

# Peptidases and peptidase inhibitors in gut of caterpillars and in the latex of their host plants

Márcio V. Ramos · Danielle A. Pereira · Diego P. Souza · Maria-Lídia S. Silva ·  
Luciana M. R. Alencar · Jeanlex S. Sousa · Juliany-Fátima N. Queiroz ·  
Cleverson D. T. Freitas

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**Abstract** Studies investigating the resistance–susceptibility of crop insects to proteins found in latex fluids have been reported. However, latex-bearing plants also host insects. In this study, the gut proteolytic system of *Pseudosphinx tetrio*, which feeds on *Plumeria rubra* leaves, was characterized and further challenged against the latex proteolytic system of its own host plant and those of other latex-bearing plants. The gut proteolytic system of *Danaus plexippus* (monarch) and the latex proteolytic system of its host plant (*Calotropis procera*) were also studied. The latex proteins underwent extensive hydrolysis when mixed with the corresponding gut homogenates of the hosted insects. The gut homogenates partially digested the latex proteins of foreign plants. The fifth instar of *D. plexippus* that were fed diets containing foreign latex developed as well as those individuals who were fed diets containing latex proteins from their host plant. In vitro assays detected serine and cysteine peptidase inhibitors in both the gut homogenates and the latex fluids. Curiously, the peptidase inhibitors of caterpillars did not inhibit the latex peptidases of their host plants. However, the

peptidase inhibitors of laticifer origin inhibited the proteolysis of gut homogenates. In vivo analyses of the peritrophic membrane proteins of *D. plexippus* demonstrate resistance against latex peptidases. Only discrete changes were observed when the peritrophic membrane was directly treated with purified latex peptidases in vitro. This study concludes that peptidase inhibitors are involved in the defensive systems of both caterpillars and their host plants. Although latex peptidase inhibitors inhibit gut peptidases (in vitro), the ability of gut peptidases to digest latex proteins (in vivo) regardless of their origin seems to be important in governing the resistance–susceptibility of caterpillars.

**Keywords** *Calotropis procera* · *Cryptostegia grandiflora* · *Danaus plexippus* · *Plumeria rubra* · *Pseudosphinx tetrio*

## Abbreviations

CgLP	Latex proteins of <i>Cryptostegia grandiflora</i>
CpCP	Purified peptidases of <i>C. procera</i> latex
CpLP	Latex proteins of <i>Calotropis procera</i>
DTT	Dithiothreitol
HT	Heat treated
LP	Latex protein
PM	Peritrophic membrane
PrLP	Latex proteins of <i>Plumeria rubra</i>

Maria-Lídia S. Silva: In memoriam.

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M. V. Ramos (✉) · D. A. Pereira · D. P. Souza ·  
J.-F. N. Queiroz · C. D. T. Freitas  
Departamento de Bioquímica e Biologia Molecular,  
Universidade Federal do Ceará, Campus do Pici,  
Cx. Postal 6033, Fortaleza, CE CEP 60451-970, Brazil  
e-mail: vramos@ufc.br; r\_marcio@hotmail.com

L. M. R. Alencar · J. S. Sousa  
Departamento de Física, Universidade Federal do Ceará,  
Campus do Pici, Fortaleza, CE, Brazil

## Introduction

An increasing number of studies investigating the resistance–susceptibility of insects to proteins that are found in latex fluids have supported the belief that latex proteins (or

**Table 1** Resistance–susceptibility relationships of caterpillars to latex proteins

Insects	Developmental stage		Characteristics		Resistance–susceptibility
	Larvae	Adult	Host plants (latex proteolytic systems)	Gut peptidases	
<i>Danaus plexippus</i> (Nymphalidae)			<i>Asclepias</i> species, <i>Calotropis procera</i> (Apocynaceae) <sup>a</sup> (cysteine peptidases)	Mostly serine peptidases are found	Resistant to LP of <i>C. procera</i> ; <i>P. rubra</i> ; <i>C. grandiflora</i>
<i>Pseudosphinx tetrio</i> (Sphingidae)			Genus <i>Plumeria</i> (Apocynaceae) <sup>a</sup> (cysteine peptidases)	Mostly serine peptidases are found	Resistant to LP of <i>P. rubra</i>
<i>Spodoptera frugiperda</i> (Noctuidae)			Generalist (maize, rice, and other crops)	Mostly serine peptidases are found	Resistant to LP of <i>C. procera</i>
<i>Anticarsia gemmatalis</i> (Noctuidae)			Generalist (soy, rice, alfalfa, peanut, peas, beans, snap beans and wheat and other crops)	Mostly serine peptidases are found	Susceptible to LP <i>C. procera</i>

<sup>a</sup> Plants possessing laticifers

their genes) would be interesting tools for use as insecticidal agents against crop insects (Wasano et al. 2009; Ramos et al. 2009, 2010). However, latex-bearing plants also host insects (Agrawal and Konno 2009). The biochemical basis underlying the resistance or susceptibility of insects to latex proteins is still the aim of research in this field (Konno 2011). This question should be carefully examined because it overcomes biological curiosity. An understanding of the biochemical basis of resistance/susceptibility could provide new insight into the obscure biological function of the highly specialized laticifer cells in plants and how specialized insects successfully overcome the elaborate defensive strategy of their target latex-bearing plants.

In this context, insect digestive enzymes are key molecules that are required to overcome the structural barriers of plants; to process food, thereby making available nutritional compounds such as free amino acids and carbohydrates; and to destroy any toxic macromolecules of the host plant (Chen et al. 2013). Accordingly, plants have developed defensive molecular mechanisms in response to herbivory that suppress the insect gut enzymatic activities, such as peptidase inhibitors (Bijina et al. 2011; Hivrale et al. 2013). Therefore, the characterization of insect digestive enzymes is a preliminary step in understanding the insect feeding abilities at least at the biochemical level (Goptar et al. 2013).

Interestingly, latex fluids are also complex enzymatic systems from which different classes of enzymes have been reported (Konno 2011). Moreover, proteolytic enzymes

(peptidases) are particularly abundant in the latex of different plants, of which cysteine peptidases seem to be the most common (Domsalla and Melzing 2008; Teixeira et al. 2008). Because of these properties, plant latex somehow mimics the digestive fluids of insects. Thus, at the time of insect feeding on latex-bearing plants, a very interesting biochemical paradox takes place as two proteolytic systems are confronted, thereby establishing a resistance–susceptibility rule between the involved organisms. This study aimed to advance this thinking by examining and revising the biochemical aspects involving the resistance–susceptibility of two latex-feeding insects as cited in Table 1 via enzymatic studies and latex protein feeding trials.

