ISJ 8: 132-142, 2011 ISSN 1824-307X

RESEARCH REPORT

Proteolytic activity in the midgut of *Ectomyelois ceratoniae* Zeller (Lepidoptera: Pyralidae), Pomegranate carob moth

M Ranjbar, JJ Sendi, A Zibaee

Department of Plant Protection, College of Agriculture, University of Guilan, 41635-1314, Rasht,Iran

Accepted July 11, 2011

Abstract

In this study, the proteolytic activity in the midgut of *Ectomyelois ceratoniae* as the major pest of pomegranate was investigated to find nature of specific proteases and their properties for adopting possible pest management procedure. It was found that fourth and fifth instar larvae had the highest proteolytic activity as well as specific proteinases including, elastase, trypsin-like, chymotrypsin-like and two exopeptidases. The optimal pH of general protease was 10 and 9 for azocasein, casein and hemoglobin as substrate. The optimal temperature of the total proteolytic activity in the midgut of *E. ceratoniae* was found 30 and 35 °C by using azocasein and casein as substrates, respectively. In case of hemoglobin, the enzyme showed the highest enzymatic activity at temperatures from 15 to 35 °C. There was no enhancement in the proteolytic activity by using different cations but SDS, citric acid and mercaptoetahnol significantly decreased the proteolytic activity in the midgut of *E. ceratoniae*. Using specific proteolytic inhibitors including PMSF, TLCK, TPCK, E-64, DTT and phenanthroline revealed presence of serine proteases as the major proteases in the midgut of *E. ceratoniae*.

Key Words: *Ectomyelois ceratoniae*; midgut; protease; characterization

Introduction

Insects obtain food to provide their necessary energy for biological processes. To obtain nutrients from ingested food, the macromolecules must be digested in the alimentary canal to smaller ones for absorption *via* midgut epithelium cells. To do so, gut is the important part of the insect to break down ingested carbohydrates, fats and proteins to their monomers. Digestion process in the midgut is catalyzed by different enzymes known by their substrates namely carbohydrases, lipases and proteases (Terra and Ferreira, 2005). In fact, enzyme pattern in the gut of each insect represent its ability to utilization of substrate according to kind of diet. Among mentioned digestive enzymes, proteases are the hydrolyzing peptidases that bind to protein a molecule from different positions for breaking-down them to more simple unites. These enzymes are divided into two groups; exopeptidases, that are separated an amino acid sequence at the end of peptide molecules and endopeptidases (proteinases) that break peptide bonds in the middle of molecule. Based on location characteristics and acidity endopeptidases are divided into the following types as: serine, cysteine, aspartic and metalloproteinases. Endopeptidases are responsible for primary digestion of proteins converting them to oligopeptides. The oligopeptides resulting from proteinase action are digested from the N-terminal end by aminopeptidases and from the C-terminal end by carboxypeptidases, both enzymes liberating one amino acid residue at each catalytic step (Terra and Ferreira, 2005).

Ectomyelois ceratoniae Zeller (Lepidoptera: Pyralidae) (Larval-instars) is a major destructive pest of pomegranate and pistachio in Iran so that its annual damage is between 15 to 90 % (Behdad, 2002). The adults are gray butterflies that emerge on May and lay their eggs on June into the crown, on the rod, anther or internal surface of sepal of pomegranate. The eggs hatch after 10 days and the larvae penetrate into the fruit and fed on their internal parts (Behdad, 2002).

Due to variation in the characteristics of insect digestive enzymes, it is necessary the better understanding of enzyme biochemistry to get knowledge on plant-herbivores associations (Wilhite *et al*., 2000). Selection of the target molecules like digestive enzymes is one of the most important aspects in the alternative pest control by providing plant protease or synthetic inhibitors. So, characterization of the digestive enzymes especially

Corresponding author:
Arash Zibaee
Department of Plant Protection
College of Agriculture
University of Guilan, 41635-1314, Rasht, Iran
E-mail: arash.zibaee@gmx.com

proteases is mandatory to reach this avenue. Based on this conception, the objectives of this study were: (i) determination of general and specific proteolytic activity by using general and specific substrates, (ii) effect of pH and temperature, (ii) effects of various cations, (iii) effect of general and specific inhibitors on proteolytic activity and demonstrating enzymatic inhibition by electrophoresis.

Materials and Methods

Ectomyelois ceratoniae rearing

Intended to provide the population of insect, first the appropriate number of developmental stages is collected from the damage gardens. To obtain same age larvae, the collected individuals were grown on pomegranate fruit exactly the same as damaged garden under 16L:8D, 30 °C and 60 % relative humidity.

E. ceratoniae dissection and sample preparation

Midguts of 150 larvae (10 larvae for each larval instar and 100 ones for enzyme characterization) were removed by dissection under a dissecting microscope in ice-cold distilled water. Samples were rinsed in ice-cold distilled water and grounded with a handling hemogenizer. Homogenates were transferred to 1.5 ml centrifuge tubes and centrifuged in 13000 rpm for 15 min at 4 °C. The supernatants for each tissue were pooled then stored at -20 °C for subsequent analyses.

Azocasein

General proteolytic activity by using azocasein 2 % was measured based on a method described by Elpidina *et al*. (2001). The reaction mixture consisted 100 µl of universal buffer solutions, 50 µl azocasein and 20 µl enzyme. After incubation at 37 °C for 60 min, proteolysis was stopped by addition of 150 µl of 10 % trichloroacetic acid (TCA). Precipitation was achieved by cooling at 4 °C for 120 min and it was centrifuged at 13,000 rpm for 10 min. An equal volume of 2 M NaOH was added to the supernatant then the absorbance was recorded at 440 nm. Blank solution consisted all mentioned portions except for enzyme solution.

Hemoglobin

Cohen‘s method (Cohen, 1993) was used to assay general proteolytic activity in midgut by using hemoglobin as substrate. Hemoglobin solution (50 µl) was added to 100 µl of appropriate buffer solution and incubation at 30 °C was initiated after addition of 20 µl of enzyme solution for 120 min. For termination of proteolysis, 150 µl of 10 % TCA was added to the reaction mixture. Precipitation was achieved by cooling at 4 °C for 45 min then the reaction mixture was centrifuged at 13000 rpm for 10 min. Blanks solution contained all mentioned portions except for enzyme. The peptides liberated from hemoglobin were estimated using Folin-phenol reagent at 650 nm (Folin and Ciocalteu, 1927).

