

# Digestive proteolytic activity in *Apodiphus amygdali* Germar (Hemiptera: Pentatomidae): effect of endogenous inhibitors

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## Abstract

The digestive proteolytic profile of *Apodiphus amygdali* was determined by using several substrates and specific inhibitors. Analysis of optimal pH and temperature showed the highest enzymatic activity at the pH range of 6-7 and temperature of 40°C when azocasein was used as a substrate. By using a negative control, the presence of several specific proteases were determined including trypsin-like, chymotrypsin-like, elastase, cathepsin B, cathepsin L, amino- and carboxypeptidases in the midgut content of *A. amygdali*, with the highest and the lowest activities of cathepsin L and carboxypeptidase, respectively. pH dependency of specific proteases revealed optimal pHs of 9, 8 and 9 for trypsin-, chymotrypsin-like, 6 for cathepsins and 5-6 for carboxy- and aminopeptidases, respectively. Specific inhibitors, including phenylmethylsulfonyl fluoride, Na-p-tosyl-L-lysine chloromethyl ketone, N-tosyl-L-phenylalanine chloromethyl ketone, L-trans-epoxysuccinyl-leucylamido-(4-guanidino)-butane, phenanthroline and ethylenediaminetetraacetic acid, significantly decreased proteolytic activity, indicating the presence of different proteases in the midgut of *A. amygdali*. Extracted inhibitors from the midgut demonstrated significant inhibition of specific proteolytic activities of *A. amygdali* except for cathepsin B and aminopeptidase. The results indicated that determination of digestive proteolytic activity could be helpful to clarify digestion

process in insects. Moreover, understanding the nature of digestive proteases might be used to develop several inhibitors for providing resistant crop varieties against pests.

## Introduction

*Apodiphus amygdali* Germar (Hemiptera: Pentatomidae) is a polyphagous and univoltine hemipteran distributed throughout Europe and the Middle East (Schuh & Slater 1995). It feeds on several fruit trees, especially plum, apricot, apple, olive, pear and pistachio as well as non-fruit trees like poplar, pine, plane-tree, elm and willow bark (Muhammed & Al-Iraqi, 2010). Nymphs and adults feed on leaves, fruits and flowers by injecting their salivary secretions into the plant tissues to liquefy nutrient materials. Feeding on stems and fruits causes host weakness and attracts other insects to feed on host plants. Feeding on fruits is the primary damage and causes complete degradation and yield loss (Schuh & Slater, 1995).

Digestive proteases are one of the crucial enzymes in the alimentary canals of insects since proteins are the key nutrients for growth, development and reproduction (Nation, 2008). The enzymes are classified based on their activity against protein molecules. In fact, proteases that attack internal bonds are called endopeptidases (proteinases) but exopeptidases refers to proteases separating amino acids from N- and C-terminal of proteins, known as aminopeptidases (EC 3.4.11.2) and carboxypeptidases (EC 3.4.11.7) (Terra & Ferreira, 2012). Endopeptidases are divided into different subclasses based on their pH dependency and catalytic sites. Serine proteases, including trypsin- (EC 3.4.21.4), chymotrypsin-like (EC 3.4.21.1) and elastase (EC 3.4.21.36), are active in alkaline pH and they have serine, histidine, and aspartic acid residues in their catalytic sites (Terra & Ferreira, 2012). Meanwhile, these enzymes have different structural features that are associated with their different substrate specificities (Terra & Ferreira, 2012). Cysteine proteases are active at acidic pH, including cathepsins B (EC 3.4.22.1) and L (EC 3.4.22.15). Cathepsin L (EC 3.4.22.15) is a true endopeptidase that preferentially cleaves peptide bonds in P<sub>2</sub> against the hydrophobic amino acid residues, but cathepsin B prefers arginine at the same position (Barrett *et al.*, 1998). Digestion of proteins is initiated by endopeptidases; due to the activity of endopeptidases, oligopeptidases are attacked from the N- and C-terminal by amino- and carboxypeptidases, resulting in dipeptides that are hydrolyzed by dipeptidases (Terra & Ferreira, 2012).

Since synthetic chemicals cause severe negative effects on the environment, non-target organisms and induce resistance in pests, numerous studies have been conducted on the use of biocontrol agents and digestive enzyme inhibitors against agricultural pests. Enzymatic inhibitors disrupt digestion process of insects and nutrient absorption, thereby decreasing reproduction and therefore pest populations. Protease inhibitors are small proteins that are present in up to 100 plant

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species and they are effective on serine and cysteine proteases (Nation, 2008). Although this approach may be suitable for decreasing economic losses caused by pests with the least environmental disruption, comprehensive experiments are required to achieve the final goal. Determination of proteolytic profiles and their properties are the first and fundamental step in this approach. The objectives of the current study were to determine digestive proteolytic profiles in *A. amygdali*, the effect of synthetic inhibitors, and to extract endogenous inhibitors.

## Materials and methods

### Insect rearing

Fifth nymphal instars of *A. amygdali* were collected from elm trees in Shiraz (Fars province, Iran) and transferred to the laboratory. The nymphs were reared on elm leaves at  $28 \pm 1^\circ\text{C}$ , 70% relative humidity and a 16L:8D of photoperiod. When adults emerged, they were allowed to feed on leaves for 48 h prior to being randomly selected for biochemical analysis.

