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VERSITA

Proteolysis on the body surface of pyrethroid-sensitive and resistant *Varroa destructor*

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Abstract

The aim of this work was to determine the activity of proteases and protease inhibitors sampled from the body surface of tau-fluvalinate-sensitive and resistant *V. destructo*r. Proteins were isolated from the tau-fluvalinate-sensitive and resistant mites, while mites untreated with tau-fluvalinate constituted the control. Subsequently, the following methodology was applied: protein concentration assay by the Lowry method – as modified by Schacterle and Pollack; assay of proteolytic activity in relation to various substrates (gelatine, haemoglobin, ovoalbumin, albumin, cytochrome C, casein) by the modified Anson method; identification of proteolytic activity in relation to diagnostic inhibitors of proteolytic enzymes (pepstatin A, PMSF, iodoacetamide, o-phenantrolin), using the Lee and Lin method; identification of acidic, neutral and basic protease activities by means of the modified Anson method; electrophoretic analysis of proteins in a polyacrylamide gel for protease detection with the Laemmli method and for protease inhibitor detection with the Felicioli method. The highest value of protein concentration was found in the tau-fluvalinate-sensitive *V. destructor*, while the highest activity levels of acidic, neutral and alkaline proteases were observed in the tau-fluvalinate-resistant mites. Aspartic, serine, thiolic and metallic proteases were found in the drug-resistant and drug-sensitive *Varroa* mites. The control samples were found to contain aspartic and serine proteases. In an acidic and alkaline environment, the results revealed a complete loss of inhibitor activities in the in vitro analyses and electrophoresis. Serine protease inhibitor activities (at pH 7.0) were high, especially in the group of tau-fluvalinate-resistant mites.

Keywords

Proteolytic system, proteins, inhibitor activities, acaracides, *Varroa destructor*

Introduction

Varroatosis is one of the most serious diseases of honey bees (*Apis mellifera*). *Varroa destructor* mites are considered to be the reason for CCD (Colony Collapse Disorder). The problem for beekeepers, including those in Poland, is the dynamic growth of the parasite and its pathogenic action. *V. destructor* mites are thought to be carriers of viruses that cause morphological deformities which reduce host vigor and longevity. They also influence flight duration and the homing ability of foragers (Schneider and Drescher 1987, Koch and Ritter 1991, Romero-Vera and Otero-Colina 2002, Garedew *et al*. 2004, Kralj and Fuchs 2006).

The *V. destructor* mite has recently displayed an ever increasing resistance to new drugs, causing a proliferation of the CCD. Despite a high efficiency of each newly formulated acaricide in the initial period of its use, the faster adaptive capacity of the *V. destructor* mite leads to the appearance of the first mites resistant to the acaricide and capable of reproduction in its presence already after a couple of years from the introduction of the agent (Mathieu and Faucon 2000). The higher the number of *Varroa* generations nurtured in the presence of an acaricide, the greater percentage of its population that becomes resistant to the acaricide with time. This is a well-known co-evolutionary phenomenon (Watkins 1997). It results in enhanced synthesis and release of the more active detoxification enzymes in the *V. destructor* organism (Watkins 1997). Resistance to tau-fluvalinate stems from a rise in the activity of the monooxygenase enzyme in the cytochrome P-450 system (Watkins 1997).

The development of these mites is being studied, including semi-chemical interaction between the parasite and the host (Colin *et al*. 2001, Salvy *et al*. 2001). What is important in *V. destructor* invasion is the secretion of enzymes, especially chitinase and protease, which may help to pierce the bee cuticle or to maintain an opening at the parasite feeding site (Colin *et al*. 2001). The following proteolytic enzymes have been de-

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tected in mite extracts: leucine arylamidase, valine arylamidase, cysteine arylamidase, trypsin and chymotrypsin (Frączek *et al*. 2009). Proteases play a prominent role in a wide array of physiological processes such as food digestion, blood clotting, embryogenesis, tissue reorganization, defense mechanisms and immune responses (Schoofs and Salzet 2002). Proteolytic enzymes are known to be present in the bee alimentary duct, hemolymph, moult liquid, venom and on the body surface (Terra and Ferreira 1994, Bode *et al*. 1999, Lima *et al*. 2000, Malone *et al*. 2004, Evans *et al*. 2006, Strachecka *et al*. 2011). Up to date, the body-surface proteolytic system of mites has not been analysed. Therefore, the aim of this work was to determine the activity of proteases and protease inhibitors sampled from the body surface of tau-fluvalinate-sensitive and resistant *V*. *destructor*.