Casein

To measure general proteolytic activity by using casein, 50 µl of substrate solution was added to 100 µl of appropriate buffer. Current solution was mixed by swirling and incubated at 37 °C for exactly 10 minutes. Then, 150 µl of 10 % TCA was added and the reaction solution was mixed by swirling and incubated at 37 °C for about 30 minutes. Precipitation was achieved by cooling at 4 °C for 45 min then the reaction mixture was centrifuged at 13,000 rpm for 10 min. Blanks solution contained all mentioned portions except for enzyme. The peptides liberated from casein were estimated using Folin-phenol reagent at 650 nm (Folin and Ciocalteu, 1927).

Determination of optimal temperature (°C) on general proteolytic activity and stability

The temperature range from 15 - 80 °C were used to find optimal temperature for general proteolytic activity in the midgut of *E. ceratoniae* by using three common substrates. The reaction mixtures were similar to described earlier but buffer solution was universal buffer at pH 9.

Specific proteolytic activity

Serine proteolytic activity

Trypsin-, chymotrypsin- and elastase-like activities (as three subclasses of serine proteases) were assayed using a concentration of 1mM BA*p*NA (Nabenzoyl- L-arginine-*p*-nitroanilide), 1 mM SAAPP*p*NA (N-succinyl-alanine-alanine-proline-phenylalanine-p-nitroanilide) and 1 mM SAAA*p*NA (N-succinyl-alaninealanine- alanine-*p*-nitroanilide) as substrates, respectively. The reaction mixture consisted 80 µl of universal buffer (pH 9), 10 µl of each mentioned substrate and 5 µl of enzyme solution. The reaction mixture was incubated at 37 °C for a period from 5 - 60 min before adding 30 % acetic acid to terminate the reaction. The absorbance of the resulting mixture was then measured spectrophotometrically at 410 nm by *p*-nitroaniline release.

Cysteine protease

For cysteine proteinase assay, benzyloxycarbonyl-Arg-Arg- *p*-nitroaniline was used as substrate. Hydrolysis of the 1 mM final concentration of the substrates was determined by measuring the absorbance by *p*-nitroaniline after 30 min of incubation. The absorbance of different concentrations of *p*-nitroaniline was read at 410 nm to find extinction coefficient for specific activity calculation. To prove the specific proteolytic activity, a negative control were provided for each substrate separately containing all mentioned components except for enzyme pre-boiled at 100 °C for 30 min.

Exopetidase activity

Leucine *p*-nitroaniline (L*p*NA) (1 mM) was used to find aminopeptidase activity in the midgut of *E. ceratoniae*. The reaction mixture consisted 80 µl of universal buffer (pH 8), 10 µl of each mentioned substrate and 5 µl of enzyme solution. The reaction mixture was incubated at 37 °C for a period from 5 - 60 min before adding 30 % acetic acid to terminate the reaction. The absorbance of the resulting mixture was then measured spectrophotometrically at 340 nm by *p*-nitroaniline release.

N-(3-(2-furyl) acryloyl)-L-phenylalanyl-L-phenylalanine was used to find aminopeptidase activity in the midgut of *E. ceratoniae*. The reaction mixture consisted 80 µl of universal buffer (pH 8), 10 µl of each mentioned substrate and 5 µl of enzyme solution. The reaction mixture was incubated at 37 °C for a period from 5-60 min before adding 30 % acetic acid to terminate the reaction. The absorbance was read at 340 nm.

Optimal pH determination of specific proteases

Universal buffer (2 mM, pH range 3 - 14) was used to obtain the optimal pH of each specific protease and find possible pH dependency of each substrate. The reaction mixtures were similar to above but the pH of used buffer was varied from 3 - 14. Also, negative controls as described above were considered in the experiment.

Effect of mono- and di-valent cations on general proteolytic activity

Different concnetrations (1, 3 and 5 mM) of cations including K^+, Na^+, Ca^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+} and Fe^{2+} were used to obtain potential alteration in general proteolytic activity in the midgut of *E. ceratoniae*. Initially, 50 µl of each cation (different concentration) was added to universal buffer solution (2 mM, pH 9) and gently stirred for 10 min then, 50 µl of casein (1 %) added and additionally stirred for 10 min. Incubation was initiated after adding of 20 µl enzyme for 60 min. Proteolysis was stopped by addition of 150 µl of 10 % trichloroacetic acid (TCA). Precipitation was achieved by cooling at 4 °C for 120 min and it was centrifuged at 13000 rpm for 10 min. An equal volume of 2 M NaOH was added to the supernatant and the absorbance was recorded at 440 nm. Blank solution consisted all mentioned portions except for enzyme solution.

Effect of general and specific inhibitors

General inhibitors

Selected general inhibitors in this experiments consisted sodium dodecylsulphate, urea, ethylendiamidetetraacetic acid and *β*-mercaptoethanol in concentrations of 1, 5 and 10 mM. Initially, 50 µl of each compound (different concentration) was added to universal buffer solution (2 mM, pH 9) and gently stirred for 10 min then, 50 µl of casein (1 %) added and additionally stirred for 10 min. Incubation was initiated after adding of 20 µl enzyme for 60 min. Proteolysis was stopped by addition of 150 µl of 10 % trichloroacetic acid (TCA). Precipitation was achieved by cooling at 4 °C for 120 min and it was centrifuged at 13000 rpm for 10 min. An equal volume of 2 M NaOH was added to the supernatant and the absorbance was recorded at 440 nm. Blank solution consisted all mentioned portions except for enzyme solution.

Specific inhibitors

Following compounds were used to find any alteration in the proteolytic activity of the midgut of *E. ceratoniae* regarding to specific used substrates; PMSF (phenylmethylsulfonyl fluoride, 1, 3, 5 mM); trypsin inhibitor, TLCK (Na-p-tosyl-L-lysine chloromethyl ketone, 1, 3, 5 mM); chymotrypsin inhibitor, TPCK (N-tosyl-L-phenylalanine chloromethyl ketone, 1, 3, 5 mM); cysteine protease inhibitor E-64 (L-trans-epoxysuccinyl-leucylamido-(4-guanidino)-butane, 1, 3, 5 mM), cystatin (1, 3, 5 mM) and metalloprotease inhibitors, phenanthroline, also, DTT (dithiothreitol, 1, 3, 5 mM) were used as cysteine activator. For E-64, cystatin and DTT no effects were observed (Data not shown).

