

Gut peptidases from a specialist herbivore of latex plants are capable of milk protein hydrolysis: Inputs for hypoallergenic milk formulas

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ABSTRACT

Transitory allergies to cow milk proteins in infants or adults have become a public health problem. Although extensively or partially hydrolyzed cow milk protein formulas are available, these products are costly. Therefore, studies into innovative enzymes to digest cow milk proteins are needed. *Danaus plexippus* gut peptidases were purified and examined with regard to cow milk protein hydrolysis. The peptidases hydrolyzed caseins and whey proteins. However, after heat treatment, there was a significant improvement in β -lactoglobulin hydrolysis. The hydrolyzed cow milk proteins were not recognized by anti-casein antibodies and only reacted slightly with antibodies against whey proteins. This performance was better than that of partially hydrolyzed formulas and similar to that of an extensively hydrolyzed formula. These results suggest that *D. plexippus* gut peptidases are suitable and innovative enzymes to produce hypoallergenic cow milk protein formulas.

1. Introduction

Milk allergy is an immunological reaction induced by undigested proteins present in milk. It is the most common food allergy during infancy and childhood (Sicherer & Sampson, 2014). Although, in more than 80% of cases the allergy is transitory, this immunological reaction can result in intensified gastrointestinal, respiratory and dermatological symptoms (Fiocchi et al., 2010). Consequently, the supply of energy and amino acids for the growth and development of children is reduced to some extent (Luyt et al., 2014). Although cow milk contains more than 25 different proteins (Hochwallner, Schulmeister, Swoboda, Spitzauer, & Valenta, 2014), caseins, β -lactoglobulin and α -lactalbumin (whey proteins) are reported as the major milk allergens (Monaci, Tregoat, van Hengel, & Anklam, 2006). Therefore, the partial or extensive cleavage of cow milk proteins (CMP) achieved by peptidases is one of the most effective methods to reduce allergic reactions to these proteins (Bu, Luo, Chen, Liu, & Zhu, 2013). Even though very effective, these formulas are expensive, making them unaffordable to many people. Children suffering from milk allergy have few alternative protein sources to supply their daily intake to minimally support their growth and development. Soy protein formulas, which are the immediate substitute for CMP, are not adequate for the nutritional needs of infants and in some cases cross-reactions between antibodies against

CMP with soy proteins have been documented (Candrea et al., 2016). To confront this complex scenario, the identification of novel enzymatic sources suitable for efficiently digesting CMP and producing hypoallergenic formulas is needed. Therefore, the study of new peptidases able to hydrolyze milk proteins should be encouraged.

The caterpillar of the Monarch butterfly is extremely well adapted to feed on the leaves of latex-producing plants, such as *Calotropis procera*. A previous study by Pereira and collaborators (2010) showed that *D. plexippus* gut peptidases are able to hydrolyze all proteins present in the latex of *C. procera* within a few minutes. Interestingly, this same protein fraction from *C. procera* latex is toxic to other insects (Ramos et al., 2007). Moreover, *D. plexippus* gut peptidases are resistant to hydrolysis by latex peptidases even after 24 h of incubation (Pereira et al., 2010). Therefore, our hypothesis was that *D. plexippus* gut peptidases can be used as powerful biomolecules to hydrolyze other proteins, including food proteins. Therefore, the aim of the present work was to purify *D. plexippus* gut peptidases by affinity chromatography and evaluate their hydrolytic action on CMP (caseins, β -lactoglobulin and α -lactalbumin) and to compare the resulting hydrolysate with commercial milk formulas.

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2. Materials and methods

2.1. Materials

Acrylamide (17-1302-02), bis-acrylamide (17-1304-02), sodium dodecyl sulfate (SDS) (17-313-01), HiTrap™ Benzamidine Sepharose 4 Fast Flow column (17-5143-01), HiTrap DEAE FF Sepharose Fast Flow (17-5055-01), HiTrap CM FF Sepharose Fast Flow (17-5056-01), HiTrap Protein A Sepharose high performance column (17-0402-01) and low molecular mass markers (17-0446-01) were acquired from GE Healthcare Life Science (São Paulo, SP, Brazil). Sequencing-grade modified trypsin was obtained from Promega (V511B) (São Paulo, SP, Brazil). Azocasein (A2765), porcine pepsin (P7012), Freund's complete (F5881) and incomplete adjuvant (F5506), goat anti-rabbit IgG conjugated with alkaline phosphatase (A6066) and *p*-nitrophenylphosphate disodium (N2765) were obtained from Sigma-Aldrich (São Paulo, SP, Brazil). All other chemicals were of analytical grade.

2.2. Gut protein extract

Gut protein extracts of *Danaus plexippus* larvae (5th instar) were obtained according to Pereira et al. (2010), dialyzed against distilled water, lyophilized and stored at -20°C until further assays were performed. Soluble proteins were quantified by Bradford's method (Bradford, 1976).

2.3. Gut peptidase purification

D. plexippus gut peptidases (DpGp) were purified by affinity chromatography using a HiTrap™ Benzamidine Sepharose 4 Fast Flow column coupled to an AKTA chromatography system (GE HealthCare). The unbound proteins (PI) were washed with equilibrium buffer (50 mM Tris-HCl pH 7.4, containing 0.5 M NaCl) and the bound proteins (PII, DpGp) were eluted with 50 mM glycine-HCl (pH 3.0) at a flow rate of 1 mL/min. Proteins were monitored at 280 nm. Both peaks (PI and PII) were dialyzed against distilled water and lyophilized.

In order to purify the peptidases, the PII fraction from the HiTrap™ Benzamidine Sepharose 4 Fast Flow column was submitted to ion exchange chromatography in DEAE-Sepharose fast flow and CM-Sepharose fast flow columns, which were previously equilibrated with 25 mM Tris-HCl buffer (pH 7.0). The proteins were eluted from the column by using a linear gradient from 0 up to 1 M NaCl in 25 mM Tris-HCl buffer (pH 7.0). Additionally, the PII fraction was also submitted to reversed-phase high-performance liquid chromatography (RP-HPLC). Samples of 50 μL (1 mg/mL) were loaded in the C2/C18 column (μRPC C2/C18 ST, bed length 100 mm, i.d. 4.6 mm, 3 μm particle size, 12 nm porosity, Amersham Bioscience) coupled to a Jasco CO-2060 Plus HPLC device. The proteins were eluted using a linear gradient from 5% to 80% acetonitrile for 30 min, containing 0.1% trifluoroacetic acid (TFA), at 0.5 mL/min flow rate. Proteins were detected at 280 nm.

2.4. Proteomic analysis

2.4.1. One-dimensional gel electrophoresis

Polyacrylamide gel electrophoresis (15%) in the presence of SDS (1D-SDS-PAGE) was performed according to Laemmli (1970). The samples were dissolved in sample buffer [0.0625 M Tris-HCl buffer (pH 6.8) containing 2% SDS] and the runs were performed at 25 mA (25 $^{\circ}\text{C}$ for 2 h) using 10×10 cm gels. Proteins were stained using Coomassie brilliant blue R-350.

2.4.2. Protein identification by mass spectrometry (LC-MS/MS)

The protein bands from the 1D-SDS-PAGE gels were removed, destained, and digested with sequencing-grade modified trypsin according to Hellman, Wernstedt, Góñez, and Heldin (1995). The tryptic peptides were analyzed using a Synapt G1 HDMS mass spectrometer (Waters,

Manchester, UK) coupled to a Nano UPLC-ESI system, as described by Freitas et al. (2016). Proteins were identified using the NCBI database and MASCOT search engine (Matrix Science Ltd., London, UK; website: <http://www.matrixscience.com>).

