were collected from alighting boards of beehives.

1. Preparation of extract from *V. destructor*

Collected parasites were washed 4 times by submergence in distilled water for 15 min, followed by gentle shaking. After drying, 30 to 90 mites were randomly taken for further analyses. They were grinded in a porcelain mortar, placed on an ice bath with 2 ml of distilled water and left at 4°C for 1 h. Homogenate was centrifuged for 10 min at 5.000 x g at 4°C. The sediment was discarded and supernatant was used for further studies.

2. Preparation of hemolymph samples of worker bees (*A. m. carnica*)

Worker bees were anaesthetized in cold condition at -15°C. Hemolymph from 125 forager bees was collected according to Chan et al. (2006). Hemolymph was diluted 2.5-fold with 0.9% NaCl. Then the mixture was centrifuged twice at 5000 x g for 10 min at 4°C.

3. Protein determination

Protein concentration was determined by the Bradford (1976) assay in supernatants.

4. Determination of hydrolytic activity of enzymes by API ZYM assay

Determination of enzymatic activity in extract of *V. destructor* was done by the API ZYM assay (Biomérieux, France), according to the manufacturer's directions. The volume of 50 µl of parasite extract was put into wells. This volume corresponds to the range of 0.75 or 2.25 mite specimen per assay for 30 or 60 mite extracts respectively. On the other side, a volume of 40 µl diluted worker bee hemolymph was put into wells. Test strips were incubated for 4.5 h at 37°C. After incubation, reagents were put inside the wells for dye reactions. Results were estimated in a range from 0 to 50 based on the colour intensity of studied samples compared to that in the control group, according to Colin et al. (2001).

RESULTS

The concentration of protein in the extract from 30 or 90 mites was very low, ranging from 3 µg/ml or 8 µg/ml, respectively. The level of protein in a single well of API ZYM assay for extracts from *V. destructor* was: 150 ng and 400 ng, respectively. This protein level was a couple of times higher for bee hemolymph 13.1 µg.

Data presented in Table 1 showed the activity of 17 out of 19 studied hydrolases in both extracts from the parasite. Within the enzymes belonging to esterases the activity of alkaline and acid phosphatase was high in the extracts. However, the activity of esterase (C4) and esterase (C8) was low, and no activity of lipase (C14) was noticed.

Within aminopeptidases, the activity of leucine arylamidase was 8-times higher in both of the extracts from mites, and 4-times higher for valine arylamidase in comparison to cysteine arylamidase. The activity of trypsin was very weak. No activity of chymotrypsin was observed in extracts from *Varroa*.

The activity of glycosidases was diverse in these extracts. In this subclass, the highest activity in both extracts had ß-glycosidases; ß-galactosidase, ß-glucosidase and N-acetyl-ß-glucosaminidases. Moreover, the activity of some α-glycosidases, α-glucosidases or α-fucosidases, especially in a sample with a higher content of studied material, was relatively high. However, α-galactosidase and α-mannosidase showed a very low activity in comparison to the rest of the glycosidases. Of the 19 enzymes presented in bee hemolymph, esterases of phosphoric esters had a very high activity. Moreover, esterases of short-chain fatty acids (C4 - C8) were also relatively active. However, there was only a trace of lipase (C14) activity. Within proteolytic enzymes, leucine arylamidase had a very high activity. Relatively high activity was found for valine arylamidase. Weak activity was observed for cystine arylamidase, trypsin and chymotrypsin. Of the glycosidases, the most active were ß-galactosidase and α-glucosidase, N-acetyl-ß-glucosamidases, ß-glucosidases and α-mannosidases. The remaining glycosidases had vestigial activity in hemolymph.