Electrophoresis zymogram

Electrophoretic detection (Laemmli, 1970) of proteolytic enzyme was performed using resolving and stacking polyacrylamide gels of 10 % and 4 %, respectively, according to the method described by Garcia-Carreno *et al*. (1993) with slight modifications. Non-reducing PAGE was carried out at 4 °C in a constant voltage of 110 mV so that gelatin (0.5 %) were added in resolving gel. When dye reached at the bottom of glace, gel carefully separated and put in universal buffer for 15 min. Then, gels were washed in water and immediately fixed and stained with 0.1 % Coomassie brilliant blue R-250 in methanol-acetic acid-water (50:10:40) overnight. Destaining was done in methanol-acetic acid-water (50:10:40) for at least 2 h. Characterization of protease classes in SDS-PAGE zymograms using specific inhibitors was done according to Garcia-Carreno *et al*. (1993) with some modifications. A total of 50 µl of the enzyme extract was mixed with 30 µl of inhibitors at 5mM concentration including PMSF, TLCK and TPCK. Electrophoresis and zymogram were carried out as described before.

Protein determination

Protein concentration was measured according to the method of Bradford (1976), using bovine serum albumin (Bio-Rad, USA) as standard.

Statistical analysis

All data obtained from a complete randomized design were compared by one-way analysis of variance (ANOVA) followed by Tukey's studentisized test when significant differences were found at $P \leq 0.05$ (SAS, 1997). Differences between samplings (n = 3) were considered statistically significant at a probability less than 5 % and marked in figures and tables.

Results

Table 1 shows general and specific proteolytic activity in the various instar larvae of *E. ceratoniae*. Although the enzyme activity was higher in 5th instars than 4th one but no significant differences was observed between them (Table 1). Similar results were obtained when specific proteolytic activity was assessed. On the other hands, 4th and 5th instar larvae of *E. ceratoniae* had the highest proteolytic activity (Table 1).

Three general substrates were used to determine the optimal pH of general proteolytic activity in the midgut of *E. ceratoniae*. it was found pH 9 and 10 (Azocasein) as the optimal pH (Fig. 1). The enzymatic activity was from pH 3 - 7 then increased and reached to its maximum at pH 9 and 10 (Fig. 1). All these mentioned substrates were used to find the optimal temperature for total

Table 1 Total and specific proteolytic activity (µmol/min/mg protein) in the different larval instars of *E. ceratoniae*

Larval instars	General protease	Elastase	Trypsin	Chymotrypsin	Aminopeptidase	Carboxypeptidase
1st instar	23±2.41d	0.96±0.033d	1.28±0.17d	0.67±0.07e	4.11±0.27d	0.27±0.022d
2nd instars	104±11.28c	2.00±0.27c	4.56±0.31c	1.52±0.11d	9.56±0.81c	1.04±0.078cd
3rd instars	163±29.45b	2.71±0.47b	10.14±0.47b	2.11±0.41c	12.21±0.67b	3.14±0.67c
4th	231±81.22a	4.59±0.77ab	15.23±1.11ab	3.02±0.72b	15.77±0.75a	7.22±0.96b
5th	250±67.24a	5.02±0.88a	17.44±2.14a	4.31±0.87a	16.00±0.80a	9.71±0.88a

[1] Different letters show statistical differences among larval instars (Tukey's test, $p \leq 0.05$).