### Sample preparation

Based on the method of Cohen (1993), adults of *A. amygdali* were randomly selected and their midgut removed by dissection in iced saline solution (NaCl, 10 mM, 1.06404.1000, Merck-Chemicals, Darmstadt, Germany). Integument and unneeded organs were removed and the midgut was gently separated and rinsed in 1 mL of iced distilled water. To obtain appropriate samples, five midguts were placed in one Eppendorff tube (Eppendorff, Hamburg, Germany) containing 1 mL of distilled water. Tissues were ground using a homogenizer and centrifuged at 13,000 rpm for 20 min at  $4^\circ\text{C}$  (Daika, Rika Kugyo Co., Tokyo, Japan). The supernatant was carefully removed, transferred to new tubes and stored at  $-20^\circ\text{C}$  for no more than one week until use in the experiments.

### General proteolytic assay

Azocasein (2%; A2765; Sigma-Aldrich Co., St. Louis, MO, USA) was used to find the general proteolytic activity in the midgut of *A. amygdali* adults. General proteolytic activity was measured by using azocasein 2% based on the method of Elpidina *et al.* (2001). The reaction mixture consisted of 50  $\mu\text{L}$  of appropriate buffer solutions (Universal buffer, 0.02 M, containing succinate, glycine and 2-morpholinoethanesulfonic acid; pH range 3-12; Frugoni, 1957), 20  $\mu\text{L}$  azocasein and 20  $\mu\text{L}$  of the enzyme solution. After incubation at  $37^\circ\text{C}$  for 60 min, proteolysis was stopped by adding 100  $\mu\text{L}$  of 30% trichloroacetic acid (TCA). Precipitation was achieved by cooling at  $4^\circ\text{C}$  for 10 min; it was then centrifuged at 13,000 rpm for another 10 min. An equal volume of 2 M NaOH was added to the supernatant and the absorbance was recorded at 450 nm (Awareness Technology Inc., Palm City, FL, USA). A blank solution consisted of all the above-mentioned portions except the for enzyme solution.

### Optimal pH and temperature ( $^\circ\text{C}$ ) for general proteolytic activity

The reaction mixture was the same as described above, but different pH ranges of universal buffer (from 3 to 12) and temperature (from 20 to  $70^\circ\text{C}$ ) were used to determine optimal pH and temperature. For the optimal pH assays, 40  $\mu\text{L}$  of buffer solution (at different pHs) was incubated with azocasein as a substrate for 10 min at  $30^\circ\text{C}$  (Terra & Ferreira, 2012). Then, 20  $\mu\text{L}$  of sample was added and the experiment continued as above. For the optimal temperature assays, 40  $\mu\text{L}$  of buffer solution (at the optimal pH found) was incubated with azocasein as a substrate for 10 min at different temperature regimes from 20 to  $70^\circ\text{C}$ .

After adding 20  $\mu\text{L}$  of the sample, the reaction mixture was incubated at each given temperature for 60 min. Other steps were conducted as described above.

## Specific protease assays

### Serine proteases

Trypsin-, chymotrypsin- and elastase-like activities (as three subclasses of serine proteases) were assayed using a 1 mM concentration of BApNA (Nabenzoyl-L-arginine-p-nitroanilide, Sigma-Aldrich, 19362), 1 mM SAAPPpNA (N-succinyl-alanine-alanine-proline-phenylalanine-p-nitroanilide, Sigma-Aldrich, S7388) and 1 mM SAApNA (N-succinyl-alanine-alanine-alanine-p-nitroanilide, Sigma-Aldrich, S4760) as substrates, respectively. The reaction mixture consisted of 35  $\mu\text{L}$  of universal buffer (pH 8, as the recommended pH for serines in the literature), 5  $\mu\text{L}$  of each substrate, and 5  $\mu\text{L}$  of enzyme solution. The reaction mixture was incubated at  $30^\circ\text{C}$  for a period of 0-10 min before adding 30% TCA to terminate the reaction. The absorbance of the resulting mixture was then measured spectrophotometrically at 405 nm by *p*-nitroaniline release. To prove the specific proteolytic activity, a negative control for each substrate was provided separately containing all the above components except for enzyme pre-boiled at  $100^\circ\text{C}$  for 30 min (Oppert *et al.*, 1997).

### Cysteine proteases

Cathepsins B and L activities (as three subclasses of cysteine proteases) were assayed using a 1 mM concentration of Z-Ala-Arg-Arg 4-methoxy- $\beta$ -naphthylamide acetate (Sigma-Aldrich, C8536) and N-Benzoyl-Phe-Val-Arg-p-nitroanilide hydrochloride (Sigma-Aldrich, B2133) as substrates, respectively. The reaction mixture consisted of 35  $\mu\text{L}$  of universal buffer (pH 5 as the recommended pH for cysteines in the literature), 5  $\mu\text{L}$  of each substrate and 5  $\mu\text{L}$  of enzyme solution. The reaction mixture was incubated at  $30^\circ\text{C}$  for a period from 0-10 min before adding 30% TCA to terminate the reaction and read at 405 nm. To prove the specific proteolytic activity, a negative control for each substrate was provided separately containing all the above components except for enzyme pre-boiled at  $100^\circ\text{C}$  for 30 min (Oppert *et al.*, 1997).