DISCUSSION

Enzymatic activity is an indicator of metabolism intensity. It changes during

Table 1

The activity of hydrolases in the extracts from *V. destructor* and hemolymph of *A. m. carnica*

No.	Enzyme	Substrate	pH	Activity (nmol)		
				V. destructor		*A. mellifera*
				(n=30)	(n=90)	(n=125)
Esterases						
1	Alkaline phosphatase	2-naphtyl phosphate	8.5	10	20	40
2	Acid phosphatase	2-naphtyl phosphate	5.4	30	30	30
3	Naphtol-AS-BI-phosphohydrolase	Naphtol-AS-BI-phosphate	5.4	30	40	40
4	Esterase (C4)	2-naphtyl butyrate	6.5	10	10	20
5	Esterase lipase (C8)	2-naphtyl caprylate	7.5	10	10	20
6	Lipase (C14)	2-naphtyl myristate	7.5	0	0	2.5
Peptidases and Proteases						
7	Leucine arylamidase	L-leucyl-2-naphtylamide	7.5	20	20	40
8	Valine arylamidase	L-valyl-2-naphtylamide	7.5	10	10	20
9	Cystine arylamidase	L-cystyl-2-naphtylamide	7.5	2.5	5	7.5
10	Trypsin	N-benzoyl-DL-arginine-2-naphtylamide	8.5	2.5	7.5	10
11	Chymotrypsin	N-glutaryl-phenylalanine-2-naphtylamide	7.5	0	0	5
Glycosidases						
12	α-galactosidase	6-Br-2-naphtyl-α-D-galactopyranoside	5.4	2.5	10	2.5
13	ß-galactosidase	2-naphtyl-ß-D-galactopiranoside	5.4	40	40	40
14	ß-glucuronidase	Naphtol-AS-BI-ßD-glucuronide	5.4	10	30	5
15	α-glucosidase	2-naphtyl-α-D-glucopiranoside	5.4	20	40	40
16	ß-glucosidase	6-Br-naphtyl-ß-D-glucopyranoside	5.4	30	30	20
17	N-acetyl-ß-glucosaminidase	1-naphtyl-N-acetyl-ß-D-glucosaminide	5.4	30	30	30
18	α-mannosidase	6-Br-2-naphtyl-α-D-mannopyranoside	5.4	10	10	10
19	α-fucosidase	2-naphtyl-α-L-fucopyranoside	5.4	10	30	2.5

ontogenesis (Żółtowska et al. 2007). This activity also depends on the system host - parasite (Nisbet and Billingsley 2000, Mira 2000). The activity undergoes changes in response to the action of other biotic and abiotic factors (Mozes-Koch et al. 2000, Sammataro et al. 2005). Studies of hydrolytic activity in *V. destructor* extract, using API ZYM assay (Colin et al. 2001) have show out activity in 12 of 19 studied hydrolases. Colin and coworkers pointed out the fact that in their findings, the negative results of the low enzymatic activity of some enzymes in mite extracts, do not have to mean the lack of their specific activity. We decided to confirm this explanation by using a higher number of parasites for extract preparation. Extracts were prepared from material that was half or 4.5-times higher than the 20 specimens used by Colin et al. (2001). To exclude morphological diversity related to the study season, material was collected at the same time and from the same source. Influence of the study season on morphological diversity was described for the Ukrainian population of Korean haplotype *V. destructor* parasitising on *A. mellifera* (Akimov et al. 2004). Probably, this procedure was the main

reason of the high repeatability of our results. Colin and coworkers (2001) demonstrated relatively high diversity regarding frequency as well as degree of enzyme activities in mites derived from different regions of France. Unfortunately, they did not mentioned information about the time of collecting specimens.