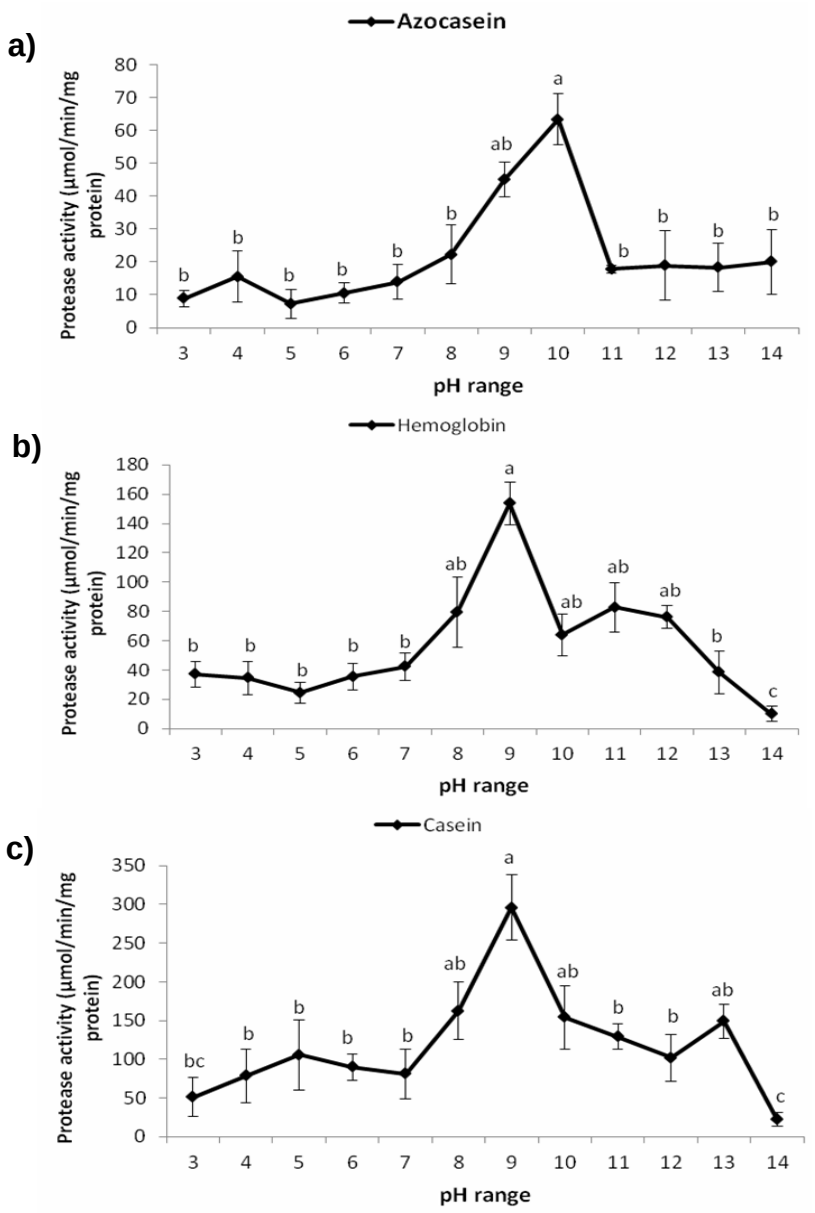

Fig. 1 Optimal pH determination for general proteolytic activity in the 5th instar larvae of *E. ceratoniae*. Azocasein (2 %), hemoglobin (20 mg/ml) and casein (1 %) were used as substrates. Statistical analysis was calculated by Tukey's test (SAS software) and showed by different letters ($p \leq 0.05$; n = 3).

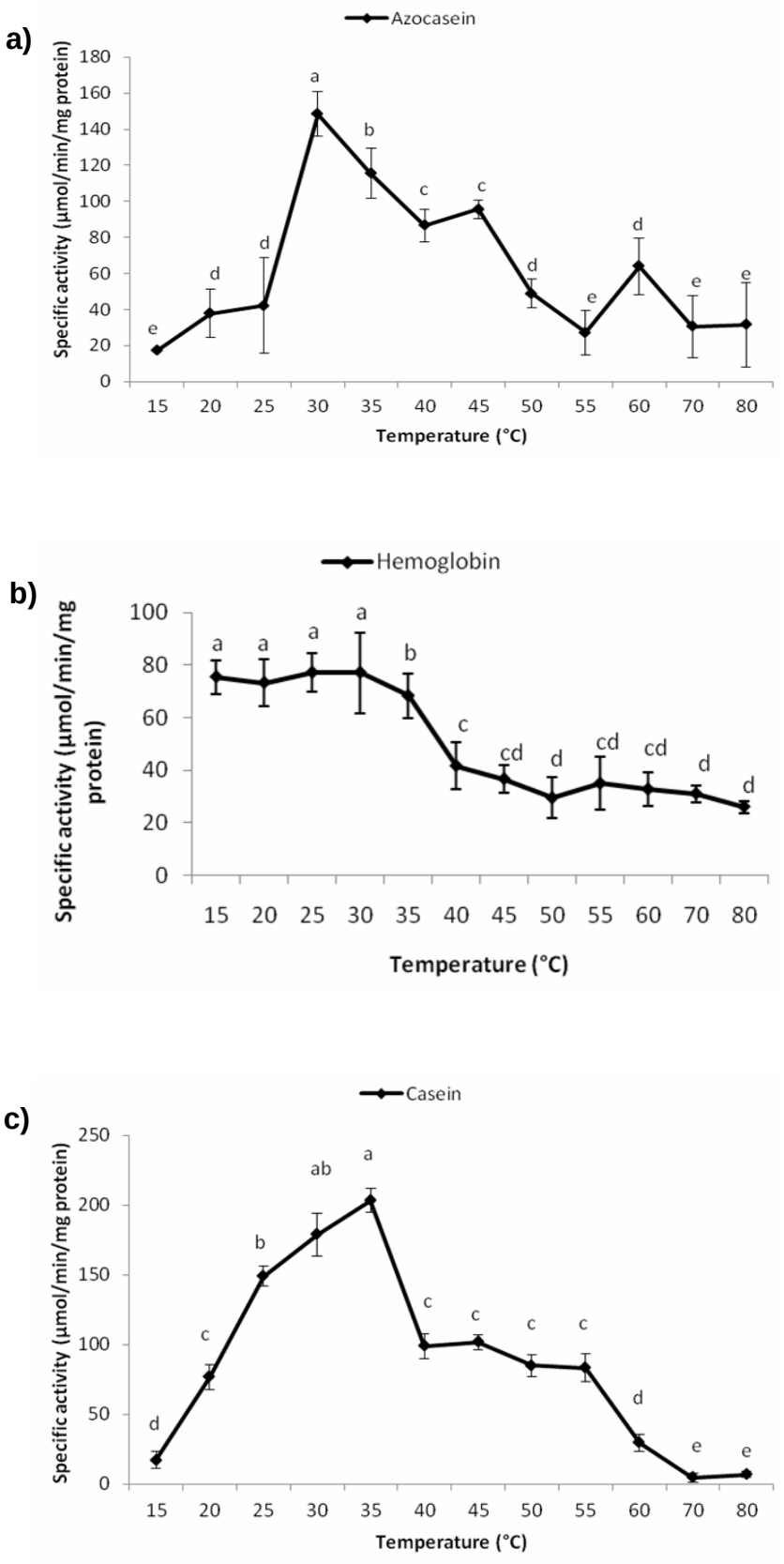

Fig. 2 Optimal temperature (°C) determination for general proteolytic activity in the 5th instar larvae of *E. ceratoniae*. Azocasein (2 %), hemoglobin (20 mg/ml) and casein (1 %) were used as substrates. Statistical analysis was calculated by Tukey's test (SAS software) and showed by different letters ($p \leq 0.05$; n = 3).