2.5. Proteolytic activity

Total proteolytic activity was evaluated using azocasein as non-specific substrate (Freitas et al., 2007). Briefly, 50 μL of total gut protein extract or peaks from affinity chromatography (1 mg/mL) were mixed with 1% azocasein (200 μL) and the final volume of the reaction was adjusted to 300 μL with 50 mM Tris-HCl (pH 9.0) or 50 mM sodium phosphate (pH 6.5). The reactions were performed at 37°C for 1 h, and then stopped with 10% TCA solution. The reaction products were measured at 420 nm.

2.6. Autolysis assays

D. plexippus gut peptidases (DpGp) [1 mg/mL in 50 mM Tris-HCl (pH 9.0) or 50 mM sodium phosphate (pH 6.5)] were incubated at different temperatures (4, 25 and 37°C) and for different time intervals (1, 2, 4 and 24 h). After incubation, aliquots (50 μL) were used to determine the residual proteolytic activity, as described in Section 2.5. Protein autolysis was verified by 15% polyacrylamide gel electrophoresis in the presence of SDS (1D-SDS-PAGE), as described previously in Section 2.4.1.

2.7. Caseins and whey protein purification

Caseins and whey proteins were purified according to Egito et al. (2007). Briefly, raw bovine milk (obtained from a local market) was centrifuged ($2100 \times g$ at 32°C for 30 min) and the supernatant (skimmed milk) was separated and acidified with 0.1 M HCl to pH 4.6. Then, caseins and whey proteins were separated by centrifugation at $1500 \times g$ at 20°C for 20 min. The supernatant (whey proteins) was collected and the precipitate (sodium caseinate) was washed three times with distilled water and centrifuged again. Both fractions were dialyzed against distilled water at 4°C for two days using membranes with an 8000 Da cut-off, and then lyophilized for further use.

2.8. β -Lactoglobulin purification

β -lactoglobulin was purified as described by Kitabatake and Kinekawa (1998). The whey protein fraction (non-lyophilized) was adjusted to pH 2.0 with 1 M HCl and porcine pepsin was added to a ratio of 1:200, enzyme:substrate (mass:mass). After 1 h at 37°C , the proteins were precipitated overnight with 75% ammonium sulfate and then centrifuged at $20,800 \times g$ for 30 min at 25°C . The supernatant (β -lactoglobulin) was dialyzed against distilled water at 4°C for two days using membranes with an 8000 Da cut-off, and then lyophilized. The homogeneity of β -lactoglobulin was checked by 15% 1D-SDS-PAGE as described in Section 2.4.1.

2.9. Hydrolysis of milk proteins

The hydrolytic potential of DpGp on the milk proteins (caseins, whey proteins and purified β -lactoglobulin) was assessed by zymograms and *in vitro* assays. For the zymograms, the gels were supplemented with the four protein substrates at 0.2% (m/m): gelatin (control), caseins, whey proteins and purified β -lactoglobulin. After the electrophoretic runs of DpGp, the gels were washed twice with 2.5% Triton X-100 for 30 min to remove all SDS. Afterward, the gels were incubated in 50 mM Tris-HCl (pH 9.0) or 50 mM sodium phosphate (pH 6.5) for 1 h at 37°C . Protein hydrolysis was detected as transparent bands in blue gels after staining with 0.1% Coomassie brilliant blue R-350.

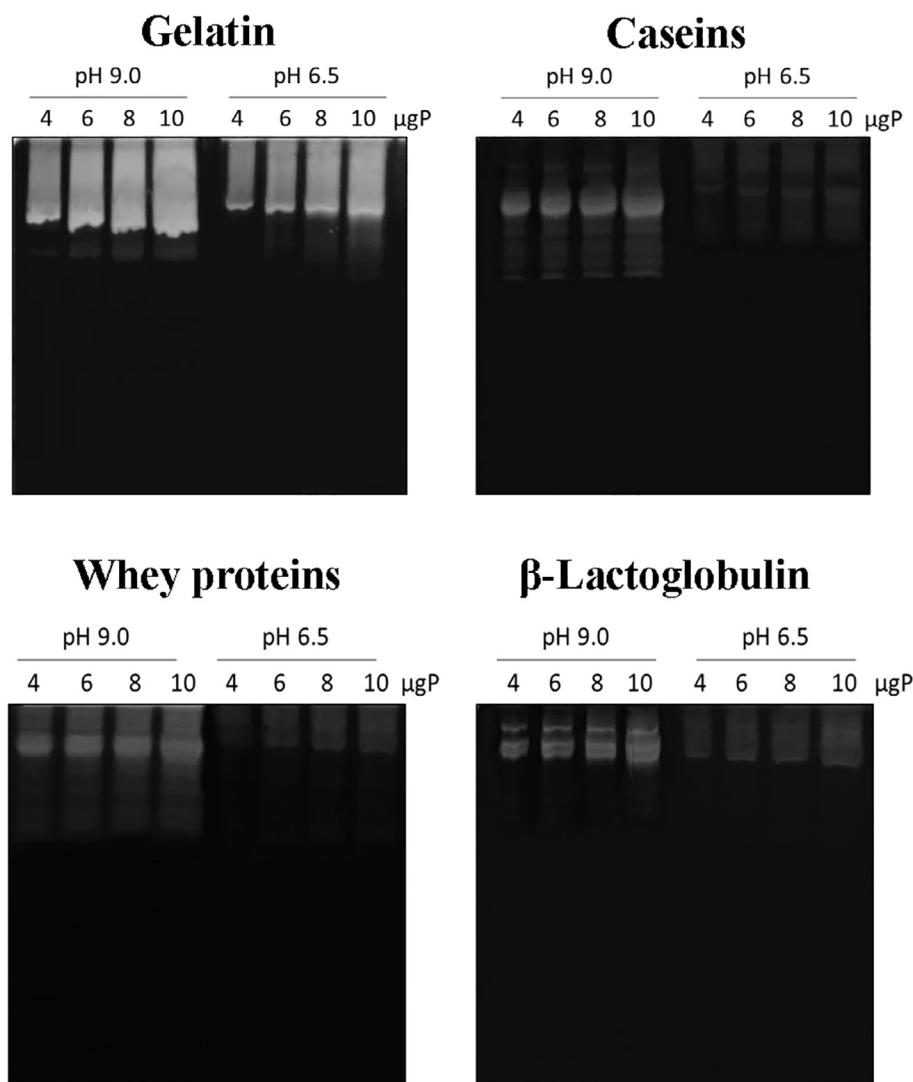


Fig. 1. Zymograms for proteolytic activity detection in gels containing gelatin, mixed caseins (α -, β -, and κ -caseins), whey proteins (α -lactalbumin and β -lactoglobulin) or purified β -lactoglobulin as substrates at 0.2%. After 1D-SDS-PAGE, the gels were washed with Triton X-100 and incubated for 1 h at 37 °C at pH 9.0 or 6.5. Transparent bands indicate protein hydrolysis by DpGp.

The *in vitro* assays were performed by incubating DpGp with the different substrates: caseins, whey proteins, native β -lactoglobulin and heated β -lactoglobulin (85 °C for 30 min) in 50 mM Tris-HCl (pH 9.0) or 50 mM sodium phosphate (pH 6.5). For the reactions, each substrate (10 mg/mL) was mixed with different aliquots of DpGp (4 mg/mL) to obtain the ratios of 1:100, 1:50 and 1:25 (enzyme:substrate, m/m). After 1, 2, 4 and 24 h of incubation at 37 °C, aliquots were taken to measure the extensiveness of protein hydrolysis. To estimate the degree of hydrolysis by absorbance, samples obtained in each time interval were mixed with 20% trichloroacetic acid (TCA) solution in a 1:1 ratio (hydrolysate:TCA solution) and centrifuged at 10,000 $\times$ g, 10 °C for 10 min. Afterward, the absorbance of the supernatants were measured at 280 nm. The degree of hydrolysis was also quantified from 1D-SDS-PAGE by using the IMAGEJ software (<https://imagej.nih.gov/ij/>).