Firstly, the determination of protein content was done in the extracts of *V. destructor* that were obtained by us from a different number of parasites. Although the protein concentration in the extract of 90 mites was almost 3 times higher in comparison to the protein level from 30 parasites, it was very low in the studied sample. Our results showed a lack of difference in many enzyme profiles in extracts from 30 as well as 90 parasites, as well as application for different protein concentrations in two simultaneous tests. What was more the test extracts prepared by us from more than 120 parasites had shown similar results in the preliminary tests as an extract made out of 90 parasites. Studied extracts were active in relation to the same 17 substrates. We found out that the extract should be prepared from at least 30 females of *V. destructor* for proper enzymes studies by the API ZYM assay. The low concentration of enzyme in the material studied by Colin et al. (2001) seemed to be a cause of their low number of positive results in comparison with our studies. In spite of the above fact most of our observations tally with each other. In the sub-class of esterases, high activity was observed for acid phosphatases, alkaline phosphatases and phosphatases which have an effect on aromatic phosphates. This set of enzymes indicates that the rate of metabolism is high in parasite's cells. Similar results were obtained for phopshatases by Nisbet and Billingsley (2000) for extracts from four species of mites from three phylogenetic orders and with differing dietary habits (*Dermanysses gallinae*, *Psoroptes* ovis, *Acarus siro* and *Tetranychus urticae*). Surprisingly the whole enzymatic profile of *V. destructor* had more in common with *A. siro*, an important pest feeding on stored produce, e.g. flour and grain, than to profiles of the animal parasitic mites *D. gallinae* and *P. ovies* (Nisbet and Billinsley 2000). Esterases, which have an effect on esters made by the short-chain fatty acids (C4 and C8), had a low activity in *V. destructor* extract. No activity of lipase C14 was observed. Researchers also connect the high activity of esterases in *Varroa* with the pesticide resistance in these parasites. They are resistant to such pesticides as Amitraza, Fluvalinate, Coumaphos (Gerson et al. 1991, Mozes-Koch et al. 2000, Sammataro et al. 2005).

Besides the relatively low activity of endoproteases, Tewarson and Jany (1982) have shown the presence of exopeptidases; carboxypeptidase A and aminopeptidases in parasite extract. Our results together with Colin et al. (2001) have confirmed a high activity of leucine aminopeptidases. The activity of valine and cystine aminopeptidases in *V. destructor* extracts was also shown by us. However, this activity was significantly lower than leucine aminopeptidase. This enzyme activity was not confirmed by Colin et al. (2001). Activity of aminopeptidases in the extract of *Varroa* suggest that aminopeptidases have an important function in the host's protein hydrolysis. This process could be a good source of amino acids for mites (Telfer and Kunkel 1991). Colin and coworkers (2001) established a lack of chymotrypsin in extracts of *V. destructor.* This was also confirmed in our studies. They did not show trypsin activity. This is contradictory to our tests. We observed very weak trypsin enzyme activity, especially in extracts from 30 parasites. Our results are consistent with

previous ones that were obtained by Tewarson and Engels (1982) regarding proteolytic activity of extracts from *V. jacobsoni*.

For most of the glycosidases our results are in accordance with the data obtained by Colin et al. (2001). The differences are only with the enzymes that have low activity; α-galactosidases and α-mannosidases. Thus, they could not be detected by Colin et al. (2001). The high activity of ß-galactosidase, ß-glucosidase, ß-glucuronidase and α-fucosidase mean an ability of *V. destructor* to hydrolyze glycoproteins. Glycoproteins are structural elements of cell surface, determinants of their histocompatibility, elements of intracellular substance, and also regulators and immune proteins of organisms. Colin et al. (2001) linked the very high activity of N-acetyl-ß-glucosaminidase in extracts of *V. destructor*, with its role in the hydrolysis of the host chitin and in keeping open-wound that serves for feeding the female-mother and her offspring.

There was a significantly higher content of protein in bee hemolymph than in the studied extracts from *V. destructor*, and all tested enzymes were active in the hemolymph of the worker bees *A. mellifera* (Table 1). By comparing our results for worker bees, with those for emerging drone (Żółtowska et al. 2007), significant differences in pattern and level of enzyme activities could be noticed. These results demonstrate metabolic differences between both casts (Hrassing and Crailsheim 2005).