The latex proteolytic systems of *Calotropis procera* and of the midgut of *Danaus plexippus* (Monarch) have been extensively and separately studied. The proteolytic system of *C. procera* is characterized by the strong activity of cysteine peptidases (Freitas et al. 2007), while that of *D. plexippus* is mostly composed of serine peptidases (Pereira et al. 2010). Even though the leaves of *C. procera* are rich in laticifers, they are fruitfully consumed by monarch. In contrast, we showed in a previous study that the latex proteins of *C. procera* were insecticidal against *Anticarsia gemmatalis* but not against *Spodoptera frugiperda*, two caterpillars that are closely related to *D. plexippus* (see Table 1). *Pseudosphinx tetrio* feeds on the leaves of *Plumeria rubra*, which also contain laticifers. However, limited information is available about both of these proteolytic systems. In this study, these proteolytic systems were examined.

## Materials and methods

### Chemicals

The following chemicals were purchased from Sigma Chemical Co. (São Paulo, SP, Brazil): azocasein, casein, *N*-benzoyl-DL-argininyl-*p*-nitroanilide (BAPNA), *N*-benzoyl-DL-arginine-*b*-naphthylamide hydrochloride (BANA), *trans*-epoxysuccinyl-L-leucylamido (4-guanidino)-butane (E-64), pepstatin, PMSF (phenylmethanesulfonyl fluoride), papain and bovine trypsin. Dithiothreitol (DTT), Iodoacetamide and molecular weight markers were obtained from GE HealthCare (São Paulo, SP; Brazil). Others chemicals were of analytical grade.

### Organisms and sample preparation

#### Caterpillars

The species that were used in this study were *Danaus plexippus* (Nymphalidae) and *Pseudosphinx tetrio* (Sphingidae). The larvae of *D. plexippus* (Monarch) were obtained from wild *C. procera* plants that were grown in the vicinity of Fortaleza, Brazil (03°43'02"S, 038°32'35"W). The fifth instar larvae of monarch were obtained under laboratory rearing conditions according to the previously established protocol (Pereira et al. 2010). The individuals who were grown under these conditions were used in the bioassays and were sources of gut homogenate and peritrophic membrane.

The larvae of *P. tetrio* were obtained from the leaves of cultivated plants (*P. rubra*) from different private gardens in Fortaleza, Brazil. These individuals were reared with fresh leaves of *P. rubra* under the appropriate conditions (12 h light/dark cycle, 27 ± 2 °C, 60–80 % relative humidity) until they reached the fifth instar. The fifth instar larvae were sources of gut homogenate.

#### Gut homogenates

Fifth instar larvae that were recently fed were used for the gut extraction. The larvae were immobilized at a low temperature (−20 °C) for 6 min, and the guts from *D. plexippus* and *P. tetrio* were dissected, homogenized in 2 ml of 150 mM NaCl and centrifuged at 4 °C, 5,000g for 10 min. The resulting supernatants were pooled, divided into aliquots and frozen at −20 °C until required. The pH values of the *D. plexippus* and *P. tetrio* gut homogenates were 7.6 and 8.0, respectively (estimated by a pH300 Analyzer).

#### Peritrophic membrane (PM) dissecting

Fifth instar larvae of *D. plexippus* that were recently fed were immobilized at a low temperature (−20 °C) for

6 min, and the guts were dissected. The gut epithelium was removed, and the PM surrounding the food bolus was thoroughly rinsed in distilled water to completely remove the gut contents (Zhang and Guo 2011). The PMs were stored at −20 °C until required.

#### Plants

The species that were used in this study were *C. procera*, *P. rubra* and *Cryptostegia grandiflora*, all members of Apocynaceae. The specimens that were sources of latex were located at the following geographic coordinates: (*C. procera*: 3°45'12"S, 38°26'59"W; *Cr. grandiflora*: 3°32'44"S, 38°49'53"W; and *P. rubra*: 3°44'38"S, 38°34'31"W).

Botanical materials were also collected and presented to the Institutional Herbarium Prisco Bezerra at Universidade Federal do Ceará for taxonomic identification. The specimens were deposited under the following catalog numbers: (*C. procera*: 32663; *Cr. grandiflora*: 40409; and *P. rubra*: 15018).

#### Latex processing and latex proteins

The latex of *C. procera*, *P. rubra* and *Cr. grandiflora* plants was obtained as previously reported (Freitas et al. 2010). After gently mixing in distilled water (1:1, v:v), the samples were centrifuged for 10 min at 4 °C and 10,000g. The insoluble phase (rubber) was discarded, and the soluble phase was exhaustively dialyzed at 8 °C in distilled water using membranes that were smaller than 8,000 Da. The latex proteins (LP) of *C. procera* were named CpLP; the latex proteins of *P. rubra* were named PrLP; and the latex proteins of *Cr. grandiflora* were named CgLP.

#### Protein content

The protein content on latex protein fractions and gut homogenates was estimated according to the Bradford method (1976) and bovine serum albumin (BSA) was used as the protein standard.

#### Proteolytic assays

##### Gut homogenate of *P. tetrio*

The total proteolytic activity of the gut homogenate from the fifth instar *P. tetrio* was first examined by zymogram containing 0.1 % gelatin. The enzymatic activity was detected as transparent bands in the gels after staining with 0.1 % Coomassie Brilliant Blue R-250 (Freitas et al. 2007). Then, the *P. tetrio* gut peptidases were characterized using azocasein, BANA and BAPNA as substrates, essentially as described by Xavier-Filho et al. (1989) and Pereira et al. (2010).

The effect of pH on the gut proteolytic activity was also evaluated (pH 2.0–9.0) using the substrates azocasein, BANA and BApNA. To determine the heat stability, the gut homogenate was incubated at temperatures ranging from 25 to 90 °C in 50 mM Tris–HCl (pH 8.0) for 15 and 30 min. The proteolytic activity was then measured at 37 °C using azocasein at pH 8.0. The inhibition of the proteolytic activity was further investigated using azocasein as a generalist substrate and known inhibitors of peptidases. Separate aliquots of gut homogenate (20 µl) were incubated with 20 µl of the following inhibitors: 1.18 mM E-64, 1 mM iodoacetamide, 5 mM PMSF, 10 mM EDTA or 10 mM pepstatin for 30 min. The remaining proteolytic activity of each aliquot was determined at 37 °C and pH 8.0. The results of all of the series of measurements were expressed as their mean value  $\pm$  SD.