2.10. Polyclonal antibody production

Rabbit polyclonal antibodies against bovine casein and whey protein fractions were produced as described by Freitas et al. (2015). The protocol was approved by the institutional ethics committee for animal experimentation of Federal University of Ceará. Briefly, two adult New Zealand male rabbits were sensitized intramuscularly, one with caseins and the another with whey proteins (1 mg, dissolved in 0.5 mL saline

and 0.5 mL of Freund's complete adjuvant) and booster injections were applied subcutaneously at 21, 35 and 42 days after the first sensitization using Freund's incomplete adjuvant containing the protein fractions. Blood samples were collected and allowed to coagulate at 37 °C for 5 h. The sera were centrifuged (2000 $\times$ g at 25 °C for 5 min) and IgGs were purified by immunoaffinity chromatography using Protein A immobilized in a Sepharose 4B column (Freitas et al., 2017).

2.11. Enzyme-linked immunosorbent assay (ELISA)

The hydrolysis of milk proteins (caseins, whey proteins and purified β -lactoglobulin) by DpGp was also estimated by ELISA using anti-casein and anti-whey protein IgGs as the primary antibodies (1:20,000 dilution) and anti-rabbit IgG conjugated with alkaline phosphatase (1:10,000 dilution) as the secondary antibody. The same samples used in the *in vitro* assays (Section 2.9, 150 μ L) were diluted in 96-well microplates. After the removal of unbound proteins, the residual binding sites were blocked with 200 μ L of gelatin (10 mg/mL) in 50 mM sodium phosphate buffer (pH 7.0). Subsequently, the primary and secondary antibodies (150 μ L) were added and the reactions were detected after adding the substrate *p*-nitrophenyl phosphate disodium and measuring absorbance at 405 nm (Freitas et al., 2015). Commercial non-hydrolyzed and hydrolyzed milk formulas were used as references: Itambé®

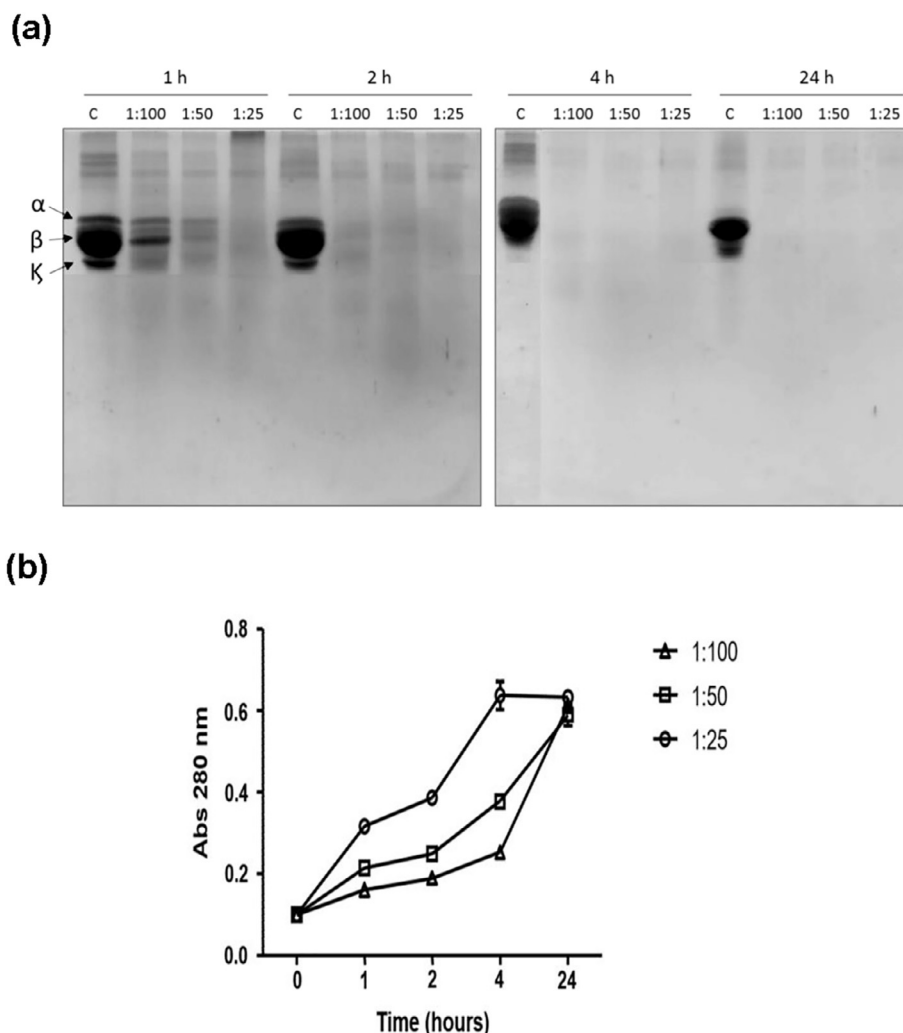


Fig. 2. Casein hydrolysis by DpGp performed at 37 °C and pH 9.0. (a) 1D-SDS-PAGE (15%) of the casein hydrolyzed at different time points and enzyme:substrate ratios (1:100, 1:50 and 1:25); 50 µg of protein was added to each well. C: non-digested casein. (b) The same samples in (a) were used to quantify the degree of hydrolysis at 280 nm. Each value represents the mean of three independent experiments and error bars indicate the standard deviation of the mean.

and Nestogeno® (Nestlé): non-hydrolyzed cow milk; Nan Supreme1® (Nestlé) and Enfamil® Gentlease® (Mead Johnson Nutrition): partially hydrolyzed cow milk; and Aptamil Pepti® (Danone): extensively hydrolyzed cow milk.

3. Results

3.1. Purification, identification and characterization of gut peptidases

Two peaks were obtained when the gut extract of *D. plexippus* was chromatographed in an affinity column with immobilized benzamidine (Supplementary Fig. 1a). The retained proteins (PII fraction) were eluted with 50 mM glycine-HCl buffer (pH 3.0). The 1D-SDS-PAGE showed that the PII fraction was rich in proteins with high molecular masses (45–70 kDa) (Supplementary Fig. 1b). All protein bands detected in the PII fraction were excised from the gels, digested with trypsin and analyzed by mass spectrometry (LC-MS/MS). All analyzed proteins matched with peptidases (Supplementary Table 1). To purify the gut peptidases, different chromatographic steps were performed, including the use of DEAE- and CM-Sepharose columns (pH 7.0) and reversed-phase high-performance liquid chromatography (RP-HPLC, C2/C18 column). However, these chromatographic steps were not effective, because only one peak was obtained in the CM-Sepharose column, and the protein peaks from DEAE-Sepharose and RP-HPLC

columns were not separated accordingly (Supplementary Fig. 2). The 1D-SDS-PAGE (15%) of different fractions from DEAE-Sepharose fast flow showed that the peptidases were not isolated (data not shown). Therefore, the PII fraction from the HiTrap™ Benzamidine Sepharose column was used in all further experiments, and termed *D. plexippus* gut peptidases (DpGp).