Materials and methods

Twenty apiaries provided the drone brood (at the turn of June) which was searched for approximately 60 *V*. *destructor* mites in order to assess mite resistance to tau-fluvalinate, using the Milani method (1995). Following assessment of their resistance, the *Varroa* mites were frozen in germ-free bags –24 C for 1–2 months. A total of 40 samples of tau-fluvalinate-sensitive and resistant mites were obtained. 20 mites untreated with tau-fluvalinate constituted the control.

Two by two, the mites from each group were successively defrozen and 1.5 ml of a 1% detergent solution (Triton X-100) were added. The mite pairs were then shaken/rinsed at 4 C for 60 min at 8000 rpm. After filtrating each of the samples through Miracloth, a solution was obtained that contained proteins. The solution was frozen in a refrigerator at –24°C. Each group provided 10 samples of this solution.

At the beginning of the experiment, the pH profiles of the samples containing washed-out proteins were determined according to the method devised by Strachecka *et al*. (2008). Optimal pH values for: 1) acidic environment, 2) neutral environment and 3) alkaline environment were selected at which the enzymes revealed the highest proteolytic activity in *V*. *destructor*. These pHs were 3.4, 7.0 and 12.2 (Fig. 1). Next, the samples containing washed-out proteins were analysed biochemically as follows:

quantitative total protein concentration assay using the Lowry method, as modified by Schacterle and Pollack (1973);

proteolytic activity test in relation to the substrates (gelatine, haemoglobin, ovoalbumin, albumin, cytochrome C, casein) according to the Anson method (1938) modified by Strachecka (2010);

determination of proteolytic activity in relation to the diagnostic inhibitors of proteolytic enzymes (pepstatin A, PMSF, iodoacetamide, o-phenantroline) according to the Lee and Lin method (1995);

determination of the activity of acidic, neutral and basic proteases by means of the Anson method (1938) modified by Strachecka *et al*. (2011);

electrophoretic analysis of proteins in a polyacrylamide gel for protease detection with the Laemmli method (1970);

determination of the levels of the natural inhibitors of acidic, neutral and alkaline proteases, based on the Lee and Lin method (1995);

electrophoretic analysis of proteins in a polyacrylamide gel in to detect aspartic and serine protease inhibitors by means of the modified Felicioli method (1997).

The statistical calculations were carried out using the SAS software (SAS Institute User's Guide Version 6.11., 1996). Statistical differences between the experimental factors were investigated using ANOVA.