### Exopeptidases

The activities of two exopeptidases were obtained by using Hippuryl-L-Arginine (Sigma-Aldrich, H2508) and Hippuryl-L-Phenylalanine (Sigma-Aldrich, H6875) for amino- and carboxypeptidases in the midgut of *A. spinidens*. The reaction mixture consisted of 35  $\mu\text{L}$  of universal buffer (pH 7 as the recommended pH in the literature for exopeptidases), 5  $\mu\text{L}$  of each substrate and 5  $\mu\text{L}$  of enzyme solution. The reaction mixture was incubated at  $30^\circ\text{C}$  for a period from 0-10 min before adding 30% TCA to terminate the reaction and read at 340 nm. To prove the specific proteolytic activity, a negative control for each substrate was provided separately containing all the above components except for enzyme pre-boiled at  $100^\circ\text{C}$  for 30 min (Oppert *et al.*, 1997).

### Optimal pH determination of specific proteases

Universal buffer was used to find the optimal pH of serine and cysteine proteases in addition to exopeptidases by using the specific substrates described. The reaction mixture and experiment procedure were the same as above.

### Effect of specific inhibitors on proteolytic activity

The following compounds were used to find any alteration of the proteolytic activity in the midgut of *A. spinidens*: phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, P7626); trypsin inhibitor, Na-p-tosyl-L-lysine chloromethyl ketone (TLCK) (Sigma-Aldrich, T5012); chymotrypsin inhibitor, N-tosyl-L-phenylalanine chloromethyl ketone

(TPCK) (Sigma-Aldrich, T7254); cysteine protease inhibitor, L-trans-epoxysuccinyl-leucylamido-(4-guanidino)-butane (E-64) (Sigma-aldrich, E3132); cystatin (Sigma-Aldrich, C8917), metalloprotease inhibitors including phenanthroline (Sigma-Aldrich, 131377) and ethylenediaminetetraacetic acid (EDTA) (Merck-Chemicals).

#### Extraction of endogenous inhibitors

Endogenous inhibitors from the midgut of *A. amygdali* were extracted by the method of Lwalaba *et al.* (2010) with slight modifications. Midguts of adults (N=10) were incubated in 500  $\mu$ L of low glucose Ringer's solution (121.5 mM NaCl; 10 mM KCl; 2.1 mM  $\text{NaH}_2\text{PO}_4$ ; 10 mM  $\text{NaHCO}_3$ ; 0.7 mM  $\text{MgCl}_2$ ; 2.2 mM  $\text{CaCl}_2$ ; pH=6.8 and 0.01 g glucose) for 30 min at 30°C. The tissues were then ground using a glass homogenizer. These samples taken from different species were heated to 90°C for 30 min. Samples were centrifuged at 13,000 rpm for 20 min at 4°C. The obtained supernatants were used as the source of inhibitors.

#### Effect of endogenous inhibitors on specific proteolytic activity

The reaction mixture contained 50  $\mu$ L of universal buffer, 20  $\mu$ L of each substrate and 20  $\mu$ L of endogenous inhibitor. After 5 min of incubation, 10  $\mu$ L of the enzyme was added and proteolytic activity was read at 405 nm after 10 min. A blank contained all components except the enzyme.

#### Protein assay

Protein concentration was measured according to the method of Lowry *et al.* (1951). In this method, peptide bonds are oxidized by Folin-Ciocalteu reagent. Briefly, 20  $\mu$ L of sample was added to 100  $\mu$ L of reagent, which was incubated for 30 min prior to reading the absorbance at 545 nm (Recommended by Ziest Chem. Co., Tehran, Iran).

#### Statistical analysis

The experimental design was based on a completely randomized design and Tukey's test was used to compare treatment means. Statistical differences were considered at  $P \leq 0.05$ . Results from the synthetic inhibitors trials were analyzed using POLO-PC software to calculate inhibitory concentration (IC) values.

## Results

### Optimal pH and temperature of general proteolytic activity

Determination of the optimal pH for general proteolytic activity resulted in two peaks in the acid and alkaline ranges. The larger peak was observed at the pH range of 6-7 and the smaller peak was found at the pH range of 9-10 (Figure 1A;  $F=40.99$ ,  $Pr<F: 0.0001$ ). General proteolytic activity increased at temperatures from 25-40°C with the highest value at 40°C, and then sharply decreased at 45-60°C (Figure 1B;  $F=32.81$ ,  $Pr<F: 0.0001$ ).

### Determination of specific proteases present

Activities of trypsin-, chymotrypsin-like, elastase, cathepsin B, L, amino- and carboxypeptidases were confirmed by using boiled midgut homogenate as a negative control (Figure 2). Chymotrypsin showed the highest activity among serine proteases and cathepsin L and aminopeptidase had the highest activity among cysteine proteases and exopeptidases, respectively ( $F=31.78$ ,  $Pr<F: 0.0001$ ). Taken collectively, the highest and the lowest enzymatic activities were observed for cathepsin L and carboxypeptidase, respectively (Figure 2;  $F=31.78$ ,  $Pr<F: 0.0001$ ).