To better characterize the hydrolytic potential of DpGp, an assay was performed to determine its proteolytic activity at two pH levels: pH 6.5 (milk pH) and pH 9.0 (optimal pH of *D. plexippus* gut peptidases) (Pereira et al., 2010). The proteolytic activity at pH 9.0 was 2.6-fold higher than at pH 6.5 (Supplementary Fig. 1c). Thus, further assays were performed at pH 9.0. The proteolytic activity of DpGp was monitored after different storage times and temperatures, both important parameters to evaluate protein autolysis and stability. The proteolytic activity of DpGp remained unaltered after 1 h at 37 °C (100% activity), with a slight decrease in activity (10%) when the sample was incubated for 2 h. Only after 24 h was there a considerable loss (55%) of activity. On the other hand, DpGp were stable at 4 °C even after 24 h, retaining 80% of the initial activity (Supplementary Fig. 2a). The evaluation of protein autolysis was observed by changes in the protein profiles and the results were in accordance with those of proteolytic activity *in vitro*. Autolysis began after 2 h and reached more than 50% after 24 h at 37 °C. In contrast, DpGp were very stable after 24 h of incubation at 4 °C (Supplementary Fig. 2b). All these results concerning the purification

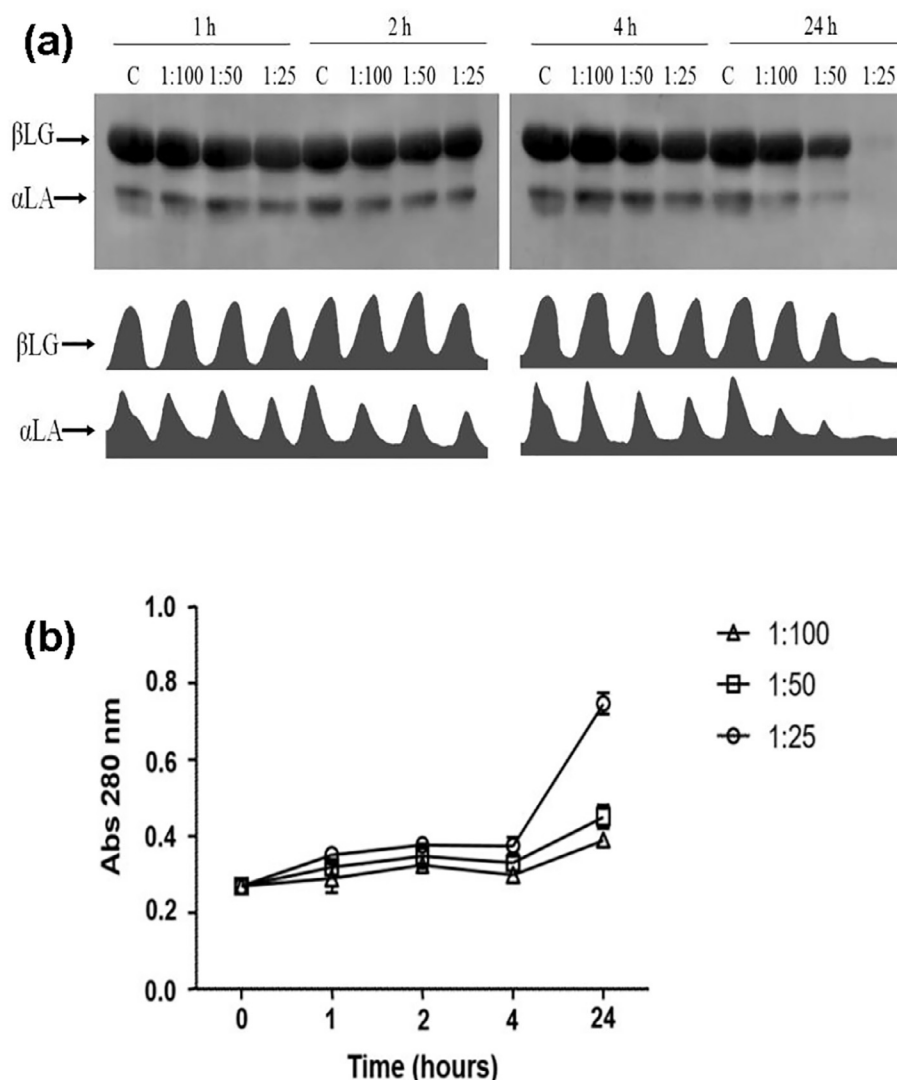


Fig. 3. Whey protein hydrolysis by DpGp performed at 37 °C and pH 9.0. (a) 1D-SDS-PAGE (15%) of the whey protein hydrolyzed at different time points and enzyme:substrate ratios (1:100, 1:50 and 1:25); 50 µg of protein was added to each well. C: non-digested whey proteins. βLG: β-lactoglobulin. αLA: α-lactalbumin. The degree of hydrolysis was quantified by densitometry using the IMAGEJ software (lower panel). (b) The same samples in (a) were also used to quantify the degree of hydrolysis at 280 nm. Each value represents the mean of three independent experiments and error bars indicate the standard deviation of the mean.

and enzymatic characterization of DpGp are detailed in the [Supplementary materials](#).

3.2. Hydrolysis of milk proteins

Once characterized, DpGp were screened for the ability to hydrolyze CMP by zymography in gels containing caseins, whey proteins and purified β-lactoglobulin as substrates. After protein migration by electrophoresis, the gels were incubated at pH 6.5 or pH 9.0 for 1 h (37 °C) and proteolytic activities were detected as transparent bands in dark gels (Fig. 1). Although all gels showed the same total proteolytic activity (determined using gelatin substrate as control), significant differences in the hydrolysis patterns were observed at pH 6.5 and pH 9.0 and among the different protein substrates (Fig. 1). In all assays, protein hydrolysis was better at pH 9.0, corroborating data from the *in vitro* assays (Supplementary Fig. 1c), and using the caseins as the substrate. Interestingly, the zymograms showed several proteolytic bands able to degrade caseins and whey proteins, including purified β-lactoglobulin (Fig. 1). These results indicate that the different gut peptidase isoforms of *D. plexippus* were able to hydrolyze the major CMP at pH 9.0.

The digestibility of milk proteins by DpGp was further assessed by *in*

vitro assays followed by electrophoresis analysis. DpGp digested caseins in a dose-dependent manner. The hydrolysis was remarkable at a 1:100 ratio (enzyme:substrate, m:m) and after 1 h of incubation at 37 °C (pH 9.0). Complete hydrolysis (100%) was obtained after 4 h using a 1:25 ratio or after 24 h using a 1:100 ratio (Fig. 2). In contrast, whey proteins were more resistant than caseins to hydrolytic action by DpGp (Fig. 3). Only at the highest ratio (1:25, enzyme:substrate) and after 24 h of incubation at 37 °C (pH 9.0) was effective hydrolysis (100%) of whey proteins (β-lactoglobulin and α-lactalbumin) observed (Fig. 3). On the other hand, when hydrolysis assays were performed at pH 6.5 (milk pH), DpGp were able to digest only caseins at highest dose tested (1:25 ratio) (Supplementary Fig. 4).

To improve hydrolysis performance, purified β-lactoglobulin was heated at 85 °C for 30 min and then incubated with DpGp at different time points and ratios at pH 9.0. Heat treatment led to an enhancement of β-lactoglobulin processing by gut peptidases of the caterpillar (Fig. 4). A comparison between native and heat denatured β-lactoglobulin hydrolysis revealed that heat denaturation supported more efficient hydrolysis even in the first hour (30%) at a 1:25 (enzyme:substrate) ratio. After 24 h, hydrolysis at the 1:100, 1:50 and 1:25 ratios were 50%, 75% and 100%, respectively (Fig. 4).