#### *Analysis of the digestibility of the LP by gut homogenates*

Aliquots of CpLP, PrLP and CgLP (in 50 mM Tris–HCl buffer, pH 8.0) were incubated with the gut homogenates of *P. tetrio* to obtain a protein ratio (µg) of 1:2 (LP:gut homogenate). The samples were incubated at 37 °C, and the aliquots were collected at 1, 5 and 10 min. To stop the reaction the samples were immediately mixed to the SDS-PAGE sample buffer and immersed in boiling water for 5 min before electrophoresis. The results were examined by SDS-PAGE as described by Laemmli (1970), and the proteins in-gel were detected after staining with 0.1 % Coomassie Brilliant Blue R-250.

#### *Inhibition of the proteolytic activity of the gut homogenates by HT-LP*

To eliminate the proteolytic activity, the latex protein samples (CpLP, CgLP and PrLP) (5 mg/ml in distilled water) were heat treated (HT) at 95 °C for 30 min, and the precipitated material was discarded after centrifugation at 10,000g for 10 min. The supernatants were checked for residual proteolytic activity using azocasein as a substrate and then freeze dried. The heat-treated samples were named HT-CpLP, HT-CgLP and HT-PrLP. The HT-LP samples were independently incubated at 37 °C with papain, trypsin and *P. tetrio* and *D. plexippus* gut homogenates using azocasein and BApNA as substrates at pH 7.5. The total proteolytic activities that were estimated in the absence of HT-LP samples were considered as 100 %.

#### *Inhibition of the LP proteolytic activity by the HT gut homogenates*

The gut homogenates of *D. plexippus* and *P. tetrio* were heat treated as reported above for the LP samples to

eliminate endogenous proteolysis. The heat-treated gut homogenates served as inhibitor sources for CpLP, CgLP and PrLP. Different aliquots of HT gut homogenates were incubated with papain, trypsin and LP samples using azocasein and BANA as substrates at pH 7.5.

#### Bioassay

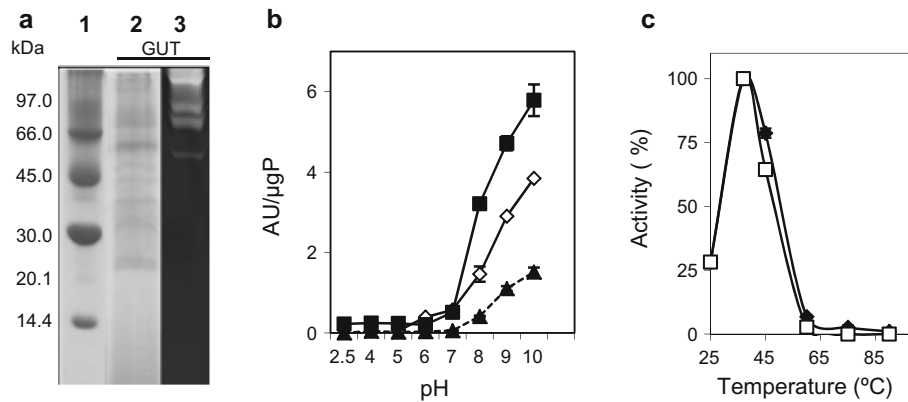
Immediately after egg hatching, the larvae of *D. plexippus* were fed an artificial diet until they reached the fifth instar, according to (Pereira et al. 2010). The fifth instar larvae ( $n = 4–10$ ) were then transferred to diets containing 1 % PrLP or 1 % CgLP by partially replacing the control protein casein (Pereira et al. 2010). The fifth instar larvae were kept in plastic vials at 28 °C and 60–70 % relative humidity in a growth chamber, and the larvae weight gain and survival rate were evaluated daily for 4 days. The control group consisted of insects that were fed an artificial diet without LP.

#### Degradation of the peritrophic membrane (PM) proteins by the LP

Two assays (in vivo and in vitro) were performed to evaluate the ability of the latex peptidases to damage the PM of *D. plexippus*. After 4 days of being fed diets containing casein, 1 % PrLP, 1 % CgLP or 1 % CpLP, the caterpillars were killed and dissected to collect the PM. The protein profiles of the PMs were analyzed by SDS-PAGE as described by Zhang and Guo (2011). In vitro assays were performed, incubating the PM of larvae that were fed fresh leaves with CpLP, CgLP, PrLP, papain and trypsin (0.5 mg/ml in 50 µl of 50 mM Tris–HCl, pH 7.5; 3 mM DTT; 2 mM EDTA). After 60 min at 37 °C, the mixtures were centrifuged at 4 °C and 10,000g for 10 min, the supernatants were discarded and 50 µl of buffer was added to the precipitate (0.0625 M Tris–HCl, pH 6.8, 2 % SDS and 5 %  $\beta$ -mercaptoethanol). The PM pieces were macerated and subjected to heating at 95 °C for 5 min, followed by agitation for 1 h and centrifugation at 25 °C and 10,000g for 10 min. The supernatants (20 µl) were analyzed by SDS-PAGE and visualized by silver staining. The overall structure and integrity of the PM were further examined by atomic force microscopy.

#### In-gel trypsin digestion and identification of the PM proteins by mass spectrometry

After SDS-PAGE, the PM proteins of the *D. plexippus* larvae that were fed fresh leaves were removed and processed for mass spectrometric analysis as described by Hellman et al. (1995). The tryptic peptides were analyzed using a Synapt HDMS mass spectrometer (Waters,



**Fig. 1** **a** Polyacrylamide (12.5 %) gel electrophoresis of the molecular weight markers (*lane 1*) and gut homogenate of the fifth-instar *P. tetrio* larvae (*lane 2*). The proteins were stained with Coomassie Brilliant Blue R-250. Twenty micrograms of protein were added. Zymogram containing 0.1 % gelatin of the gut homogenate (*lane 3*) and 60 ng of protein were added. **b** Effect of pH on the gut proteolytic

activity of *P. tetrio* using azocasein (*open diamond*), BApNA (*filled square*) and BANA (*filled triangle*) as substrates. **c** Thermo-stability of the gut proteolytic activity using azocasein as a substrate at pH 8.0. The gut proteins were heated for 15 min (*filled diamond*) or 30 min (*open square*) prior to performing the assays at 37 °C. The data are the means of three independent determinations

Manchester, UK) coupled to a NanoUPLC-ESI system. The results were submitted to the NCBI database using MASCOT (Matrix Science Ltd., London, UK, <http://www.matrixscience.com>) search engine.