### Optimal pH determination of specific proteases

Figures 3-5 demonstrate the pH dependency of proteases with specific substrates. The highest activity of trypsin-like protease was found at a pH range of 7-9, with the highest activity at pH 9 (Figure 3;  $F=27.53$ ,  $Pr<F: 0.0001$ ). Chymotrypsin-like and elastase proteases had the highest activity at pHs of 8 and 9, respectively (Figure 3B and C;  $F=9.21$ ,  $Pr<F: 0.0001$ ;  $F=10.95$ ,  $Pr<F: 0.0001$ ). The highest activity of cathepsin B was obtained at pH 6 (Figure 4;  $F=17.17$ ,  $Pr<F: 0.0001$ ) but cathepsin L showed the highest activity at a pH range of 4-6 (Figure 4;  $F=17.75$ ,

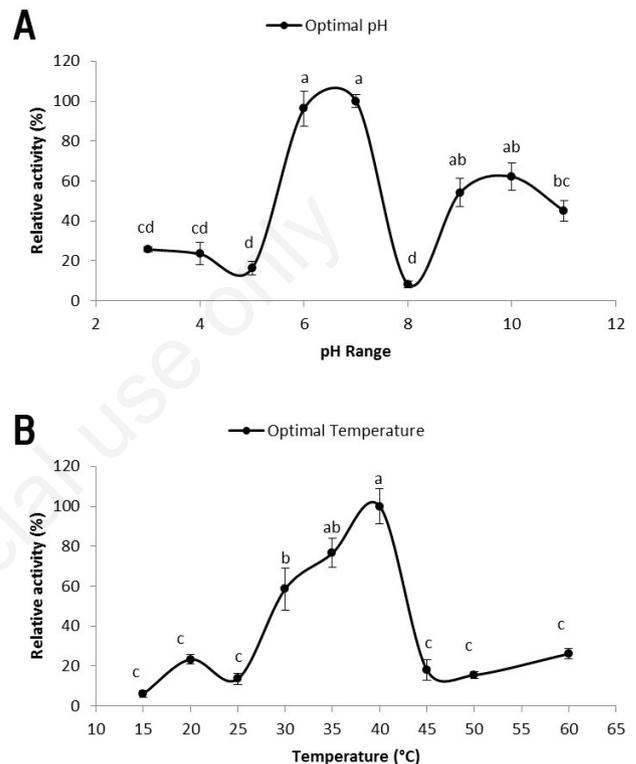


Figure 1. Optimal pH (A) and temperature (B) for general proteolytic activity in the midgut of *A. amygdali*. Letters show statistical differences among values (Tukey's test,  $P \leq 0.05$ ).

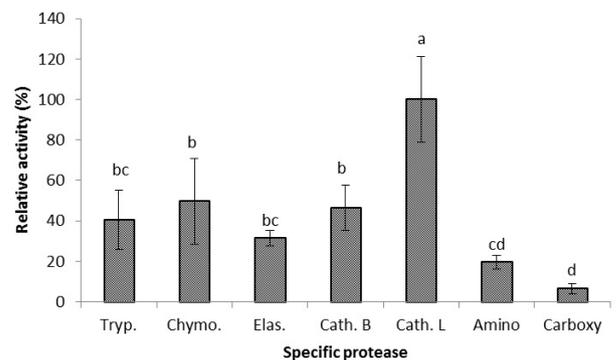


Figure 2. Presence of specific proteases in the midgut of *A. amygdali* by using specific substrates and negative control. Letters show statistical differences among values (Tukey's test,  $P \leq 0.05$ ).

Pr<F: 0.0001). Amino- and carboxypeptidases showed the highest activity at pHs of 6 and 5, respectively, although there was a slight non-statistical difference between these values for aminopeptidase (Figure 5A and B; F=28.08, Pr<F: 0.0001; F=20.34, Pr<F: 0.0001).

### Effect of synthetic and endogenous inhibitors on proteolytic activity

PMSF, TLCK and TPCK, as specific inhibitors of serines, trypsin and chymotrypsin proteases, significantly decreased proteolytic activity in the midgut of *A. amygdali* (Figure 6A; Pr<F: 0.0001, F=1547.84; Pr<F: 0.0001, F=16.83; Pr<F: 0.0001, F=54.56). Among these inhibitors, PMSF had the most significant effect on proteolytic activity, with the result that the enzymatic activity sharply decreased to lower than 20% (Figure 6A; Pr<F: 0.0001, F=1547.84). Proteolytic activity in the midgut of *A. amygdali* was significantly affected by cystatin and E-64, with cystatin decreasing the enzymatic activity to lower than 5% (Figure 6B; Pr<F: 0.0001,

F=74.76; Pr<F: 0.0001, F=54.36; Pr<F: 0.0001, F=68.44). Phenanthroline and EDTA also significantly decreased proteolytic activity to 20% (Figure 6C; Pr<F: 0.0001, F=25.58; Pr<F: 0.0001, F=48.75). Table 1 shows IC<sub>50</sub> values of the above mentioned inhibitors in which PMSF had the lowest IC<sub>50</sub> on proteolytic activity, while TLCK, phenanthroline and EDTA had the highest value. Extracted inhibitor from the midgut of *A. amygdali* significantly decreased specific proteolytic activity except for cathepsin B and aminopeptidase (Table 2; F=35.64, Pr>F: 0.0001).