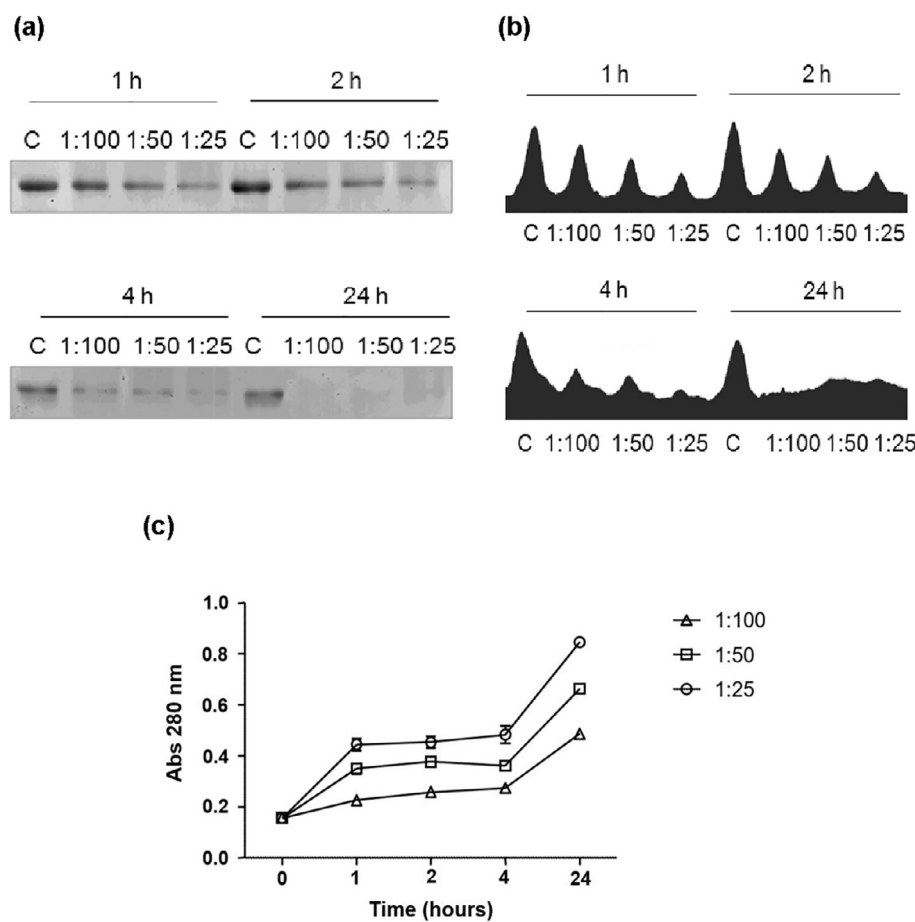


Fig. 4. Heated β -lactoglobulin hydrolysis by DpGp performed at 37 °C and pH 9.0. (a) 1D-SDS-PAGE (15%) of the heated β -lactoglobulin hydrolyzed at different time points and enzyme:substrate ratios; 50 μ g of protein was added to each well. C: non-digested heated β -lactoglobulin. (b) Densitometry of the protein bands in (a) using the IMAGEJ software. (c) The same samples in (a) were also used to quantify the degree of hydrolysis at 280 nm. Each value represents the mean of three independent experiments and error bars indicate the standard deviation of the mean.

3.3. Residual antigenicity of peptides from hydrolyzed milk proteins

To confirm the hydrolytic potential of DpGp, whole skimmed milk (Itambé®) was used as the substrate (Fig. 5) and the resulting hydrolysis was compared with commercial non-hydrolyzed (Itambé® and Nestogeno®), partially hydrolyzed (Nan Supreme® and Enfamil®) and extensively hydrolyzed (AptamilPepti®) milk formulas. After 24 h of incubation at 37 °C (pH 9.0), caseins and whey proteins were completely hydrolyzed by DpGp, using the 1:25 (enzyme:milk proteins) ratio. This pattern was very similar to those observed for all hydrolyzed commercial formulas (Fig. 5a) and corroborated the previous results.

The possible residual antigenicity of peptides from hydrolyzed milk by DpGp was measured by ELISA, using anti-casein and anti-whey protein antibodies (Fig. 5b). The detection of casein peptides was minimal for milk hydrolyzed by DpGp when compared to non-hydrolyzed milk (Itambé® and Nestogeno®), and very similar to partially and extensively commercial milk (Nan Supreme®, Enfamil® and AptamilPepti®). On the other hand, antigenic peptides from whey proteins were detected in milk hydrolyzed by DpGp, although at a much lower level than in non-hydrolyzed milk (Itambé® and Nestogeno®) and the partially hydrolyzed milk Enfamil® (Fig. 5b). The extensively hydrolyzed milk formula (Aptamil Pepti®) did not show any residual antigenicity for caseins and whey proteins.

4. Discussion

An important industrial application of peptidases is in the hydrolysis of food proteins, particularly to reduce their allergenicity. Food allergies are important medical and social problems. Around 8% of infants and 2% of adults in the world have some type of food allergy (Gendel, 2013). Therefore, it is estimated 700 million people (considering a

current population of 7 billion) have some kind of food allergy, representing a major global health problem. In particular, cow milk allergy constitutes the main food allergy (Fiocchi et al., 2010). Therefore, peptidases from microbial, animal and plant sources have been studied for the hydrolysis of CMP (Patel, Singhania, & Pandey, 2016; Tavano, 2013). However, gut peptidases from insects have not received much attention. Here, the gut peptidases of *D. plexippus* larvae were purified, identified, partially characterized and evaluated to digest CMP.

Because previous studies have reported predominance of serine peptidases in the *D. plexippus* larval gut extract (Pereira et al., 2010), these enzymes were cleaned from the extract using an affinity column containing immobilized benzamidine, which is a reversible competitive inhibitor for serine peptidases. The efficiency of the affinity chromatography was confirmed by proteomic analysis, since only peptidases were identified (Supplementary Table 1). Accordingly, the same method was successfully used in the purification of peptidases from other insects (Rascón, Gearin, Isoe, & Miesfeld, 2011). Because additional chromatographic steps failed to purify the gut peptidases to homogeneity (Supplementary Fig. 2), the mixture of peptidases (PII fraction) from the HiTrap™ Benzamidine Sepharose column was used in all further experiments.

Beyond high activity, industry demands peptidases that do not easily undergo inactivation or autolysis during the proteolytic process. Thus, two biochemical parameters are prerequisites for new alternative enzymes: the effect of reaction time and temperature on peptidase stability. *D. plexippus* gut peptidases remained stable even after two hours at 37 °C. Only after 24 h at 37 °C was there a considerable loss of activity and autolysis (Supplementary Fig. 2). Similar results were obtained for a thermo active alkaline serine peptidase from alkaliphilic *Bacillus* sp. (Ibrahim, Al-Salamah, El-Badawi, El-Tayeb, & Antranikian, 2015). Although the peptidases still exhibited the problems of