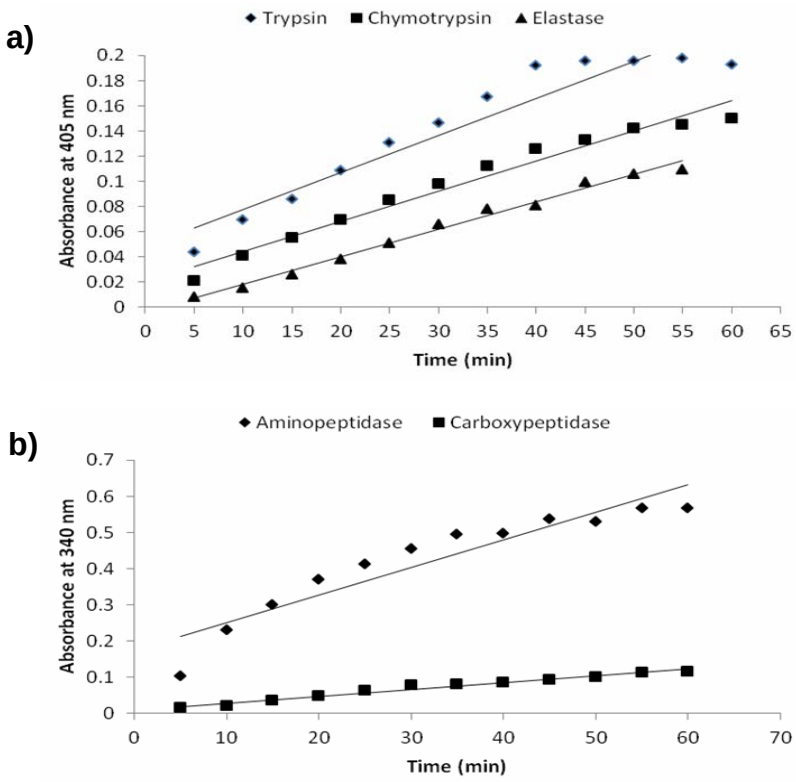

Fig. 3 Time course determination of the specific proteolytic activity in the midgut of *E. ceratoniae*. First, 80 µl appropriate buffer (in all range) incubated with 20 µl substrate at 35 °C as standard temperature in enzymatic assessment. After 10 min, 7 µl of enzyme added and allowed the reaction to continue for 60 min. At each time interval, absorbance was read at 405 nm for serine proteases and 340 nm for exopeptidases.

proteolytic activity. In the case of azocasein, the enzyme activity sharply increased at pH 9 and 10 then decreased (Fig. 1). When hemoglobin and casein were used as substrates, the enzyme activity increased at pH 8 and 9 with the highest activity at pH 9 then decreased so that the lowest enzymatic activity was obtained at pH 14 (Fig. 1).

The optimal temperature of the general proteolytic activity in the midgut of *E. ceratoniae* was found 30 and 35 °C by using azocasein and casein as substrates, respectively (Fig. 2). In the case of hemoglobin, the enzyme showed the highest activity at temperatures 15 to 35 °C then decreased (Fig. 2).

Maximal specific proteinases activity of the midgut extract was obtained in case of trypsin-like protease with the initial velocity of 40 at 405 nm/min (Fig. 3). The initial velocities of activity in midgut extract on elastase and chymotrypsin-like were found 30 and 40 at 405 nm/min, respectively (Fig. 3). In case of amino- and carboxypeptidases, the initial velocities were observed 30 at 340 nm/min for both enzymes (Fig. 3).

By using specific substrates and universal buffer, it was found alkali condition to optimal activity of specific proteases in the midgut of *E. ceratoniae* (Fig. 4). The optimal pH for elastase, trypsin-like and chymotrypsin-like activity were obtained 9, 10 and 8, respectively while it was found 10 for amino and carboxypeptidases (Fig. 4).

Tables 2 and 3 show the effects of some cations and general compounds on the general proteolytic activity in the midgut of *E. ceratoniae*. In case of cations, not only no enhancement of proteolytic was observed but also some cations decreased the enzymatic activity (Table 2). In case of general compounds, urea and EDTA had no significant effects on the proteolytic activity but SDS, citric acid and mercaptoethanol significantly decreased the enzymatic activity so that the most inhibition was observed in case of mercaptoethanol (Table 3).

Different specific inhibitors were used to show the nature of proteases in the midgut of *E. ceratoniae* (Table 4). E-64 (Cysteine inhibitor), DTT (Cysteine activator) and phenanthroline (Metalloproteinase inhibitor) had no effect on the proteolytic activity so being cysteine and matalloproteinases was denied in the midgut of *E. ceratoniae* (Table 4). Instead, PMSF (Serine protease inhibitor), TLCK (Trypsin-like protease inhibitor) and TPCK (Chymotrypsin-like protease inhibitor) significantly decreased the proteolytic activity so it was confirmed that serine proteases were the major proteases in the midgut of *E. ceratoniae* (Table 4).