### Discussion and conclusions

A critical role of proteases could be expected in the midgut of *A. amygdali*, as this is a polyphagous hemipteran that feeds on different trees. In the current study, several proteases were found in the midgut, although cysteine proteases, mainly cathepsin L, showed the highest activity among the proteases assayed. Results of other studies partially correspond with our findings. Stamopoulos *et al.* (1993) and Bell *et al.* (2005) demonstrated trace activities of trypsin and chymotrypsin in the gut of *Podisus maculiventris* Say (Hemiptera: Pentatomidae). Silva & Terra (1994) reported the presence of cysteine proteases in the midgut of *Dysdercus peruvianus* Guérin-Ménéville (Hemiptera: Pyrrhocoridae). Pascual-Ruiz *et al.* (2009) reported serine, cysteine, aminopeptidase and carboxypeptidase as the main proteases involved in protein digestion of *P. maculiventris*, although their activities can differ due to variability of prey. Zhu *et al.* (2003) and Wright *et al.* (2006) found high activities of cysteine and serine proteases in the midgut of *Lygus lineolaris* and *L. hesperus* Knight (Hemiptera: Miridae). Bigham & Hosseiniaveh (2010) found the presence of cathepsins B and L in the

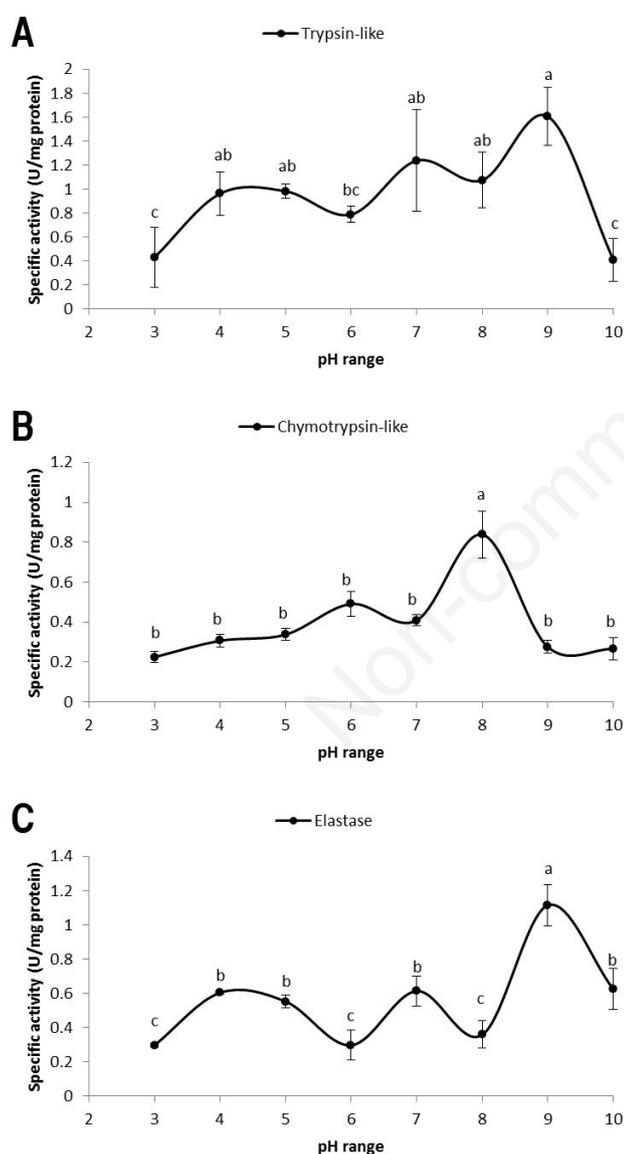


Figure 3. Optimal pH for serine proteases (A-C) in the midgut of *A. amygdali*. Letters show statistical differences among values (Tukey's test, P<0.05).

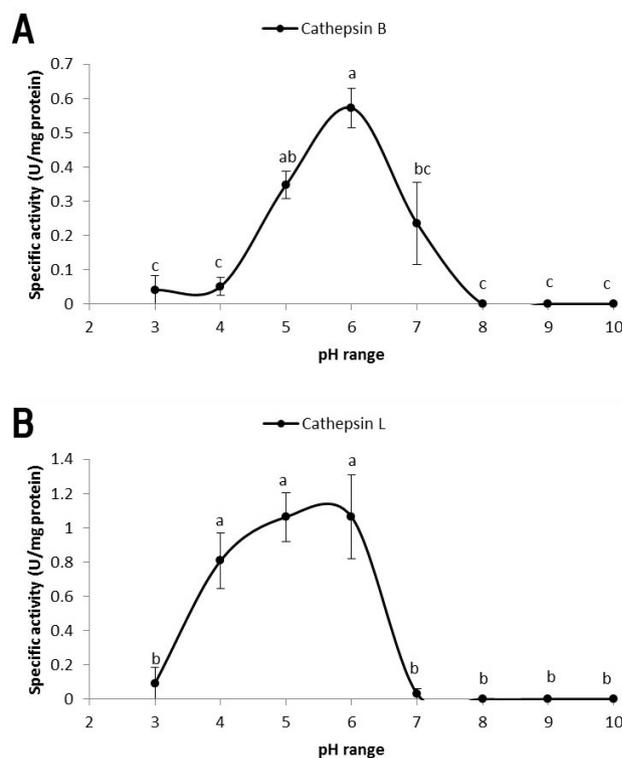


Figure 4. Optimal pH for cysteine proteases (A and B) in the midgut of *A. amygdali*. Letters show statistical differences among values (Tukey's test, P<0.05).

midgut of *Brachynema germari* Kolenati (Hemiptera: Pentatomidae). Fialho *et al.* (2012) obtained cathepsin L and aminopeptidase as the major proteases from the midgut of *Podisus nigrispinus* Dallas (Hemiptera: Pentatomidae). Sorkhabi-Abdolmaleki *et al.* (2013) reported both serine and cysteine proteases through the significant role of cysteines in the midgut content of *Andrallus spinidens* Fabricius (Hemiptera: Pentatomidae).