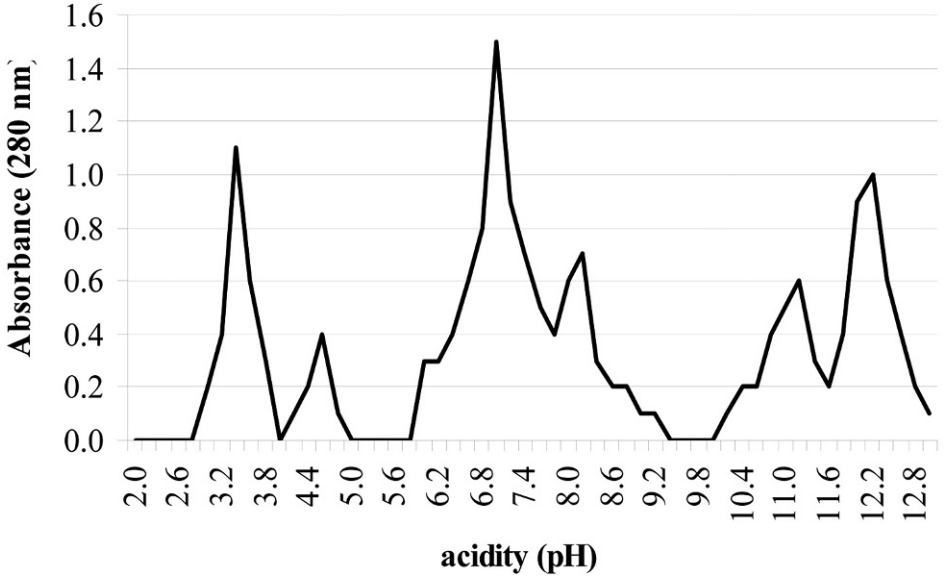

Fig. 1. The proteolytic activity on the body surface of *Varroa destructor* reflects the activity of the enzyme and substrate bond

Table I. Protein concentration (C µg x ml^{-1}) and proteolytic activity (As U x mg^{-1} protein) in the samples rinsed from the body surface of *Varroa* mites in an acidic (pH 3.4), neutral (pH 7.0) and basic (pH 12.2) environment

Groups of *Varroa* mites	Protein concentration C	pH 3.4		pH 7.0		pH 12.2	
		$\bar{\chi}_{As}$ ± se	C_p ± se	$\bar{\chi}_{As}$ ± se	C_p ± se	$\bar{\chi}_{As}$ ± se	C_p ± se
Control	46.10^{b} ± 0.07	5.50^{b} ± 0.11	12.00 ± 0.01	30.03^{b} ± 0.12	94.00 ± 0.02	3.39^{c} ± 0.09	9.30 ± 0.05
tau-fluvalinate-sensitive	48.90^{a} ± 0.11	4.49^{c} ± 0.09	13.25 ± 0.04	27.91^{c} ± 0.09	93.50 ± 0.05	4.21^{b} ± 0.11	12.50 ± 0.03
tau-fluvalinate-resistant	44.20^{c} ± 0.09	5.80^{a} ± 0.12	12.25 ± 0.03	31.33^{a} ± 0.13	92.50 ± 0.04	5.17^{a} ± 0.09	10.00 ± 0.03

Key: various lowercase letters – the differences are statistically significant for comparisons in the columns between the groups (control and tau-fluvalinate-sensitive and tau-fluvalinate-resistant mites) at $P \leq 0.05$; $\bar{\chi}_{As}$ – mean value of the protease activities; ± se – standard error; C_p – mean concentration values of proteins which exhibit protease activity in µg x ml^{-1} from the Eppendorf Bio-Photometer; shaded – the highest values among the proteolytic activity groups.

Table II. Protease inhibitor activities (As U x mg^{-1} protein) in the samples rinsed from the body surface of *V. destructor* in an acidic (pH 3.4), neutral (pH 7.0) and basic (pH 12.2) environment

Groups of *Varroa* mites	pH 3.4		pH 7.0		pH 12.2	
	$\bar{\chi}_{As}$ ± se	C_p ± se	$\bar{\chi}_{As}$ ± se	C_p ± se	$\bar{\chi}_{As}$ ± se	C_p ± se
Control	0.00 ± 0.00	66.00 ± 0.04	19.73^{b} ± 0.11	9.00 ± 0.06	0.00 ± 0.00	58.00 ± 0.13
tau-fluvalinate-sensitive	0.00 ± 0.00	68.00 ± 0.11	18.86^{c} ± 0.09	8.00 ± 0.09	0.00 ± 0.00	67.00 ± 0.11
tau-fluvalinate-resistant	0.00 ± 0.01	64.00 ± 0.09	22.00^{a} ± 0.12	11.01 ± 0.12	0.00 ± 0.00	55.00 ± 0.16

Key: various lowercase letters – the differences are statistically significant for comparisons in the columns between the groups (control and tau-fluvalinate-sensitive and tau-fluvalinate-resistant mites) at $P \leq 0.05$; $\bar{\chi}_{As}$ – mean value of the protease inhibitor activities; ± se – standard error; C_p – mean concentration values of proteins which exhibit protease inhibitor activity in µg x ml^{-1} from the Eppendorf Bio-Photometer.