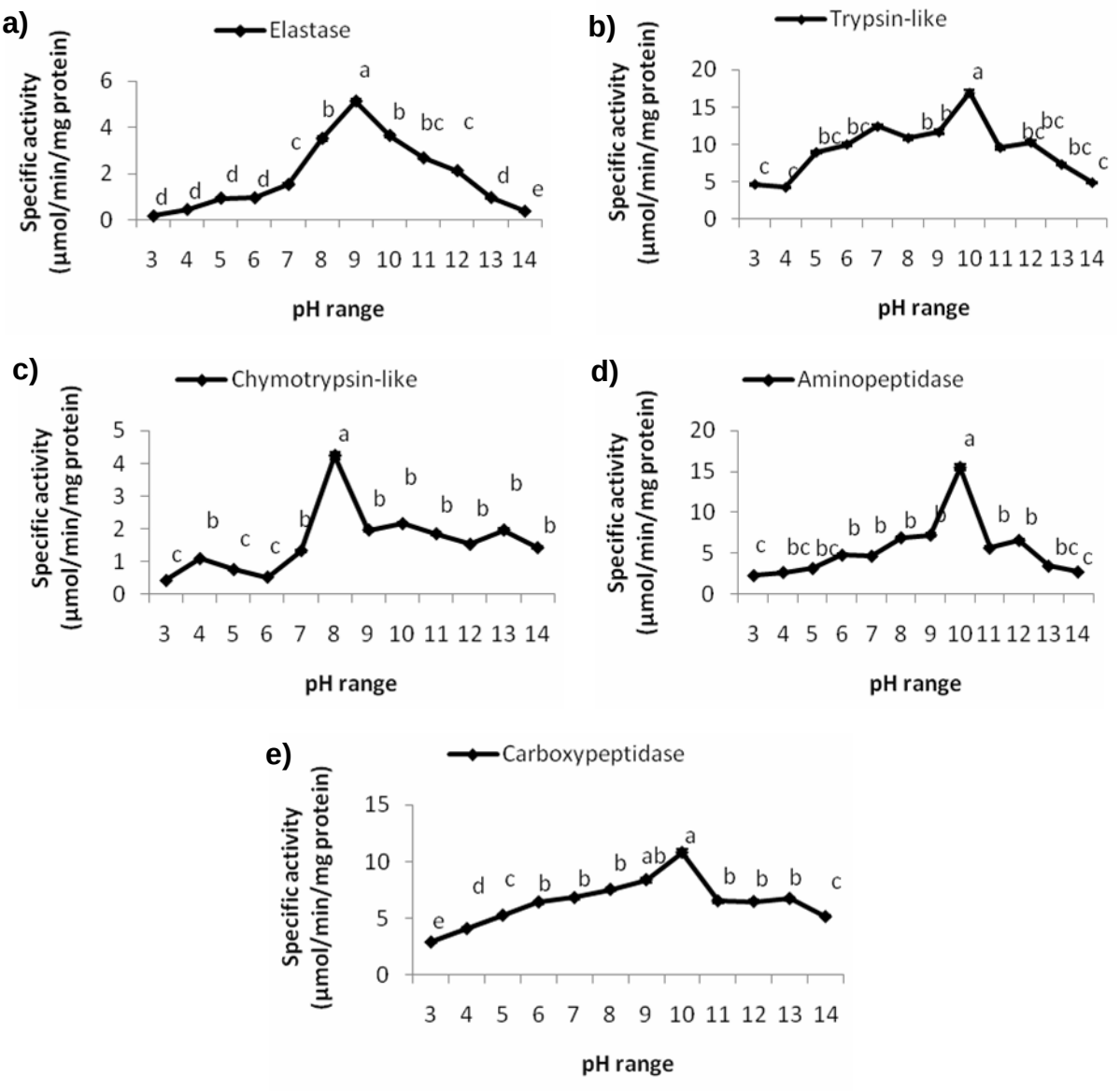

Fig. 4 Optimal pH determination of the specific proteolytic activity in the midgut of *E. ceratoniae* by using specific substrates. One way analysis (Anova, Tukey's test) was used to determine statistical differences by various letters ($p \leq 0.05$; n = 3).

Figure 5 demonstrates zymogram analysis of proteinase inhibitors effects on gelatin (0.5 %) hydrolytic activity in the midgut of *E. ceratoniae*, by using 8 % non-reducing SDS-PAGE. In control, there were three protease bands namely P1, P2 and P3 that P1 band was sharper than other ones (Fig. 5). When PMSF was used as serine protease inhibitor, P1 change to a narrow band and P2 almost disappear and P3 completely removed. By using TLCk as trypsin-like inhibitor, P3 completely disappeared and P1 and P2 were less sharp in comparison with control (Fig. 5). TPCk as chymotrypsin-like protease inhibitor decreased the sharpness P1 but made no significant changes in P2 and P3 (Fig. 5).

Discussion

This study depicted different proteases in the midgut of a serious pomegranate pest, *E. ceratoniae*. Detailed experiments to clarify the nature of specific proteases revealed being serine proteases including trypsin, chymotrypsin and elastase types as well as two types of exopeptidases namely amino and carboxypeptidases. The higher activity of serine proteases in the midgut of *E. ceratoniae* showed a typical fact for lepidopteran larvae (Johnston, 1993, 1995; Broadway, 1995; Gatehouse *et al*., 1999; Hegedus *et al*., 2003; Terra and Ferriera, 2005; Chougule *et al*., 2008).

Table 2 Effect of mono- and di-valent cations on the proteolytic activity in the midgut of *E. ceratoniae*

Cations	Concentration (mM)	Relative activity (%)
Control	-	100
K^+	1	4.5*
	3	6.5*
	5	3.7*
Na^+	1	93.4
	3	41.6*
	5	13.3*
Ca^{2+}	1	103.7
	3	108.8
	5	100.7
Mn^{2+}	1	17.1*
	3	3.3*
	5	0*
Mg^{2+}	1	49.5*
	3	9.7*
	5	0*
Zn^{2+}	1	105.8
	3	98.8
	5	104.8
Fe^{2+}	1	53.1*
	3	43.1*
	5	21.4*

1. Casein 1 % was used as substrate.
2. Asterisks show statistical differences among used concentrations of cations (Tukey's test, $p \leq 0.05$).

Table 3 Effect of some general inhibitors on the proteolytic activity in the midgut of *E. ceratoniae*

Concentration (mM)	Urea	SDS	EDTA	Citric acid	Mercaptoethanol
Control	100a	100a	100a	100a	100a
1	92a	28b	85a	99a	25b
5	82a	14c	92a	26b	38b
10	83a	14c	101a	19b	10c

1. All experiments were made at pH 9 and temperature of 35 °C.
2. Different letters show statistical differences among used concentrations (Tukey' test, $p \leq 0.05$).

pH and temperature are the two main factors for characterization of enzymes in the biochemistry. Both of these parameters provide suitable conditions to better affinity between enzyme and substrate complex as well as higher rate of breaking down of the complex. Also, these parameters prepare a better situation of substrate accessibility for enzyme site of action (Zibaee *et al*., 2011). In this study we found the optimal pH of proteolytic activity at 9 and 10 for both general and specific substrates except for elastase that it was obtained the highest activity at pH 8. The results of optimal pH in the current study confirm Terra and Ferreira (1994) on attribution the high pH of the lepidopteran gut to an adaptation of leaf-eating lepidopteran ancestors for extracting hemicellulose from plant cell walls. The alkaline pH is typical for the highest activity of proteases in the midgut of lepidopteran larvae. Different earlier reports confirmed this finding including: *Spodoptera littoralis* Fabricius (Lepidoptera: Noctuidae) pH 11 (Ishaaya *et al*., 1971); *S. litura* Fabricius (Lepidoptera: Noctuidae),