Atomic force microscopy

The structural integrity of the PM was further assessed by atomic force microscopy. The PMs were collected from the individuals who were fed fresh leaves of *C. procer*, in vitro exposed to CpLP, CgLP, PrLP and purified peptidases of *C. procer* latex (CpCP) (1 mg/ml in 50 mM Tris–HCl pH 7.5; 3 mM DTT and 2 mM EDTA) and then examined. The CpCP were obtained as described by Ramos et al. (2013). The samples were maintained in distilled water until examination. The set of images was acquired by a Multimode Nanoscope IIIa equipment (Bruker, Santa Barbara, CA, USA) using the intermittent (or tapping) mode scan using a NCH tips (NanoWorld) with a nominal spring constant of 42 N/m. The measurements were performed with a scan rate of 0.5 Hz. The three-dimensional images that were presented were obtained in the height mode in different scan areas with a resolution of 512 per 512 lines (maximum resolution). The images were acquired at air and room (25 ± 2 °C) temperature.

Statistical analysis

The data corresponding to the proteolytic assays and the inhibition of proteolytic activities are presented as the mean ± SD (*n* = 3) of at least three independent measurements. The data corresponding to the effects of latex proteins on the fifth instar monarch larvae that were fed the

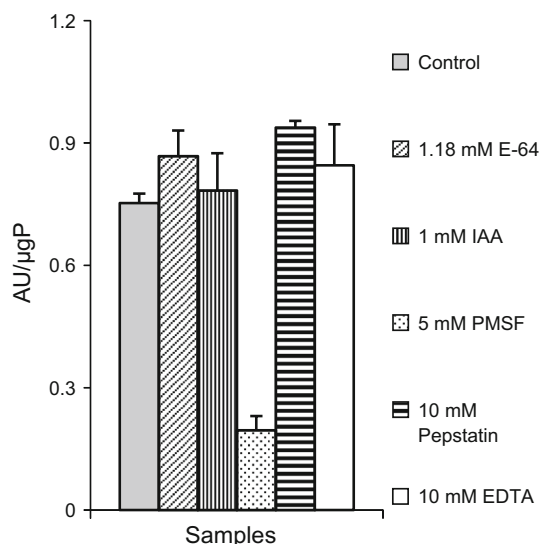
artificial diets were analyzed by a one-way analysis of variance (ANOVA). Dunnett’s test (using the Prism 4.0 software) was performed to identify the means that differed when the ANOVA test was significant. A *P* value of <0.05 was considered to indicate a significant difference.

Results

Characterization of proteolytic activity of the *P. tetrio* gut homogenates

The total proteolytic activity of the gut homogenates in the fifth instar larvae was first observed by zymogram. The protein profile of gut homogenate of *P. tetrio* as well the total proteolytic activity is seen in Fig. 1a. The stronger proteolytic activity was observed upon BapNA (Fig. 1b). Moderate activity was observed upon azocasein and the lowest activity was upon BANA. Azocasein is an unspecific protein substrate used to determine the total proteolytic activity. BANA and BApNA are very similar and chromogenic substrates for trypsin-like peptidases. However, some works also show that papain-like cysteine peptidases are able to hydrolyze these substrates (Abe et al. 1992; Freitas et al. 2007, 2010; Teixeira et al. 2008; Torres et al. 2010). As a result the use of different substrates did not help to identify the proteolytic specificity in gut extract of *P. tetrio*. This question was thus approached by inhibition of the proteolytic activity using inhibitors specific to each peptidase class.

No proteolysis was observed at pH values less than 7.0, suggesting that the proteolytic system of *P. tetrio* is largely active under alkaline pH conditions (Fig. 1c). This result is



**Fig. 2** Effect of peptidase inhibitors on the gut proteolytic activity of the fifth-instar *P. tetrio* larvae. The data are the means of three independent determinations. The error bars indicate the standard errors of the mean. Abbreviations: *E-64* trans-epoxysuccinyl-l-leucylamido-(4-guanidino)butane, *IAA* iodoacetamide, *PMSF* phenylmethylsulfonyl fluoride, and *EDTA* ethylenediaminetetraacetic acid

consistent with the endogenous pH value (8.0) of the gut homogenate. It is, however, interesting to mention that the proteolytic activity of the gut homogenate increased in all of the tested substrates under the highest pH conditions. At the temperature of 37 °C was the best proteolytic activity in the gut homogenates (Fig. 1c). Therefore, the proteolytic system of the gut homogenate of *P. tetrio* shares very similar characteristics to that of *Danaus plexippus* (Pereira et al. 2010). These similarities were also confirmed when the proteolytic activity of the gut homogenate of *P. tetrio* was inhibited (Fig. 2). Attempts to improve the proteolytic activity of the gut homogenate by introducing known reducing agents such as DTT did not at all alter the activity (not shown), suggesting an absence of cysteine peptidases in the gut of *P. tetrio*, in agreement with the data of *D. plexippus* (Pereira et al. 2010). As only PMSF (specific serine peptidase inhibitor) was able to inhibit the proteolytic activity, it was established that only serine peptidases were present in gut extract of *P. tetrio* (Fig. 2). These results indicate that serine peptidases predominate as the major proteolytic systems of the digestive fluid of *P. tetrio*, again similar to the data of *D. plexippus*.

#### Gut digestive fluid largely overcomes the latex proteolytic systems

The set of electrophoreses that is shown in Fig. 3 suggests that PrLP underwent extensive proteolysis after mixing with the gut homogenate of *P. tetrio*. Consequently, it is