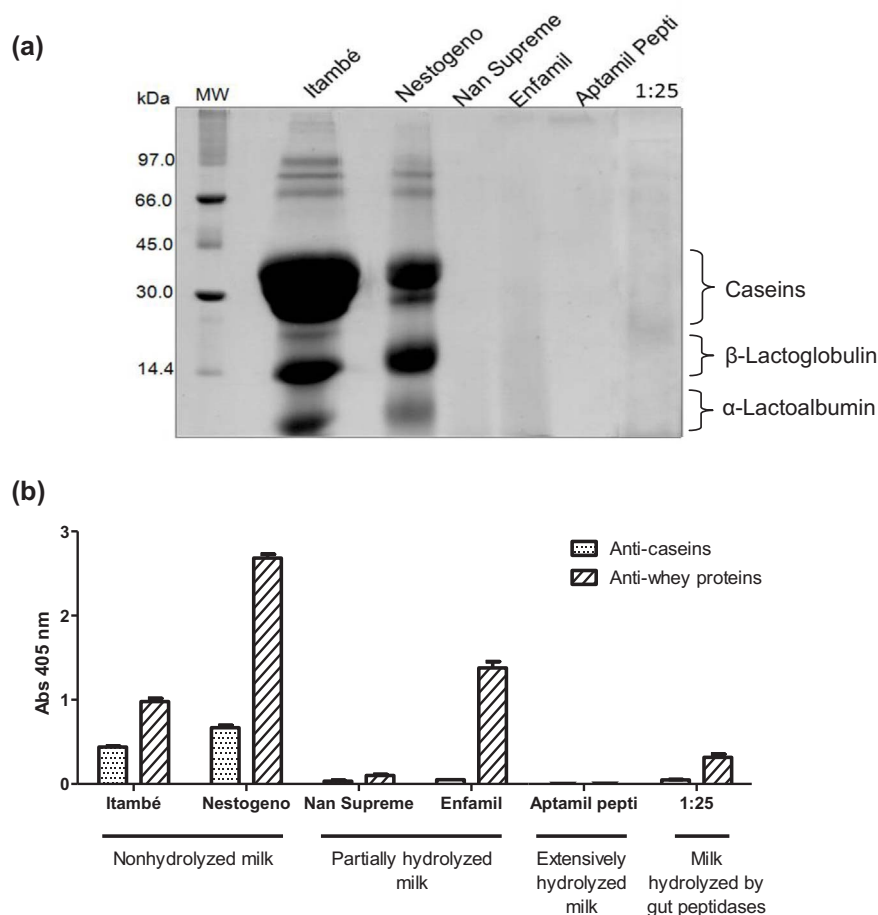


Fig. 5. Comparison between commercial hydrolyzed milk formulas with hydrolysate produced by DpGp (1:25 ratio). (a) Protein profile of all formulas was performed by 15% 1D-SDS-PAGE. The same amount of protein was added protein to each well (40 μ L, 20 mg/mL). (b) ELISA comparing the residual immunogenicity of the milk formulas using anti-caseins or anti-whey protein antibodies. Itambé® milk (non-hydrolyzed milk) was incubated with DpGp (1:25 ratio) for 24 h at 37 °C and pH 9.0. This sample represents the well 1:25 in (a) and milk hydrolyzed by gut peptidases in (b).

inactivation and autolysis, with additional understanding of their three-dimensional structures and the specific amino acids involved in the mechanisms of action, it may be possible to manipulate and modify them to optimize their use in hydrolytic processes (Olivera-Nappa, Reyes, Andrews, & Asenjo, 2013).

The hydrolysis of caseins by DpGp was very effective. In a similar work, Mika, Gorshkov, Spengler, Zorn and Rühl (2015) tested gut extracts from six insects for the hydrolysis of different food proteins (casein, gluten and rice proteins). The gut peptidases of the beetles *Tribolium castaneum* and *Sitophilus granarius* exhibited no activity, while the enzymes from *Rhyzopertha dominica*, *Oryzaephilus surinamensis*, *Tenebrio molitor* and *Alphitobius diaperinus* were able to digest casein (Mika et al., 2015). Because caseins constitute the most abundant CMP, corresponding to almost 80% of all proteins, they have been identified as major milk allergens (Docena, Fernandez, Chirido, & Fossati, 1996). By analyzing 80 milk-atopic patients on the basis of the presence of cow milk-specific IgE antibodies in serum and compatible clinical history, Docena and collaborators (1996) showed that casein-specific IgE antibodies were present in all sera examined (100%), whereas 12% were reactive to β -lactoglobulin and 6% to α -lactalbumin. Consequently, peptidases able to extensively hydrolyze caseins are potentially good biotechnological tools for producing hypoallergenic milk formulas.

Although to a lesser extent when compared to caseins, whey proteins (α -lactalbumin and β -lactoglobulin) can also be considered cow milk allergens (Fiocchi et al., 2010). In particular, β -lactoglobulin has been studied because this protein is not present in human milk. Moreover, it is very resistant to enzymatic action (Fiocchi et al., 2010). In the present study, only after 24 h at 37 °C and using the highest concentration (1:25, peptidase:substrate ratio) were DpGp able to totally digest whey proteins (α -lactalbumin and β -lactoglobulin) (Fig. 3). Similar results were observed with other enzymatic sources, such as

trypsin, chymotrypsin, pepsin, chymosin, rat gastric juice and acid peptidases from the fungus *Monascus pilosus* (Kitabatake & Kinekawa, 1998; Lakshmana et al., 2011; Salami et al., 2008). The high resistance of whey proteins to enzymatic digestion can be explained by the stability of the tertiary structure (Reddy, Kella, & Kinsella, 1988). Therefore, some researchers have added physical treatments, such as pressure and heating, in order to increase the hydrolysis of whey proteins (Patel et al., 2016). Here, we showed that the hydrolytic action of DpGp on β -lactoglobulin was significantly increased after heat treatment (Fig. 4). Accordingly, the hydrolysis of whey proteins by trypsin and pepsin were slightly increased after the same treatment (Adjou, Doran, Torley, & Agboola, 2013). It is worth noting that heat treatment can also promote the formation of aggregates of β -lactoglobulin, thereby decreasing the hydrolysis performance by hindering the cleavage sites. Therefore, increasing/decreasing β -lactoglobulin hydrolysis during thermal treatment is affected by the temperature and the duration of heating (Leeb, Götz, Letzel, Cheison, & Kulozik, 2015).

The pH is another important parameter to be considered for effective protein hydrolysis by peptidases. DpGp showed better hydrolytic action on milk proteins at pH 9.0 (optimal pH) than at pH 6.5 (milk pH), corroborating other studies that showed better hydrolytic action when the assays were performed at optimal pH of enzymes. Lakshmana et al. (2011) tested an acid peptidase from *Monascus pilosus* to reduce the antigenicity of milk whey protein and the pH of the reaction mixtures was maintained at 3.0. In another work, whey proteins were hydrolyzed by pepsin, chymotrypsin and trypsin always using enzyme working pH (Adjou et al., 2013). Therefore, there is no discrepancy if the optimal pH of enzyme is different from milk pH, because after the hydrolysis, the milk pH can be adjusted to neutrality with NaOH or HCl.

Different commercial hypoallergenic milk formulas were used as standards to compare with the milk protein hydrolysate obtained by

DpGp (Fig. 5). Residual peptides from the casein fraction were not detected by the ELISA at all, similar to commercial hydrolyzed milk formulas. However, peptides from whey proteins were detected, although in a much lower proportion when compared to commercial non-hydrolyzed milk and the partially hydrolyzed milk formula Enfamil® (Fig. 5). Rosendal and Barkholt (2000) studied potentially allergenic peptides in 12 commercially available hydrolyzed milk formulas, all of which are listed by the European Society of Pediatric Allergy and Clinical Immunology. They concluded that proteins with molecular masses above 20 kDa were detected in some products, including extensively hydrolyzed formulas. Additionally, they found residual antigenic β -lactoglobulin in all products by ELISA. Therefore, after performing hydrolysis with peptidases, it is very important to use complementary techniques such as ultrafiltration to eliminate any potential remaining allergenic peptides (Quintieri et al., 2017).

In this study, the gut peptidases of the Monarch caterpillar were found to be a suitable source of proteolytic activity for CMP processing. The genomic data of the Monarch is available; therefore, given the availability of genomic information on their peptidases, heterologous expression in desired cellular systems would be feasible.

5. Conclusion

Gut peptidases of the Monarch caterpillar efficiently digested cow milk proteins and would be suitable for producing partially hydrolyzed milk formulas. Further studies could extend their usefulness to produce fully hydrolyzed milk formulas and other related applications.

Conflict of interest

The authors confirm that the contents of this article impose no conflicts of interest.

Contributions

JPBO and CDTF performed the main research work in this study, including gut peptidase purification, gel electrophoresis, proteolytic activity assays and hydrolysis assays. FESL, ICS and JSO produced polyclonal antibodies and performed ELISA. MDPL and ACOMM performed the proteomic analysis. CDTF and MVR contributed to data analysis, discussion and wrote the manuscript.