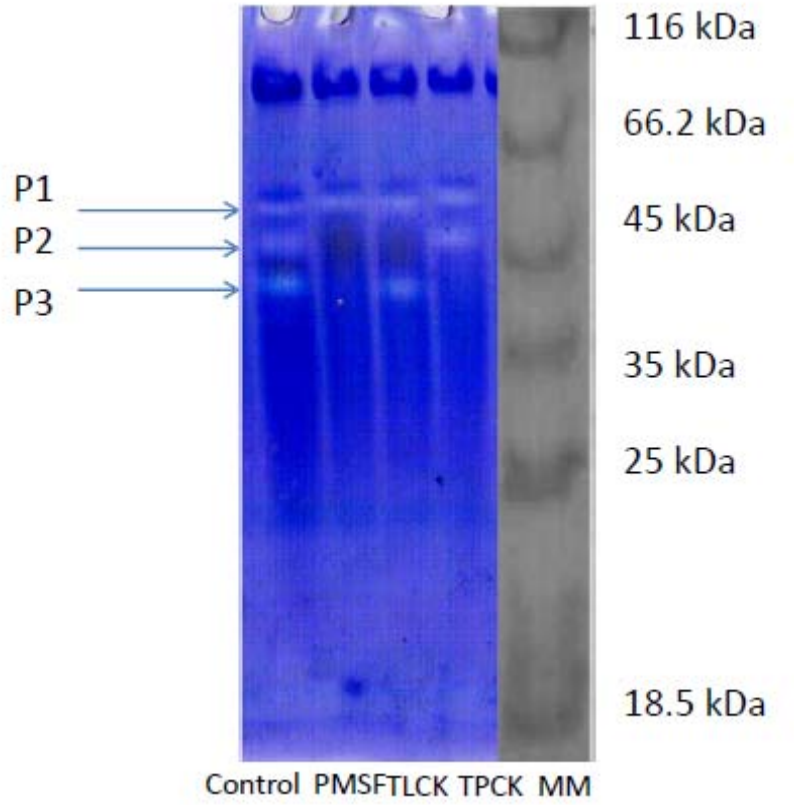

Fig. 5 Zymogram analysis of the effect of proteinase inhibitors on gelatin hydrolytic activity in the midgut of *E. ceratoniae*, by using 8 % non-reducing SDS-PAGE. A: control (no inhibitor), B: PMSF 5mM, C: TLCK, 5mM, D: TPCK 5mM and E: Molecular weight marker.

pH 9, 10.5, and 11.0 (Ahmad *et al*., 1976, 1980); *Heloithis zea* Stral (Lepidoptera: Noctuidae), pH 11 (Klocke and Chan, 1982); *G. mellonella* Walker (Lepidoptera: Pyralidae), pH 10.5 and 11.2 (Hamed and Attias, 1987); *Helicoverpa armigera* Fabricius (Lepidoptera: Noctuidae), pH 9.5 and 10 (Johnston *et al*., 1991); *Phtorimaea opercula* Zeller (Lepidoptera: Gelechidae), pH > 9.0 (Christeller *et al*., 1992); *Manduca sexta*, pH 8.5 (Samuels *et al*., 1993); *Helothis virescens*, pH 10 - 11 (Johnston *et al*., 1995); *Lacanobia oleracea* L (Lepidoptera: Noctuidae) pH < 11 (Gatehouse *et al*., 1999) and *Mamestra brassicae* L. (Lepidoptera: Noctuidae), pH 11 (Chougule *et al*., 2008).

Temperatures of 30 and 35 °C were found as the optimal temperatures for activity of proteases in the midgut of *E. ceratoniae* by using three general substrates. To obtain the optimal temperature of proteases, it is mandatory to use different substrates because of the specificity of each substrate for temperature. For example, azocasein and hemoglobin have been kept at 4 °C but casein at 25 or 30 °C. So, using all these substrates could be attributed to real enzymatic activity not denaturation of the available substrates. Biological reactions occur faster with increasing temperature up to the point of enzyme denaturation, above which temperature, enzyme activity and the rate of the reaction decreases sharply (Zibaee *et al*., 2011). Extremes in temperatures can also disrupt the hydrogen bonds that hold the enzyme in its three-dimensional structure, denaturing the enzyme (Zibaee *et al*., 2011).

In this study, different concentrations of ions, general and specific inhibitors were used to verify the type of specific proteases and the nature of their activity. It was found no enhancement of the proteolytic activity by using cations and surprisingly the enzyme activity decreased. These findings have been proved by molecular analysis in the midgut proteolytic activity of Lepidoptera. On the other hands, although insect serine proteases having structural features resembling vertebrate trypsins, they differ from these because they are not activated or stabilized by ions especially calcium (Terra and Ferriera, 1994). Also, our results by using EDTA as the general chelating agents of ions in the active sites of enzymes showed no inhibition on proteolytc activity in the midgut of *E. ceratoniae*. Another experiment that proved this finding was to use phenenthroline as the metalloproteinase inhibitor that showed no significant difference on the

proteolytic activity. The important point in using inhibitors was citric acid inhibitory effects. Citric acid is a specific binding molecule to amino acids in the active site of the enzyme (especially histidine) and it makes inaccessible for enzymatic activity.

As the important point, it was shown that PMSF, as the serine-protease inhibitor, and TLCK, as the trypsin-like protease inhibitor as well as TPCk as the chymotrypsin-like protease inhibitor, significantly decreased the proteolytic activity in the midgut of *E. ceratoniae*. Because TLCK is known to act as a specific inhibitor of trypsin-like proteases by binding to the histidine residue within the active site of the enzyme (Anwar and Saleemudin, 2002), the inhibition of the *E. ceratoniae* protease by TLCK indicates that a histidine residue might be at the enzyme active site. These results correspond with the finding of Anwar and Saleemudin (2002), *Spilosoma oblique*, Josephrajkumar *et al*. (2006) in *Conogethes punctiferalis* and Chouglue *et al*. (2008) in *Mamestra brassicae*.

Digestive enzymes of insects play dual functions in digestion and defense (Zibaee and Bandani, 2010) so that in response to inhibitor challenge, many insects undergo a series of adaptive changes to regain their normal feeding and growth, including over-expression of inhibitor-insensitive proteases. Also, they could be a reliable target to develop pest management programs by considering host plant resistance having digestive enzyme inhibitor genes. Using biotechnological approaches to target these enzymes, such as transgenic plants, may be a winning strategy for the development of effective bio-pesticides. This could be achieved through traditional breeding programs to select plants possessing higher degrees of resistance factor that are not easily degraded.