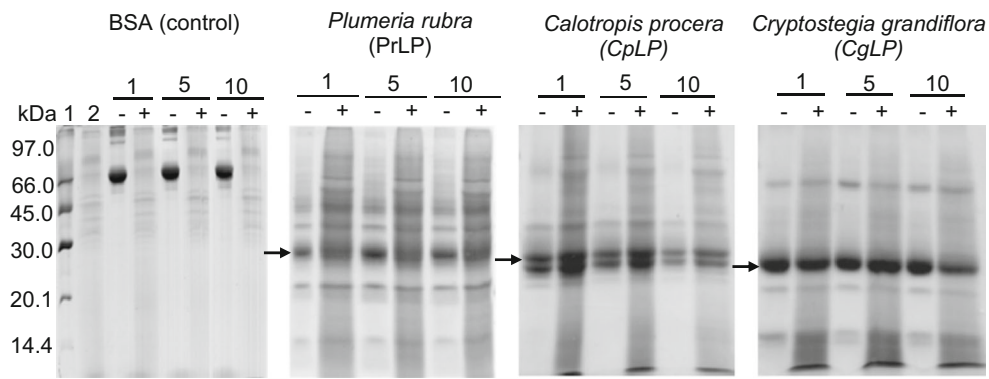
assumed that the proteolytic enzymes of the caterpillar digestive system overcame the latex proteolytic system of its host plant. This proteolysis was not restricted to PrLP. CpLP and, to a lesser extent, CgLP were both partially digested by the gut homogenates of *P. tetrio* (Fig. 3). To better characterize this observation we altered the experimental conditions. Modifying the kinetic parameters by reducing temperature of hydrolysis (25 °C instead 37 °C) or altering LP:GH rate, also reduced the effectiveness of gut homogenates to digest latex proteins, however, confirmed that gut proteins are resistant to the latex proteolytic system and progressively digest latex proteins along the time (Online resource 1). Almost complete digestion of latex proteins was reached after 8 h (not shown). This result begs the question as to whether *P. tetrio* larvae can grow adequately when fed diets containing CpLP or CgLP and whether *D. plexippus* larvae can grow adequately when fed diets containing PrLP or CgLP.

#### Performance of the caterpillars that were fed diets with added latex proteins

Attempts to cultivate *P. tetrio* under laboratory conditions failed. Even collecting individuals at the same developmental stage to statistically support the assays could not be accomplished. Therefore, bioassays to evaluate the performance of the fifth instar larvae of *P. tetrio* that were fed artificial diets are not reported here. Instead, the fifth instar larvae of *D. plexippus* (which were easy to obtain) were fed an artificial diet containing 1 % PrLP or 1 % CgLP. It is worth noting that caterpillars grew fastest when fed diets with added latex proteins compared to those that were fed the control diet with casein (Online resource 2). A similar trend was observed previously when monarchs were fed diets containing the latex proteins (CpLP) of their own host plant (Pereira et al. 2010).

#### Caterpillars overcame the latex proteolytic inhibitory machinery

In this study, we mostly focused our efforts on determining whether peptidase inhibitors play important roles when both of the proteolytic systems (plant latex versus caterpillar gut) are confronted. First, the aliquots of all of the studied proteolytic systems (latex and gut homogenates) were submitted to heat treatment (HT) to eliminate the activity of their own proteolytic enzymes. Furthermore, these HT samples were tested to inhibit the purified peptidases (papain and trypsin) that were introduced as controls and then tested to inhibit the proteolytic activity of the latex and gut homogenates that had not been submitted to heat treatment. This protocol could help us to detect the occurrence of peptidase inhibitory activity in all of the



**Fig. 3** Polyacrylamide (12.5 %) gel electrophoresis of bovine serum albumin (BSA), PrLP, CpLP and CgLP incubated with the gut peptidases of the fifth-instar *P. tetrio* larvae for different durations. Lane 1 molecular weight markers, lane 2 gut homogenates. The latex

proteins (LP) and BSA were controls (-); the gut homogenates were incubated with BSA or LP (+). A total of 30 µg of protein was added to each well. The arrows indicate the predominant digestion

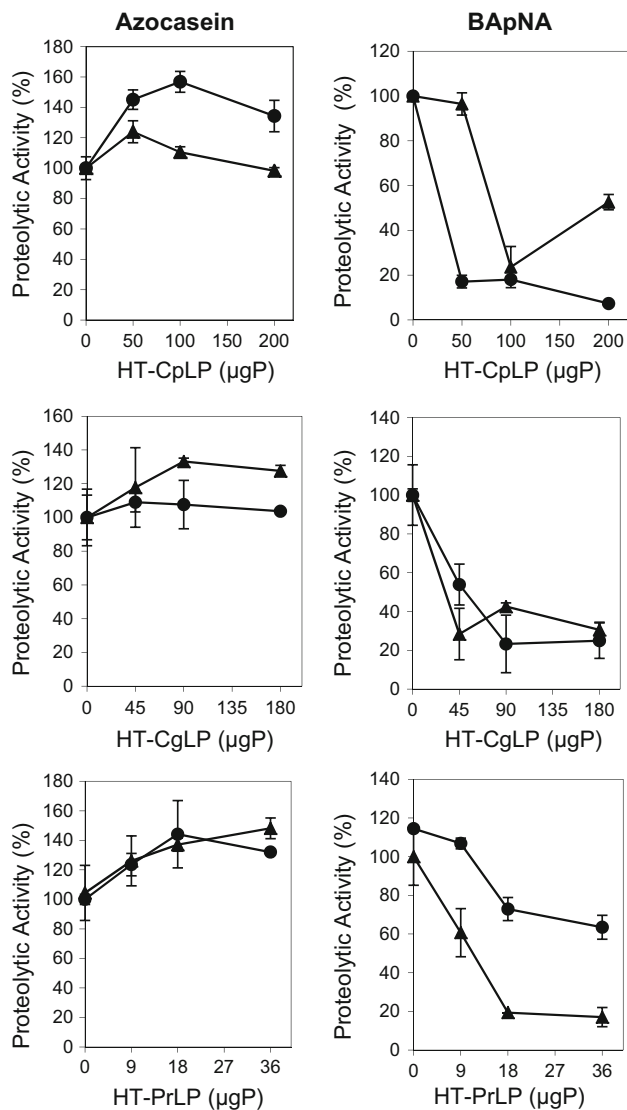
samples and gain insight into whether latex peptidase inhibitors can inhibit the peptidases of gut homogenates or gut peptidase inhibitors can inhibit latex peptidases. Unspecific (azocasein) and specific (BApNA or BANA) substrates were adequately used in all of the assays. The results concerning the ability of the HT samples to inhibit the purified peptidases (trypsin and papain) are presented as supplementary material (Online resource 3). The inhibitory activity upon azocasein hydrolysis was always discrete or null in the HT latex samples (not shown). However, HT-CpLP and HT-CgLP inhibited papain, mostly when BApNA was used as a substrate. HT-PrLP exhibited only a discrete inhibition of papain when BApNA was the substrate. This result suggests that cysteine proteinase inhibitors are found in latex. Therefore, it is also concluded that cysteine and cysteine peptidase inhibitors are concomitantly found in latex. The presence of inhibitory activity on papain by HT-CpLP was merely confirmed, as it was previously reported (Ramos et al. 2010). The physiological relevance of this co-existence is currently being evaluated but is outside of the scope of the present study. The HT-latex samples (CpLP and CgLP) exhibited a discrete inhibition of trypsin activity, while PrLP was completely fruitless. Therefore, the presence of trypsin-like serine peptidase inhibitors in these LP is unlikely.