Results

The highest value of protein concentration was found in the tau-fluvalinate-sensitive *V. destructor* (Table I), while the highest activity levels of acidic, neutral and alkaline proteases were observed in the tau-fluvalinate-resistant mites. Very high protease activities were observed in all the groups in neutral environment. Proteolytic activity was observed only in relation to ovoalbumin and gelatin in the case of the three groups of mites. Aspartic, serine, thiolic and metallic proteases were found in the drug-resistant and drug-sensitive *Varroa* mites (activity in relation to pepstatin A, phenylmethylsulfone fluo-

Table III. PAGE zymography of protease activity in a neutral environment on the body surface of *Varroa* mites from the control, tau-fluvalinate-sensitive and tau-fluvalinate-resistant groups

MW kDa	Control groups		tau-fluvalinate-sensitive mites		tau-fluvalinate-resistant mites	
	bands	OD	bands	OD	bands	OD
200	–		–		–	
120	–		–		+	0.14
116	–		–		+	0.15
97	+	0.14	–		+	0.10
85	+	0.20	+	0.20	+	0.10
65	+	0.30	+	0.15	+	0.12
50	+	0.15	–		–	
42	+	0.20	+	0.12	+	0.10
28	+	0.26	+	0.15	+	0.12
14	+	0.20	+	0.14	+	0.10

Key: minus "–" – proteases with this molecular weight were not detected; plus "+" – proteases with this molecular weight were detected; MW – molecular weight; OD – width of the bands (mm).

Table IV. PAGE zymography of protease inhibitor activity in a neutral environment on the body surface of *Varroa* mites from the control, tau-fluvalinate-sensitive and tau-fluvalinate-resistantgroups

MW kDa	Control groups		tau-fluvalinate-sensitive mites		tau-fluvalinate-resistant mites	
	bands	OD	bands	OD	bands	OD
97	–		–		–	
85	–		–		–	
65	–		–		–	
50	–		+	0.10	+	0.20
42	–		–		+	0.25
28	+	0.26	+	0.20	+	0.25
14	+	0.20	+	0.25	+	0.30

Key: minus "–" – protease inhibitors with this molecular weight were not detected; plus "+" – protease inhibitors with this molecular weight were detected; MW – molecular weight; OD – width of the bands (mm).

ride – PMSF, iodoacetamide and o-phenantroline). The control samples were found to contain aspartic and serine proteases (activity in relation to pepstatin A and phenylmethylsulfone fluoride – PMSF).

At an acidic and alkaline pH, SDS-PAGE electrophoresis revealed few, narrow, not very prominent and predominantly low-molecular bands of proteases whose molecular weights ranged from 10 kDa to 42 kDa. Electrophoresis of the control group at pH 7.0 revealed 7 bands of neutral serine proteases (Table III, Fig. 2). We observed a lack of the 50 kDa-heavy fraction of these enzymes in the two treated groups, present in the control group. The resistant mites were observed to have additional bands of high-molecular proteins of about 116 kDa and 120 kDa.

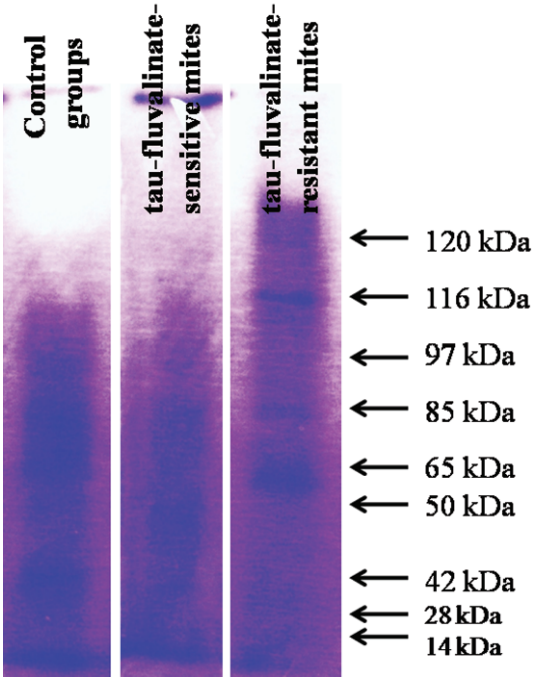

Fig. 2. An electrophorogram (example) depicting protease activity in a neutral environment on the body surface of *Varroa* mites from the control, tau-fluvalinate-sensitive and tau-fluvalinate-resistant groups