Acknowledgement

This study was supported by a university of Guilan grant. The authors highly appreciate H Tabatabaee and M Allahyari for their assistance.

References

Ahmad Z, Saleemuddin M, Siddiqui M. Alkaline protease in the larvae of the army worm *Spodoptera litura*. Insect Biochem. 6: 501-505, 1976.

Ahmad Z, Saleemuddin M, Siddiqui M. Purification and characterisation of three alkaline proteases from the larva of army worm *Spodoptera litura*. Insect Biochem. 10: 667-673, 1980.

Anwar A, Saleemudin M. Purification and characterization of a digestive alkaline protease from the larvae of *Spilosoma oblique*. Arch. Insect Biochem. Physiol. 51: 1-12, 2002.

Behdad E. Major plant pests in Iran. Yadbood publishing, 2002.

Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt. Biochem. 72: 248-254, 1976.

Broadway RM. Are insects resistant to plant proteinase inhibitors? J. Insect Physiol. 41: 107-116, 1995.

Chougule NP, Doyle E, Fitches E, Gatehouse JA. Biochemical characterization of midgut digestive proteases from *Mamestra brassicae* (cabbage moth; Lepidoptera: Noctuidae) and effect of soybean Kunitz inhibitor (SKTI) in feeding assays. J. Insect Physiol. 54: 563-572, 2008.

Christeller JT, Liang WA, Markwick NP, Burgess EPJ. Midgut protease activities in 12 phytophagous lepidopteran larvae: dietary and proteases inhibitory interactions. Insect Biochem. Mol. Biol. 22: 248-254, 1992.

Cohen AC. Organization of digestion and preliminary characterization of salivary trypsin like enzymes in a predaceous Heteropteran, *Zelus renadii*. J. Insect Physiol. 39: 823-829, 1993.

Doijode SD. Seed storage of horticultural crops. New York: Food Products Press, 2001.

Elpidina EN, Vinokurov KS, Gromenko VA, Rudenskaya YA, Dunaevsky YE, Zhuzhikov DP. Compartmentalization of proteinases and amylases in Nauphoeta cinerea midgut. Arch. Insect Biochem. Physiol. 48: 206-216, 2001.

Folin O, Ciocalteu V. On tyrosine and tryptophane determinations in proteins. J. Biol. Chem. 73: 627-650, 1927.

Garcia-Carreno FL, Dimes LE, Haard NF. Substrate-gel electrophoresis for composition and molecular weight of proteinases or proteinaceous protease inhibitors. Analyt. Biochem. 214: 61-69, 1993.

Gatehouse AMR, Norton E, Davison GM, Babbe SM, Newell CA, Gatehouse JA. Digestive proteolytic activity in larvae of tomato moth, *Lacanobia oleracea*; effects of plant proteinase inhibitors in vitro and in vivo. J. Insect Physiol. 45: 545-558, 1999.

Hamed MMB, Attias J. Isolation and partial characterization of two alkaline proteases of greater wax moth *Galleria mellonella* L. Insect Biochem. 17: 653–658, 1987.

Hegedus DD, Baldwin M, O'Grady L, Braun S, Gleddie A, Sharpe D, *et al*. Lydiate M. Midgut proteases from *Mamestra configurata* (Lepidoptera: Noctuidae) larvae: characterization, cDNA cloning and expressed sequence tag analysis. Arch. Insect Biochem. Physiol. 53: 30-47, 2003.

Ishaaya I, Moore I, Joseph D. Protease and amylase activity in the larvae of Egyptian cotton worm *Spodoptera littoralis*. J. Insect Physiol. 17: 945-953, 1971.

Johnston KA, Lee MJ, Gatehouse JA, Anstee JH. The partial purification and characterization of serine protease activity in the midgut of larval *Helicoverpa armigera*. Insect Biochem. 21: 389-397, 1991.

Johnston KAJA, Gatehouse JH, Anstee, JH. Effects of soybean protease inhibitors on the growth and development of larval *Helicoverpa armigera*. J. Insect Physiol. 39: 657-664, 1993.

Johnston KAM, Le C, Brough VA, Hilder AMR, Gatehouse JA. Protease activities in the larval midgut of *Heliothis virescens*: evidence of trypsin and chymotrypsin-like enzymes. Insect. Biochem. Mol. Biol. 25: 375-383, 1995.

Josephrajkumar A, Chakrabarty R, Thomas G. Midgut proteases of the cardamom shoot and

capsule borer *Conogethes punctiferalis* (Lepidoptera: Pyralidae) and their interaction with aprotinin. Bull. Entomol. Res. 96: 91-98, 2006.

Klocke JA, Chan BG. Effects of cotton condensed tannin on feeding and digestion in the cotton pest *Heliothis zea*. J. Insect Physiol. 28: 911-915, 1982.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685, 1970.

LeOra software, Polo-PC. A user guide to probit or logit analysis, LeOra software, Berkeley, CA, 1987.

Samuels RI, Charnley AK, Reynolds SE. A cuticle degrading proteinase from the moulting fluid of the tobacco hornworm, *Manduca sexta*. Insect Biochem. Mol. Biol. 23: 607-614, 1993.

SAS institute. SAS/STAT User's guide for personal computers. SAS Institute, Cary, Nc, 1997.

Terra WR, Ferreira C. Insect digestive enzymes: properties, compartmentalization and function. Comp. Biochem. Physiol. 109B: 1-62, 1994.

Terra WR, Ferreira C. Biochemistry of digestion. In: Gilbert LI, Iatrou K, Gill SS (eds), Comprehensive molecular insect science (Vol. 3), San Diego, Elsevier, pp 171-224, 2005.

Wilhite SE, Elden TC, Brzin J, Smigocki AC. Inhibition of cysteine and aspartyl proteinases in the alfalfa weevil midgut with biochemical and plant-derived proteinase inhibitors. Insect Biochem. Mol. Biol. 30: 1181-1188, 2000.

Zibaee A, Bandani AR, Malagoli D. Purification and characterization of phenoloxidase from the hemocytes of *Eurygaster integriceps* (Hemiptera: Scutelleridae). Comp. Biochem. Physiol. 158B: 117-123, 2011.