The optimal pH for general proteolytic activity in the midgut of *A. amygdali* was represented as two peaks at both acidic and alkaline pHs, indicating the presence of all the determined proteases (see above). Zhu *et al.* (2003) found optimal proteolytic activity of *Lygus lineolaris* Knight (Hemiptera: Miridae) at pH 4.25 and 8.5. Wright *et al.* (2006) found acidic and neutral pHs for caseinolytic activity in *L. lineolaris* that might be attributed to cysteine and serine proteases. Bigham & Hosseinaveh (2010) observed the presence of general protease activity by acidic and alkaline optima in the midgut extract of *B. germari* using hemoglobin as a substrate. Sorkhabi-Abdolmaleki *et al.* (2013) found an alkaline pH for general proteolytic activity, serine proteases and two exopeptidases with another peak at pH 6, as with the cysteine proteases. The optimal pH of specific proteases corresponds with the expected values, as noted in the literature (Terra & Ferreira, 2012). A pH peak was observed for all specific proteases except for cathepsin L, for which the enzyme was active at a pH range of 4-6. This could be attributed to the presence of several isozymes and higher activity of cathepsin L. Variations in optimal pH of general proteolytic activity in insects could be attributed to feeding habits as either monophagous or polyphagous. Since polyphagous insects feed on various hosts, the presence of different proteases and the difference in general protease pH could be attributed to host materials or secondary metabolites of plants.

An optimal temperature of 40°C was found for general proteolytic

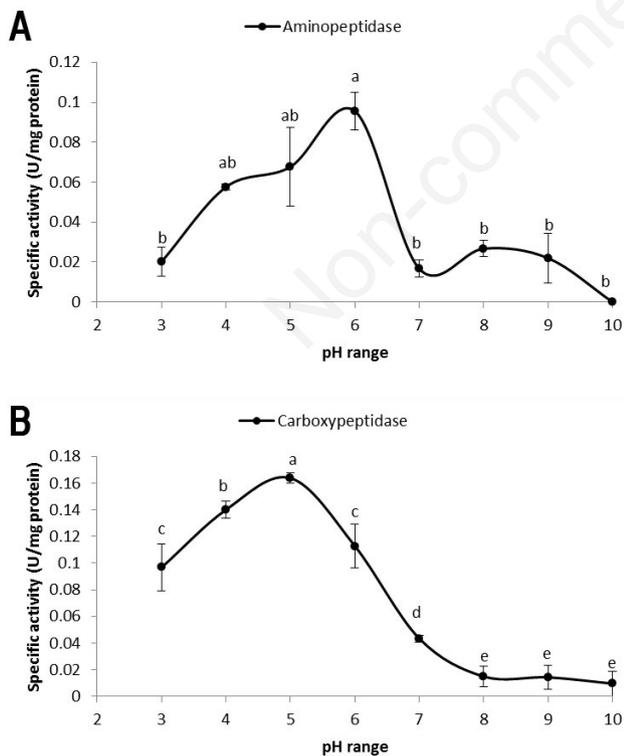


Figure 5. Optimal pH for exopeptidases (A and B) in the midgut of *A. amygdali*. Letters show statistical differences among values (Tukey's test,  $P \leq 0.05$ ).

activity, although there was slightly lower but not statistically different activity at 35°C. Generally, optimal temperature of an enzyme *in vitro* assays reflects the temperature of the environment where the organism feeds on its hosts. Increasing temperature can disrupt hydrogen bonds in the three-dimensional structure of the molecule, leading to denaturation of the protein (Zeng *et al.*, 2000). At the same time, biological reactions occur faster with increasing temperature, up to the point of enzyme denaturation, where the enzymatic activity and the rate of the reaction decrease sharply (Zeng *et al.*, 2000). Swart *et al.* (2006) found 37°C as the optimal temperature for proteolytic activity in the salivary secretion of two belostomatid bugs. Sorkhabi-Abdolmaleki