Interestingly, the HT gut homogenates of the caterpillars strongly inhibited both papain and trypsin, suggesting that inhibitory activity towards both classes of peptidases is present in caterpillar guts (Online resource 3).

It is worth noting that all of the HT latex samples strongly inhibited the proteolytic activity of both of the caterpillars. This result suggests that the serine peptidases that are present in the gut homogenates of both of the caterpillars are not universally trypsin like. In fact, other serine peptidases, such as chymotrypsin and elastase, are also commonly found in the guts of insects; this could be

the case for *D. plexippus* and *P. tetrio* (Christeller et al. 1992). Therefore, further search into these types of inhibitors in latex will help to better understand these complex systems. The inhibition of the HT latex samples in the gut homogenates was only observed, however, when BApNA instead of azocasein was used as a substrate (Fig. 4). In most of the cases, a higher proteolytic activity of gut homogenates was often obtained when azocasein was assayed as a substrate. Azocasein is by far structurally closer to the proteins than are either of the synthetic substrates (BANA and BApNA). This result may explain the extensive hydrolysis of azocasein by the gut peptidases of *D. plexippus* and *P. tetrio* and may explain, at least in part, the failure of inhibition by the HT latex samples when azocasein was a substrate. The complexity of insect proteolytic systems and their performance facing different (synthetic) inhibitors has been previously recognized (Novillo et al. 1997).

The HT gut homogenates of *D. plexippus* and *P. tetrio* consistently inhibited the proteolytic activity of CpLP and CgLP when azocasein was used as a substrate (Fig. 5). Thus, the presence of cysteine-like peptidase inhibitors in both of the gut homogenates is once more supported. These results are consistent with those that are shown in Online resource 3. The inhibition of the proteolytic activity of PrLP was considered null, even when BANA was the substrate (Fig. 5). The proteolytic activity of CpLP unexpectedly increased when BANA was the substrate, regardless the source of the tested inhibitor. The proteolytic activity of CpLP strongly increased in the presence of the HT gut homogenate of *D. plexippus* and exhibited unusual activity toward BANA in the presence of increasing concentrations of the HT gut homogenate of *P. tetrio*. This trend was consistently confirmed by four independent experimental determinations. Although it is difficult to discuss this kinetic activity, the inability of both of the HT

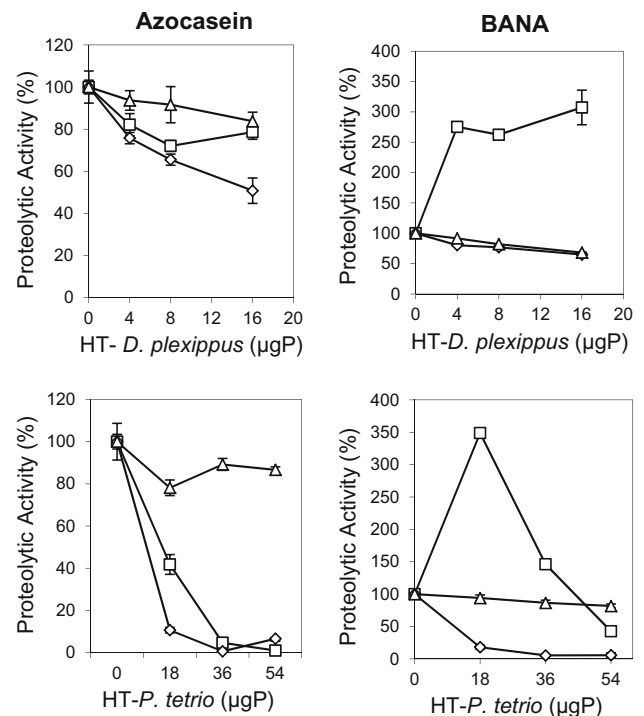


**Fig. 4** Inhibition of the proteolytic activity of the gut homogenates of *D. plexippus* (filled circle) and *P. tetrio* (filled triangle) by HT-CpLP, HT-CgLP and HT-PrLP using azocasein and BApNA as substrates at pH 7.5

gut homogenates to inhibit CpLP when BANA was used as substrate is clear.

#### Effect of latex peptidases on the peritrophic membrane (PM)

The insecticidal effect of proteins is frequently associated with damage to the peritrophic membrane (PM) of insects. Therefore, we performed two assays (in vivo and in vitro) to evaluate the ability of latex peptidases to damage the PM of *D. plexippus*. The PM proteins of *D. plexippus* larvae that were fed diets containing casein (control), 1 % PrLP or CgLP (Online resource 2) were analyzed by SDS-PAGE (Fig. 6, lane a). The major proteins of the PM (97 and

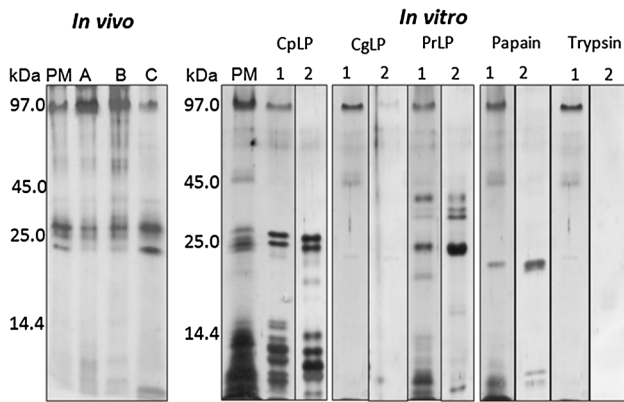


**Fig. 5** Inhibition of the proteolytic activity of (open square) CpLP, (open diamond) CgLP and (open triangle) PrLP by the HT gut homogenates of *D. plexippus* and *P. tetrio* using azocasein and BANA as substrates at pH 7.5

25 kDa) were resistant to the in vivo exposure to all of the LPs. However, in vitro assays showed that CgLP and PrLP, in addition to CpLP, papain and trypsin (which were introduced as controls), hydrolyzed the PM proteins (45 and 25 kDa). The more extensive hydrolysis that was observed in vitro could be explained by all of the gut proteins, including peptidases and peptidase inhibitors, being removed, which did not occur in the in vivo assays. Therefore, the structure of the PM seems to be vulnerable to action of latex peptidases only under in vitro conditions.