Acknowledgments

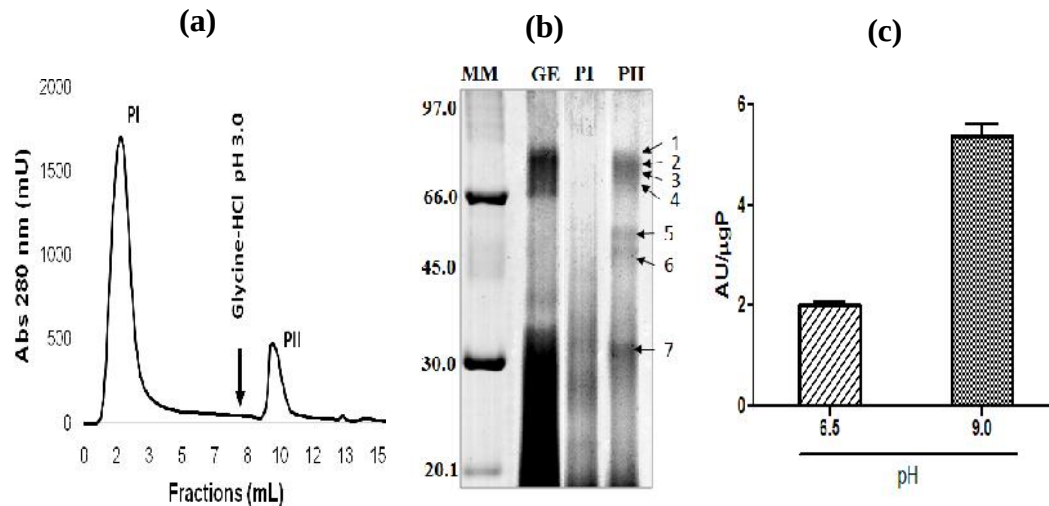
This work was supported by grants from the following Brazilian Agencies: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico (FUNCAP). This study is part of the consortium “Molecular Biotechnology of Plant Latex”.

Appendix A. Supplementary data

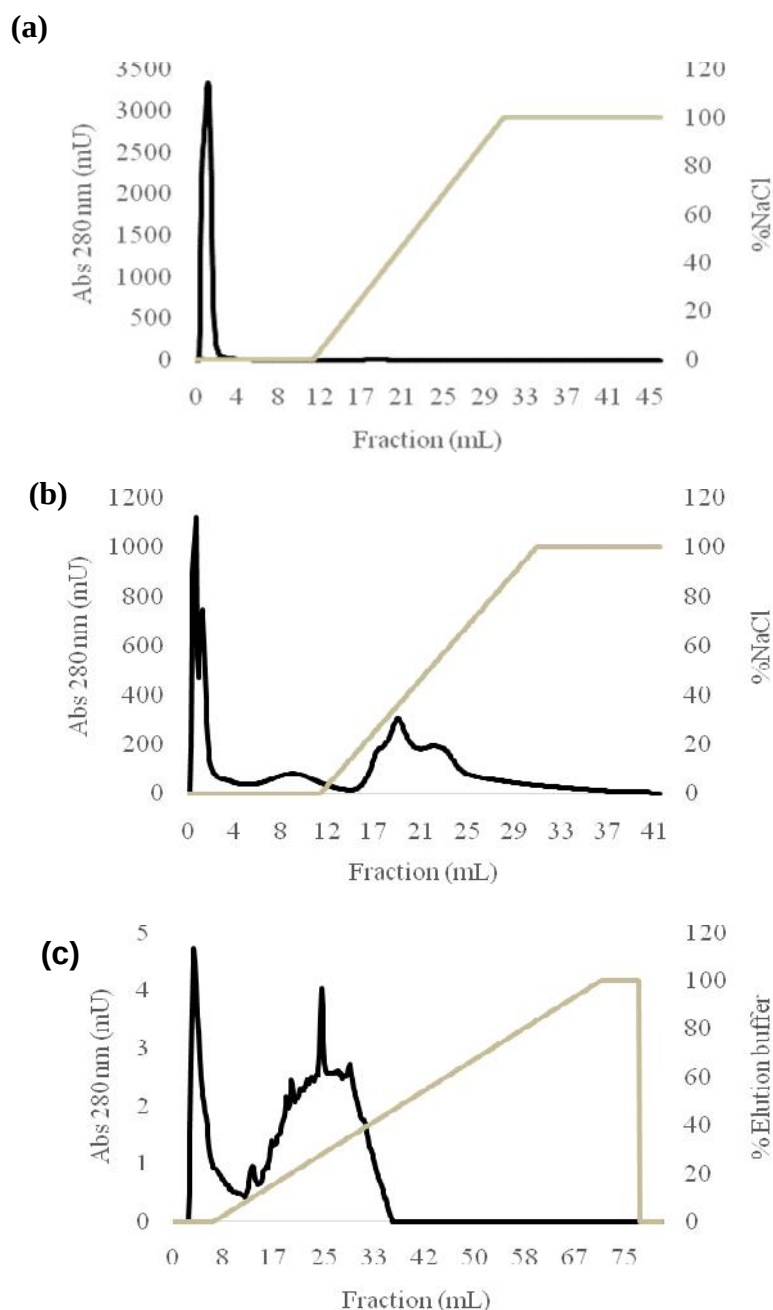
Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2018.02.032>.

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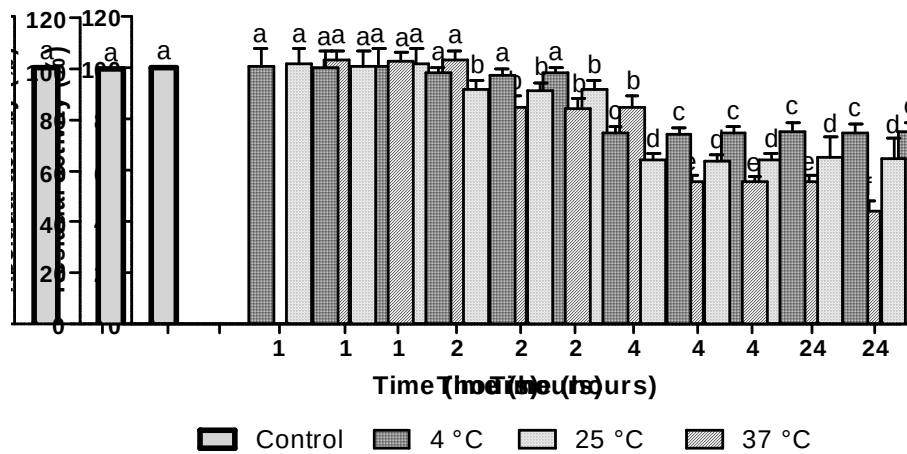


Supplementary Fig. 1. (a) Chromatographic profile on a HiTrap™ Benzamidine Sepharose 4 Fast Flow column of the gut protein extract from *Danaus plexippus*. (b) 1D-SDS-PAGE (15%) of the gut protein extract (GE) and fractions (PI and PII) obtained by affinity chromatography. Numbers represent the bands removed, destained and digested with sequencing-grade modified trypsin for identification by mass spectrometry. (c) Proteolytic activity of the purified peptidases (PII peak) at pH 6.5 or pH 9.0, using 1% azocasein as the substrate at 37 °C. In (a), the arrow indicates where the 50 mM glycine-HCl (pH 3.0) buffer was added to elute the peptidases. In (c), the values represent the mean of three independent experiments and the standard deviation of the mean.

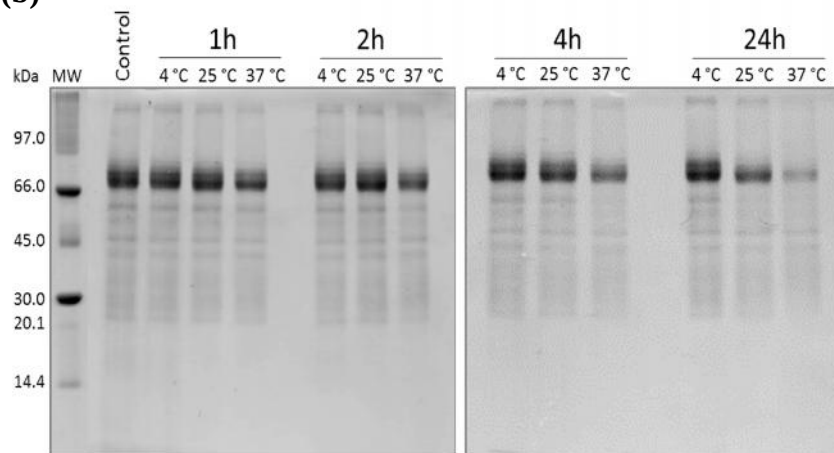


Supplementary Fig. 2. Chromatographic profiles of the PII fraction from HiTrap™ Benzamidine Sepharose 4 Fast Flow column on CM-Sepharose (A), DEAE-Sepharose (B), and C2/C18 columns (C). In A and B, the columns were equilibrated with 25 mM Tris-HCl buffer (pH 7.0) and the proteins were eluted from the column by using a linear gradient from 0 up to 1 M NaCl at 1 mL/min flow rate. In C, the proteins were submitted to reversed-phase high-performance liquid chromatography (RP-HPLC). The proteins were eluted using a linear gradient from 5% to 80% acetonitrile for 30 min, containing 0.1% trifluoroacetic acid (TFA), at 0.5 mL/min flow rate.