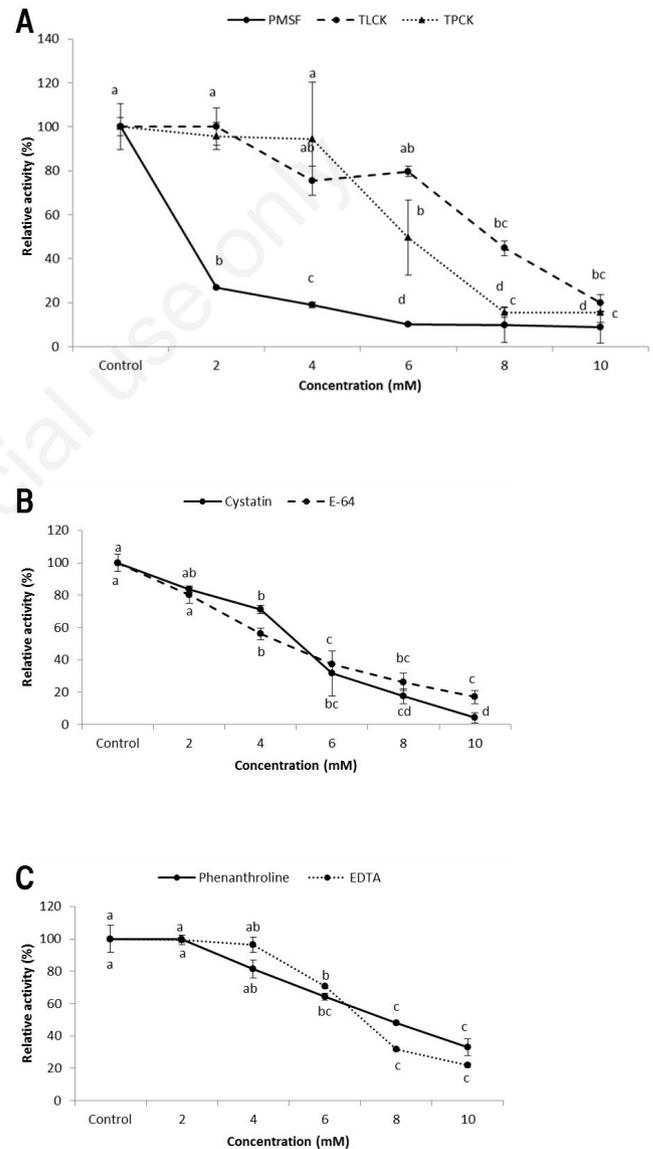


Figure 6. Effect of specific inhibitors on general proteolytic activity in the midgut of *A. amygdali*. A) Specific inhibitors of serine proteases; B) Specific inhibitors of cysteine proteases; C) Specific inhibitors of metalloproteinase. Letters show statistical differences among values (Tukey's test,  $P \leq 0.05$ ). PMSF, phenylmethylsulfonyl fluoride; TLCK, Na-p-tosyl-L-lysine chloromethyl ketone; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone; E-64, L-trans-epoxysuccinyl-leucylamido-(4-guanidino)-butane; EDTA, ethylenediaminetetraacetic acid.

& Zibae (2013) reported an optimal temperature of 25°C for proteolytic activity in the midgut of *A. spinidens* that corresponded with laboratory conditions of mass-rearing.

Our results demonstrated inhibitory effects of PMSF, TLCK and TPCK on the proteolytic activity in the midgut of *A. amygdali*. PMSF showed the highest inhibition of enzymatic activity with the lowest IC<sub>50</sub> value (0.574 mM), in comparison with TLCK (7.13) and TPCK (5.19). These results corresponded with previous results on the presence of serine proteinases, especially chymotryptic activities, in the midgut of *A. amygdali*, which have been supported by using a negative control and specific substrates. Zhu *et al.* (2003) found serine proteases as the major enzymes in the gut of *L. lineolaris* by TLCK (Zhu *et al.*, 2003). Bigham & Hosseiniaveh (2010) and Sorkhabi *et al.* (2013) found significant inhibition of digestive proteases by PMSF, TLCK and TPCK in *B. germanica* and *A. spinidens*. Presence of cysteine proteases was confirmed by using specific substrates, cystatin, E-64 and DTT, although the experiment with a negative control showed cathepsin L as the major protease in the midgut of *A. amygdali*. Houseman and Dowe (1983) found cathepsin B and L activities in the posterior midgut of *Rodnius prolixus* L. (Hemiptera: Reduviidae). Overney *et al.* (1997) reported cysteines as the major proteases in the midgut of *Perillus bioculatus* Fabricius (Hemiptera: Pentatomidae), showing inhibition of 90% by E-64. Proteolytic activity in the midgut of *L. lineolaris* was significantly decreased with E-64 (Wright *et al.*, 2006). Bigham & Hosseiniaveh (2010) found negative effects of E-64 on the proteolytic activity of *A. spinidens*. Sorkhabi-Abdolmaleki *et al.* (2013) observed that E-64 and cystatin decreased proteolytic activity in the midgut of *A. spinidens*.

EDTA as general chelating agent and phenanthroline as metalloproteinase inhibitor significantly decreased proteolytic activity in the midgut of *A. amygdali*, indicating the presence of metal ions in the active sites of aminopeptidase. Aminopeptidases are divided into aminopeptidase A and N based on the presence of Zn<sup>2+</sup> and Mn<sup>2+</sup> in their active sites (Terra & Ferreira, 2012). There is little knowledge on the characterization of aminopeptidases' active site. Ferreira & Terra (1986) employed multiple inhibition analysis to find the possible role of EDTA on inactivation of aminopeptidase in *Rhynchosciara americana* Wideman (Diptera: Sciaridae) larvae. The authors found two sub-

sites in the active center of the enzyme: a hydrophobic subsite to which isoamyl alcohol binds, exposing the metal ion, and a polar subsite, to which hydroxylamine binds (Ferreira & Terra, 1986). Exposure of the metal ion after isoamyl alcohol binding may be analogous to the situation when a part of the substrate occupies the hydrophobic subsite to cause conformational changes associated with the catalytic step (Ferreira & Terra, 1986). Cristofolletti & Terra (2000) found that the catalysis property of aminopeptidase in *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) depends on a metal ion, a carboxylate and a protonated imidazole group. Detailed experiments revealed that the enzyme is a zinc metalloproteinase like mammalian aminopeptidase N, but it differs in some details (Cristofolletti & Terra, 2000).