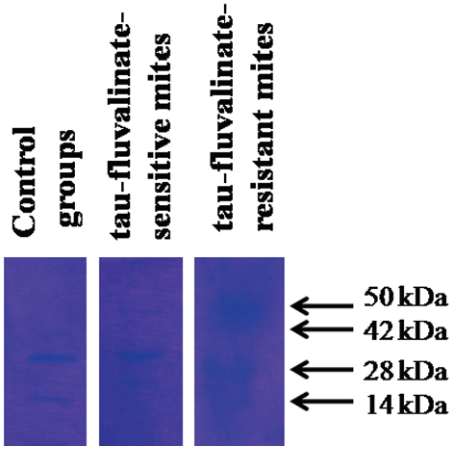

Fig. 3. An electrophorogram (example) depicting protease inhibitor activity in a neutral environment on the body surface of *Varroa* mites from the control, tau-fluvalinate-sensitive and tau-fluvalinate-resistant groups

In an acidic and alkaline environment, the results revealed a complete loss of inhibitor activities in the in vitro analyses and electrophoresis (especially in the case of aspartic and serine protease inhibitors at pH 12.2). Simultaneously, proteins with protease inhibitor activities had high concentrations (Table II). Serine protease inhibitor activities (at pH 7.0) were high, especially in the group of tau-fluvalinate-resistant mites, but the concentrations of these inhibitors were low. These in vitro activities corresponded with numerous electrophoretic bands (Table IV, Fig. 3). In the electrophorograms, it can be observed that tau-fluvalinate treatment increased the number of bands of neutral serine protease inhibitors and their molecular weights to about 50 kDa.

Discussion

The resistant *V. destructor* had lower protein concentrations and higher body-surface protease activity (Table I). The metabolism

of tau-fluvalinate is dominated by hydrolysis followed by oxidation and conjugation of the metabolites. The major metabolites included conjugates with the following amino acids: glicyne, serine, threonine and valine and with cholic acid, taurocholic acid and taurochendeoxycholic acid (Roberts and Hutson 1999). These amino acids are part of the third- and fourth-order protease structure and form complexes with taufluvalinate that render the cuticle stiff and make it less permeable to other compounds, including acaricides.Alternatively, the proteolytic system may break down tau-fuvalinate which is made up of valine ((RS)-cyano-(3-phenoxyphenyl)methyl N-(2-chloro-4-trifluoromethylphenyl)-D-valinate) (Roberts and Hutson 1999), thus making the compound non-toxic to the mites. To be more specific, the more active body-surface proteins in *Varroa* did not allow the active agent inside the mite organism, thanks to which the mites could gain more resistance. Thus, the sealing-up/encystment was more effective. This is also confirmed in the numer of bands and in the molecular mass of the proteins (Tables III and IV). More diversified proteins appear in the resistant mites (116 kDa, 120 kDa – proteases, 42 kDa – protease inhibitors). They form additional protection on the mite carapace. Such protection was not observed in the vulnerable and control mites. These proteins may coagulate in contact with tau-fluvalinate, which results in better mechanical resistance to the compound for the mites. Proteases play an important role in the degradation of the host cuticle and in *V. destructor* resistance (Mira 2000, Colin *et al*. 2001). The intensification of the activity of these enzymes under the influence of the acaricide may lead to a greater infestation of bees with the parasite. Thus, a vicious cycle appears: bee-keepers apply taufluvalinate to which the mites become resistant and more readily attack bees. As a result, apiarists increase the acaricide dosis etc. Frączek *et al*. (2009) observed very weak trypsin activity and their results were consistent with previous ones that were obtained by Tewarson and Engles (1982) regarding proteolytic activity of extracts from *V. jacobsoni*. In our study, trypsin activity corresponded with the activities of serine protease inhibitors. These activities were the highest in the taufluvalinate-resistant mites at a neutral pH. Serine protease inhibitors safeguard the mites against pathogens (Bania and Polanowski 1999).

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