The PM 97 kDa abundant protein exhibited remarkable resistance to proteolytic hydrolysis in vivo and, to a lesser extent, in vitro. This protein was further identified by mass spectrometry as the peritrophic matrix intestinal mucin of *D. plexippus*. The mass spectrometry analysis of the PM also identified other proteins, such as serine peptidases, lipase and actin (Online resource 4, 5). A proteomic study of the PM of *Bombyx mori* larvae also showed the presence of serine peptidases, lipases and actin, in addition to alkaline phosphatases, peroxiredoxin and cytochrome P450, among others (Hu et al. 2012). The strong resistance of *D. plexippus* peritrophic mucin (the major protein in the structure of the PM) to CpLP, PrLP and CgLP agrees with others assays that showed no effect of these latex proteins on the development of the fifth instar of *D. plexippus* (Online resource 2).





**Fig. 6** Electrophoresis analysis of the PM proteins (left) of *D. plexippus* larvae (fifth instar) that were fed diets containing casein (lane PM), 1 % CpLP (lane A), 1 % CgLP (lane B) and 1 % PrLP (lane C). In vitro analyses of protein degradation (right) in the PMs that were treated with 50 mM Tris–HCl buffer, pH 7.5, with 3 mM DTT and 2 mM EDTA (lane PM, control) and treated with 1 mg/ml LP, 0.1 mg/ml papain and 0.1 mg/ml trypsin. The PMs were incubated with LP or purified peptidases (lane 1) and LP alone or peptidases as controls (lane 2). The proteins were stained with silver

As a final analysis, the PMs were treated in vitro with CpLP, CgLP, PrLP or peptidases that were purified of *C. procera* latex (CpCP). Atomic force microscopy (AFM) is a type of scanning probe microscopy used to obtain images from a wide variety of samples, at extremely high (nanometer) resolution (Meyer 1992). AFM can resolve the structure of molecules; therefore, it has been extensively used to study several kinds of biological samples, such as membrane proteins and their interactions with ligands under physiological conditions or cell morphological modifications induced by drugs (Pillet et al. 2014; Whited and Park 2014). However, up to now there is no work that used AFM to analyze damage on peritrophic membrane of insects. On the other hand, scanning electron microscopy has been used to examine the effect of plant cysteine protease on the structure of the caterpillar peritrophic membrane (Pechan et al. 2002). In our study AFM was applied to examine the peritrophic membranes exposed to latex proteins and purified latex peptidases. The images recorded were compared to those of the peritrophic membrane unexposed to latex proteins.

Atomic force microscopy of the PM revealed altered areas on the PM that were treated with LP or CpCP (Fig. 7). The black regions indicated by arrows in Fig. 7 represent local disruptions on the membrane structure. The measurements of roughness under the superficial area of the sections scanned increased in the PM that were treated with LP (i.e., CpLP: 2.945  $\mu\text{m}$ ; CpCP (1.800  $\mu\text{m}$ ) when compared to the PM of the control (0.582  $\mu\text{m}$ ), also suggesting that latex peptidases damage the PM.

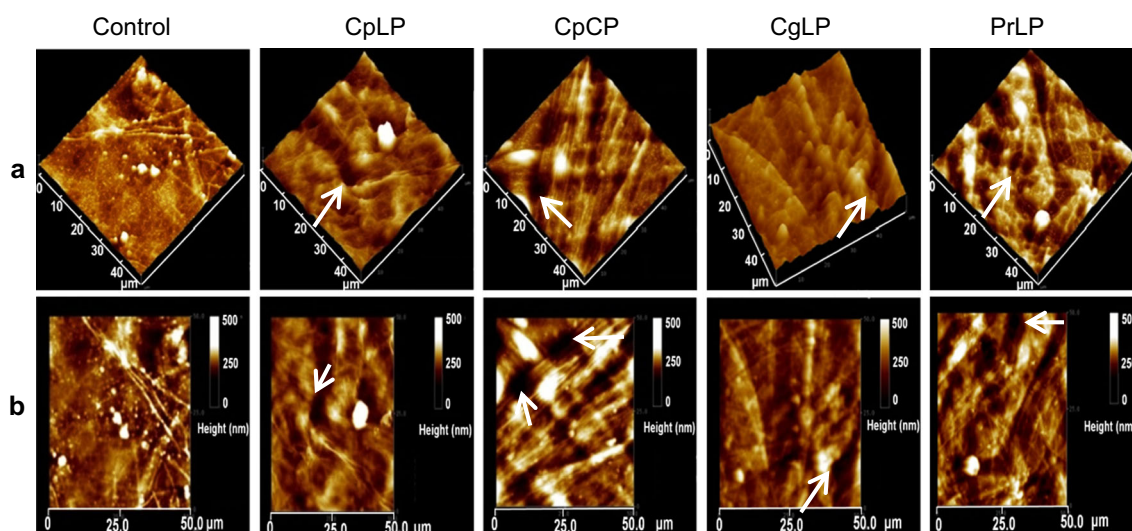
## Discussion

We have previously demonstrated through in vitro assays that the gut homogenates of *D. plexippus* promptly digested the latex proteins of its host plant *Calotropis procera* (CpLP) and, to a lesser extent, the latex proteins of *Plumeria rubra* (PrLP) and *Cryptostegia grandiflora* (CgLP) (Pereira et al. 2010). In the present study, we repeated the experimental strategy and examined the ability of the gut homogenates of *P. tetrio* to digest the latex proteins of its host plant (PrLP) and those of CpLP and CgLP through a time-course protocol. The results suggest that, in every case, the ability of the gut proteolytic systems to quickly digest the latex proteins seems to be the determinant factor supporting the success of caterpillars. This finding explains, at least in part, why the defensive strategies of the host plants, which includes peptidases, peptidase inhibitors, and chitinases, among other recognized defense proteins, are unable to protect themselves against their hosted insects.