Extraction of endogenous inhibitors from the midgut of *A. amygdali* revealed diverse effects on specific proteolytic activities. In serine proteases, inhibition was more significant in the case of trypsin and elastase than chymotrypsin. In the case of cysteine and exopeptidases, cathepsin B and aminopeptidase were not affected by the endogenous inhibitor. Endogenous inhibitors may have several functions, such as protection of  $\alpha$ -amylase from proteolytic activity (Stein & Fischer, 1958), attacking fungi and bacteria in gut (Taranushenko *et al.*, 2009) and protection of epithelial cells from digestive enzymes when food is not present in the midgut (Lwalaba *et al.*, 2010). Engelmann & Geraert (1980) reported extraction of the midgut lumen content in *Leucophaea maderae* Fabricius (Blattodea: Oxyhaloidae) as an endogenous inhibitor. Lwalaba *et al.* (2010) extracted an endogenous inhibitor from the midgut of *Spodoptera frugiperda* Hubner (Lepidoptera: Noctuidae) larvae. It was found that the ability of the endogenous inhibitor to decrease trypsin activity was similar to the results from ingestion of the exogenous inhibitor SBTI. Zibae *et al.* (2012) and Sorkhabi-Abdolmaleki *et al.* (2013) extracted endogenous inhibitors from *Chilo suppressalis* Walker (Lepidoptera: Crambidae), *Naranga aenescens* Moore (Lepidoptera: Noctuidae), *Pieris brassicae* L. (Lepidoptera: Pieridae), *Hyphantria cunea* Drury (Lepidoptera: Arctiidae) and *Ephesia kuhniella* Zeller (Lepidoptera: Pyralidae). The authors found different effects of inhibitors on salivary and midgut proteases of *A. spinidens* (Zibae *et al.*, 2012; Sorkhabi-Abdolmaleki & Zibae, 2013). Specifically, extracted inhibitors from *C. suppressalis*, *N. aenescens* and *E. kuhniella* signifi-

**Table 1. Determination of inhibitory concentrations (mM) of specific inhibitors on proteolytic activity in the midgut of *A. amygdali*.**

Compound	IC <sub>10</sub>	IC <sub>30</sub>	IC <sub>50</sub>	Slope±SF	$\chi^2$
PMSF	0.045 <sup>e</sup>	0.574 <sup>f</sup>	0.574 <sup>e</sup>	1.16±0.27	0.568
TLCK	3.57 <sup>b</sup>	5.38 <sup>a</sup>	7.13 <sup>ab</sup>	4.27±0.40	25.27
TPCK	3.29 <sup>b</sup>	4.65 <sup>b</sup>	5.91 <sup>b</sup>	5.04±0.39	37.46
Cystatin	1.99 <sup>c</sup>	3.18 <sup>c</sup>	4.41 <sup>c</sup>	3.71±0.29	17.44
E-64	1.39 <sup>cd</sup>	2.75 <sup>cd</sup>	4.41 <sup>c</sup>	2.55±0.25	0.293
DTT	0.59 <sup>d</sup>	1.56 <sup>e</sup>	3.07 <sup>d</sup>	1.79±0.24	39.21
Phenanthroline	3.44 <sup>b</sup>	5.43 <sup>a</sup>	7.46 <sup>a</sup>	3.81±0.38	2.79
EDTA	4.33 <sup>a</sup>	5.80 <sup>a</sup>	7.10 <sup>ab</sup>	5.95±0.52	21.49

IC, inhibitory concentrations; PMSF, phenylmethylsulfonyl fluoride; TLCK, N-p-tosyl-L-lysine chloromethyl ketone; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone; E-64, L-trans-epoxysuccinyl-leucylamido-(4-guanidino)-butane; DDT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid. <sup>a,b,c,d,e,f</sup>Letters show statistical differences among values (Tukey's test, P≤0.05).

**Table 2. Effect of endogenous inhibitor on specific proteolytic activity in the midgut of *A. amygdali*.**

	Trypsin	Chymotrypsin	Elastase	Cathepsin B	Cathepsin L	Aminopeptidase	Carboxypeptidase
Control	1.59±0.02*	0.82±0.01*	1.10±0.11*	0.55±0.05	1.04±0.024*	0.094±0.009	0.16±0.003*
Treatment	0.15±0.025	0.23±0.06	0.046±0.003	0.65±0.05	0.015±0.012	0.17±0.001*	0.024±0.001

\*Asterisks show statistical differences among values (Student's t-test, P≤0.05).

cantly decreased trypsin and chymotrypsin activities. All extracted inhibitors significantly decreased elastase and cathepsin activities of *A. spinidens* except for *C. suppressalis* (Sorkhabi-Abdolmaleki & Zibae, 2013). Extracted inhibitors from all species significantly decreased cathepsins B and L activities, but this decrease was not significantly different in the case of *C. suppressalis*. In the case of exopeptidases, endogenous inhibitors from *E. kuehniella* and *H. cunea* on aminopeptidase and all inhibitors on carboxypeptidase significantly decreased enzymatic activities (Sorkhabi-Abdolmaleki & Zibae, 2013).

In conclusion, the presence of different specific proteases involved in protein digestion was found in *A. amygdali* using specific substrates and inhibitors. These enzymes had different pH profiles and extracted endogenous inhibitors from the insect's midgut significantly decreased the majority of the proteases. These results were expected, as *A. amygdali* feeds on several host plants. Sometimes, *A. amygdali* is used as a rearing host for *Trissolcus* spp. parasitoids (a biocontrol agent of wheat sunn pest), so these findings could be helpful for efficient mass-rearing. *A. amygdali* occasionally occurs as a secondary pest of agricultural products, so the possible use of protease inhibitors could be a promising method to avoid the use of pesticide sprays.

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