Most of the studied enzymes had a similar profile and level of relative activity, both in host and in parasite (Table 1). Undetected mite activity of lipase (C14) and chymotrypsin were very weak in bee hemolymph. Similar low activities were observed for the peptidases; cystine arylamidase and trypsin, and one glycosidase (α-mannosidase) in both organisms.

Differences concern mainly activity of glycosidases; α-fucosidase,ß-glucuronidase and α-galactosidase. These enzymes were significantly more active in the parasite than in the host. This observation confirmed the importance of these enzymes for mite in the hydrolysis of the host's glycoproteins. This is especially true in hydrolysis of proteoglycans that also occur in larvae cuticle as well in adult insects. Colin et al. (2001) drew attention to special glycoproteins (hexamerins) that occur in high concentration in bee hemolymph (Danty et al. 1998) and can be equally important for the parasite as a source of nitrogen and carbon.

The obtained results conclude that we confirmed and extended Colin et al. (2001) results to include the activities of five enzymes to the list of hydrolases present in *V. destructor*. Our data are in agreement with the finding of Nisbet and Billingsley (2000). They found that mites from sub-class Acari are adapted to their different food sources. *V. destructor* contains enzymes with the high activity necessary for the digestion of carbohydrates and glycoprotein present in honey bee hemolymph.

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AKTYWNOŚĆ DZIEWIĘTNASTU HYDROLAZ W EKSTRAKTACH Z *Varroa destructor* ORAZ W HEMOLIMFIE ROBOTNIC *Apis mellifera carnica*

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Streszczenie

Badano aktywność hydrolaz rozkładających białka, tłuszcze i cukry w wyciągu z roztoczy *Varroa destructor* oraz w hemolimfie robotnic pszczoły miodnej podgatunku *Apis mellifera carnica* stosując półilościowy test API ZYM. W ekstraktach z pasożyta stwierdzono aktywność 17 z 19 badanych hydrolaz. Wśród esteraz pasożyta wysoką aktywność miały fosfatazy alkaliczna i kwaśna zaś esterazy C4 i C8 niską. W wyciągach z pasożyta nie wykazano aktywności lipazy C14 oraz chymotrypsyny. Wśród proteaz wyższą aktywność miały aminopeptydazy leucynowa i walinowa niż cystynowa, aktywność trypsyny była bardzo niska,. W podklasie glikozydaz najwyższą aktywność miały kolejno: ß-galaktozydazy, ß-glukozydazy i N-acetylo-ß-glukozaminidazy, α-glukozydazy i α-fukozydazy, natomiast niską aktywność α-galaktozydaza i α-mannozydaza

W hemolimfie robotnic pszczoły miodnej aktywne były wszystkie badane enzymy (19), wśród nich wysoką aktywność miały esterazy estrów fosforanowych oraz esterazy C4 i C8, zaś aktywność lipazy C14 była śladowa. Wśród peptydaz bardzo wysoką aktywność miała aminopeptydaza leucytowa, wysoką aminopeptydaza walinowa, niską aminopeptydaza cysteinowa, trypsyna i chymotrypsyna Wśród glikozydaz najbardziej aktywne były: ß-galaktozydaza, α-glukozydazy i N-acetylo-ß-glukozaminidazy, niższą aktywność miały ß-glukozydaza i α-mannozydaza, zaś aktywność ß-glukuronidazy i α-fukozydazy była bardzo niska.

Poziomy aktywności większości badanych enzymów były zbliżone u żywiciela i pasożyta. Główne różnice dotyczą α-fukozydazy, ß-glukuronidazy i α-galaktozydazy, które były znacznie bardziej aktywne w ekstraktach z *V. destructor* niż w hemolimfie pszczół. Sugeruje to, że enzymy te mogą mieć duże znaczenie w rozkładzie glikoprotein pszczoły przez roztocza, w tym w hydrolizie proteoglikanów kutikuli żywiciela.

Słowa kluczowe: *Apis mellifera carnica*, *Varroa destructor*, enzymy, hydrolazy.