As a whole, the results of the in vitro digestibility assays suggest that both of the studied caterpillars can grow when fed latex protein-containing diets, regardless of the origin of the latex. This hypothesis was positively supported by the results of Fig. 4 and agrees with those of our previous studies (Pereira et al. 2010). However, this hypothesis may be restricted only to caterpillars that are hosted by latex-bearing plants. In our previous study, we reported that experiments with artificial diets containing CpLP-fed *Anticarsia gemmatalis* and *Spodoptera frugiperda* (Lepidoptera: Noctuidae) produced opposite results. Both of these caterpillars are generalists (Table 1) but do not feed on latex-bearing plants. *A. gemmatalis* that were fed diets containing 0.1 % CpLP underwent a reduction in their body mass, while diets containing 1 % CpLP had no observed effect on *S. frugiperda* (Ramos et al. 2007). Therefore, while an overall view of the resistance–susceptibility to latex-bearing plants and their specialist caterpillars can most likely be constructed, a broader picture including non-eating latex caterpillars seems unlikely.

The proteolytic digestive systems of both of the Noctuidae species (at larvae stage) that are mentioned in Table 1, as well as both of the caterpillars that were studied here (Nymphalidae and Sphingidae), are mostly formed by serine peptidases (Paulillo et al. 2000; Oliveira et al. 2005). Therefore, this shared characteristic cannot explain the different performances of these insects when fed diets with added latex proteins. More detailed information on the specific set of serine peptidase types (i.e., trypsin, chymotrypsin and elastase) that are present in each species is needed to further address this question.

Despite proteolytic enzymes frequently representing the major enzymatic fraction of laticifer fluids; other recognized proteins that have been implicated in plant defense



**Fig. 7** Atomic force microscopy images of PMs that were treated with 50 mM Tris-HCl buffer, pH 7.5, with 3 mM DTT and with 2 mM EDTA (*control*) and treated with LP (1 mg/ml) or the purified peptidases of *C. procera* latex (*CpCP*). The proteins were diluted in

the same buffer that was used in the control. **a** Three-dimensional images. **b** Height representation of the three-dimensional images of PMs. The *white arrows* emphasize the depressions that formed in the PMs that were treated with the latex proteins

have also been previously described (Konno 2011). For instance, other known insecticidal proteins that are involved in plant defense and that are found in latex include chitinases and peptidase inhibitors that are thought to play important roles in the established rule of resistance-susceptibility. Chitinases were detected in the latex of *C. procera*, *Cr. grandiflora* and *P. rubra* (Freitas et al. 2010). These enzymes may be involved in latex defense against non-latex-eating caterpillars or insects belonging to other taxa, and this proposal remains to be evaluated. For instance, CpLP at 0.1 % added to the diet has also been shown to be toxic to larvae and adult insects of *Callosobruchus maculatus* (Coleoptera: Chrysomelidae) (Ramos et al. 2010). In the light of the results reported here, it is unlikely that latex chitinases play a pivotal role in latex protection against latex-eating caterpillars.

All of the HT latex samples strongly inhibited the proteolytic activity of *D. plexippus* and *P. tetrico* when BApNA was used as a substrate. This result suggests that the host plants have molecular strategies to fight against predators. Why then are these putative inhibitors incapable of avoiding caterpillar attack? In vitro conditions do not faithfully reproduce the results of in vivo conditions. The biological milieu includes other important chemical/biochemical parameters that were not considered in our study. Therefore, the overall view of this finding should be seen with reserve. Even under in vivo conditions, enzymatic kinetics is governed by intrinsic conditions that are unlikely to be experimentally reproduced. It is concluded that although peptidase inhibitors occur in the lattices of *C. procera*, *Cr. grandiflora* and *P. rubra* they cannot prevent caterpillar attack on leaves.

The peritrophic membrane (PM) is a semi-permeable and non-cellular structure lining the guts of insects. The PM is mainly composed of chitin microfibrils and specific proteins named peritrophins (Terra 2001). Several physiological functions have been proposed to this structure, including protection against mechanical and chemical damage, and it serves as a barrier to invasion by microorganisms and parasites (Hegedus et al. 2009). The integrity of the PM is essential for the survival and development of insects, and any damage to this structure results in reduced growth or insect death. Intestinal mucins are the most abundant chitin-binding proteins in the PM of lepidopteran insects. Intestinal mucins are also present in humans and other mammals (Wang and Granados 2001). Insect intestinal mucins display important physiologic roles, such as lubricating the passage of food and protecting the midgut epithelium against pathogenic or toxic molecules from the diet (Toprak et al. 2010). Mucins are highly resistant to peptidase degradation and, in this context, can be tooled as targets of prospecting enzymes for insect biocontrol (Zhang and Guo 2011).

A 97-kDa mucin that was identified in the PM of *D. plexippus* was only slightly digested in vitro by CpLP, PrLP and CgLP. However, no evidence for toxicity was documented by in vivo assays. Li et al. (2009) reported the complete in vitro digestion of the insect intestinal mucin of *Trichoplusia ni* (Lepidoptera) by the latex proteolytic fraction of *Asclepias syriaca*. However, an analysis of this protein from animals that were fed this protein in the diet revealed the full integrity of the protein. This result is consistent with that of our in vivo assays, which showed no effect of CgLP and PrLP on the development of *D.*

*plexippus*, suggesting that no damage to the PM occurs in vivo.

Atomic force microscopy suggests that damage to the PM structure occurs when the PM is directly exposed to latex proteins or purified latex peptidases in vitro. This observation once more demonstrates the ability of the latex defensive machinery. However, as previously evoked, the observed partial hydrolysis and, consequently, the damage to the PM could be explained by the removal of all of the gut proteins. The performance of the larvae that were fed diets containing latex proteins indubitably demonstrates the adaptive success of these insects to feeding on latex-bearing plants.

## Conclusion

The molecular basis underlying the resistance–susceptibility of plants and their hosted insects is still an exciting area of research. The specific case involving laticifer plants represents a very special model for approaching this question. In this study, we report the biochemical aspects of two complex proteolytic systems that govern, at least in part, the resistance–susceptibility rules involving latex and specialist caterpillars that fed on latex-bearing plants. It is concluded that both plants and insects possess known defensive molecular strategies based on proteolysis and the inhibition of proteolysis. The ability of insect proteolytic systems, which hydrolyze latex proteins, seems to be an important event favoring caterpillars overcoming plant defense.

**Author contribution** Márcio V. Ramos: scientific hypothesis; experimental design, writing. Danielle A. Pereira: performed bioassays. Diego P. Souza: latex collecting and processing. Maria-Lídia S. Silva: performed bioassays. Luciana M. R. Alencar and Jeanlex S. Sousa: performed atomic force microscopy analysis, Juliany-Fátima N. Queiroz: performed enzymatic measurements. Cleverson D. T. Freitas: performed mass spectrometry analysis. Maria-Lídia S. Silva In memoriam.

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