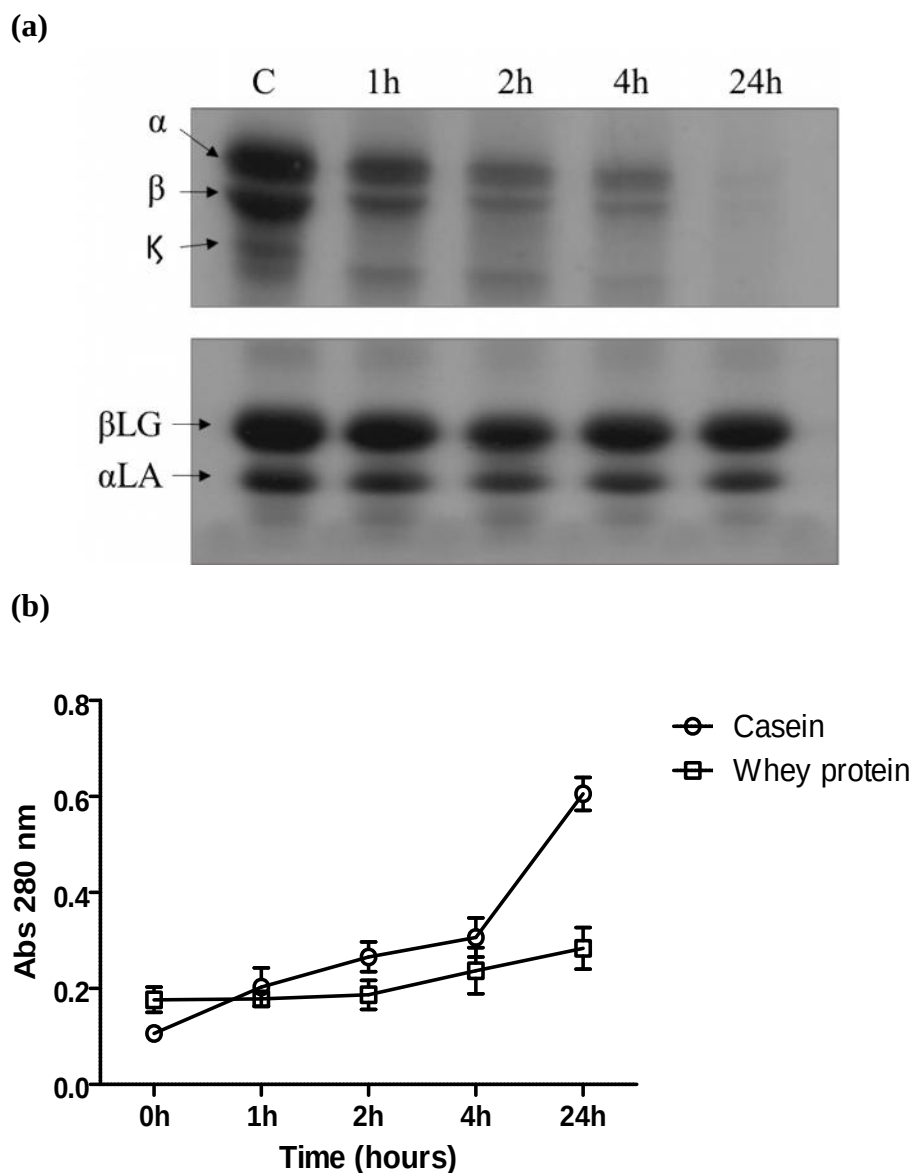
(a)



(b)



Supplementary Fig. 3. Effect of time and temperature on proteolytic activity and autolysis of *Danaus plexippus* gut peptidases (DpGp) analyzed by *in vitro* assays (a) and 15% 1D-SDS-PAGE (b). DpGp, dissolved at pH 9.0, were incubated at different temperatures and aliquots were taken at different time points. The residual proteolytic activity was assessed using 1% azocasein as the substrate at pH 9.0 and 37 °C. Each value represents the mean of three independent experiments and the standard deviation of the mean. Different letters represent significant differences ($p < 0.05$) between the indicate group and the control. The same samples from the *in vitro* assays (a) were analyzed by electrophoresis (b).



Supplementary Fig. 4. Milk protein hydrolysis by DpGp performed at 37°C and pH 6.5. (a) 1D-SDS-PAGE (15%) of the caseins and whey proteins hydrolyzed at different times and an enzyme:substrate ratio of 1:25. Each well received 50 μ g of proteins. C: non-digested proteins. α : α -casein. β : β -casein. κ : κ -casein. β LG: β -lactoglobulin. α LA: α -lactalbumin. (b) The same samples in (a) were also used to quantify the degree of hydrolysis at 280 nm. Each value represents the mean of three independent experiments and error bars indicate the standard deviation of the mean.

Supplementary Table 1. List of proteins identified in gut extract from *Danaus plexippus* by ESI-QUAD-TOF.

¹ The indicated numbers were from 1D SDS-PAGE (Supplementary Fig. 1b). ²The theoretical molecular masses and pI values were those of the proteins represented in the ID (NCBI) column.

¹ Spots	² Theoretical		Protein Score	Sequences of all identified peptides	ID (NCBI)	Protein description [Organism]
	Molecular mass (kDa)	pI				
1	26.549	6.8	140	RLGEHNIDVLEGNEQFINAAKI KLSSPATLNSRV	NP_001156363.1	Trypsin precursor [<i>Sus scrofa</i>]
1	26.748	4.9	83	KLSSPATINSRV	XP_004661211.1	Anionic trypsin-like [<i>Jaculus jaculus</i>]
2	26.549	6.8	140	RLGEHNIDVLEGNEQFINAAKI KLSSPATLNSRV	NP_001156363.1	Trypsin precursor [<i>Sus scrofa</i>]
2	26.748	4.9	83	KLSSPATINSRV	XP_004661211.1	Anionic trypsin-like [<i>Jaculus jaculus</i>]
3	27.749	8.2	99	RTSIPAQGSVIPANLPVVHAGWGTSSGGQLSSVLRD	OWR45697.1	Serine protease [<i>Danaus plexippus plexippus</i>]
4	90.481	8.7	145	KLLTENLDWIIHPLANPDGYEYSINEDRL RNFDFDWGSRPDSNIACSHIYEGPSPFSEPETRI	OWR48083.1	Carboxypeptidase [<i>Danaus plexippus plexippus</i>]
5,6	46.251	4.7	70	KIVLYDPIFTTYGETVVYRS	KPJ10556.1	Carboxypeptidase [<i>Papilio machaon</i>]
5,6	47.436	3.7	65	KYVQYNDLIQPIRI	OWR55096.1	Trypsin serine proteinase [<i>Danaus plexippus plexippus</i>]
7	27.749	8.2	99	RTSIPAQGSVIPANLPVVHAGWGTSSGGQLSSVLRD	OWR45697.1	Serine protease [<i>Danaus plexippus plexippus</i>]
7	31.952	6.5	130	RIITTDVVTHPLWIPQLTFNDIAMVRL KGD TGGLVVMRN	OWR49146.1	Chymotrypsin protein [<i>Danaus plexippus plexippus</i>]