PROTEOLYTIC ACTIVITY IN THE MIDGUT OF THE CRIMSON SPECKLED MOTH *UTETHESIA PULCHELLA* L. (LEPIDOPTERA: ARCTIIDAE)

Maryam Ajamhassani[1], Arash Zibaee[1], Jalal Jalali Sendi[1], Hassan Askary[2], Nasser Farrar[3]*

[1] Department of Plant Protection, College of Agriculture, University of Guilan, 41635-1314, Rasht, Iran
[2] Department of Biological Control, Iranian Research Institute of Plant Protection, 19395-1454, Tehran, Iran
[3] Department of Entomology, Natural Resources Research, Research Center of Agricutural and Natural Resources, 1731, Bushehr, Iran

Received: January 26, 2012
Accepted: May 8, 2012

Abstract: Samples were prepared from the midgut of 4th instar larvae of the crimson speckled moth *Utethesia pulchella* L. to find proteolytic activity and properties. Result revealed the presence of high proteolytic activity in the midgut when taking into account specific proteinases including trypsin-like, chymotrypsin-like, elastase and two exopeptidase (aminopeptidase and carboxipeptidase). The optimal pH of general protease was 8 and 7 when using azocasein and hemoglobin as general substrates, respectively. The optimal temperature of the total proteolytic activity in the midgut of *U. pulchella* was 25°C and 30°C when using azocasein and hemoglobin, respectively. Proteolytic activity was inhibited significantly by soybean trypsin inhibitor (SBTI), phenylmethylsulfonyl fluoride (PMSF), trypsin inhibitor (TLCK), chymotrypsin inhibitor (TPCK) and Phenanthroline. These results provide evidences for the presence of serine proteinases as the major proteases in the midgut of *U. pulchella;* a key rangeland pest in warm climates. The interaction between digestive proteases and protease inhibitors have potentially important consequences for pest management programs.

Key words: *Utethesia pulchalle*, midgut, protease, inhibitor

INTRODUCTION

The crimson speckled moth *Utetheisa pulchella* L. (Lepidoptera: Arctiidae) is one of the important pests of rangelands in the southern provinces of Iran. This moth causes defoliation of such major plants as: *Malva neglecta*, *Crotalaria persic, Echium khuzistanicum* and *Anchusa iranica***.** The highest activity of this insect on rangelands takes place especially in February and March as an aggregative population. Larvae of the insect feed intensively on leaves of host plants and prevent growth of the infected hosts. The damage from feeding results in the leaves being useless and allows fungal and bacterial pathogens to grow and penetrate host tissues. Biological and chemical methods are two main ways to decrease the damage of the pest, but there is currently no control procedure to decrease the pest population.

Genetic engineering enables the transfer of novel genes to economically important plants in order to produce resistant plants (Gatehouse *et al*. 1999). These plants are then able to suppress insect growth (Ozgur *et al*. 2009). Genes encoding inhibitors that target digestive proteolytic enzymes of herbivorous insects are candidates for plant transformation (Ryan 1990). Proteinase inhibitors are found in many plants. The inhibitors are believed to have an essential role in the defense against pests (Broadway and Duffey 1986.). The potential uses of these inhibitors as resistant factors have been showed in many studies (Hilder *et al*. 1987; Gatehouse *et al*. 1999). Binding of these inhibitors to digestive proteinases causes protein digestion by the enzymes to be postponed. Induction of protein synthesis and consequently, the pernicious hyper-secretion of digestive enzymes appears to compensate for the depletion of essential amino acids. Negative amino acid balance may result in delayed development and eventually death. Proteases are divided into exo- and endo-peptidases (Terra and Ferriera 2005). Exopeptidases remove amino acids from N-terminal and C-terminal of protein molecules namely amino- and carboxy-peptidases (Terra and Ferriera 2005). Endo-proteases or proteinases are classified into four classes based on the nature of the catalytic site which cleaves peptide bonds consisting of serine, cysteine, aspartic, and metalloproteases (Terra and Ferriera 2005). Except for some hemipteran and coleopteran species, in most insect groups initial protein digestion relies on serine-proteinases, particularly trypsin- and chymotrypsin-likes. In Lepidopteran larvae, these enzymes have been shown to have a high pH optimum 8–11, which is consistent with the alkaline conditions in their midgut (Applebaum 1985). Serine proteinases are the best-studied, and are found in viruses, prokaryotes and eukaryotes, suggesting that serine proteinases are vital for the survival of organisms. Serine proteinases, trypsin- and chymotrypsin-like act in a wide range of physiological processes including digestion, protein activation in the melanization cascade, antibacterial activity and insect immune response (Nakajima *et al*. 1997; Gorman

*Corresponding address:
arash.zibaee@gmx.com, arash.zibaee@guilan.ac.ir

et al. 2000a, b; Ma and Kanost 2000). Information about the activity of these enzymes and their sensitivity to inhibitors is fundamental for future pest control programs. In the present study, we report the partial characterization of proteolytic activities in the midgut of *U. pulchella* larvae by using general and specific substrates as well as inhibitors.

MATERIALS AND METHODS

Insect rearing

After eggs hatched, high numbers of larvae were collected from the regions of the Bushehr province. These were regions which had suffered damage. Then, the larvae were grown on C*henopodium* sp (chenopodiaceae) in laboratory conditions (14L:8D, 25°C and 60% relative humidity) to reach 4th instar larvae.

Insect dissection and sample preparation

Midguts of 150 larvae were removed by dissection under dissecting microscope in ice-cold saline buffer (NaCl 10 mM). 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 13,000 rpm for 15 min. at 4°C (Zibaee 2012). The supernatants of each tube were pooled then stored at –20°C for subsequent analyses.

Azocasein

General proteolytic activity was measured using azocasein 2%, based on the method described by Elpidina *et al*. (2001). The reaction mixture consisted of 100 µl of Tris-HCl buffer solutions (20 mM), 50 µl azocasein and 20 µl enzyme. After incubation at 37°C for 60 min., proteolysis was stopped by adding 100 µl of 10% trichloroacetic acid (TCA). Precipitation was achieved by cooling at 4°C for 5 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 450 nm. Blank solution consisted of all the mentioned portions except for the enzyme solution.

Hemoglobin

Cohen's method (Cohen 1993) was used to assay general proteolytic activity in the midgut by using hemoglobin as a substrate. Hemoglobin solution (50 µl) was added to 100 µl of Tris-HCl buffer solution (20 mM) and incubated at 30°C after adding 20 µl of enzyme solution for 120 min. For termination of proteolysis, 150 µl of 30% TCA was added to the reaction mixture. Precipitation was achieved by cooling at 4°C for 5 min., then the reaction mixture was centrifuged at 13,000 rpm for 10 min. Blanks solution contained all the mentioned portions except for the enzyme. The peptides liberated from hemoglobin were estimated using Folin-Phenol reagent at 650 nm (Folin and Ciocalteu 1927).

Determination of optimal pH on general proteolytic activity and stability

A pH range from 3–12 was used to find the optimal pH for general proteolytic activity in the midgut of *U. pulchella* by using two general substrates. The reaction mixtures were similar to those described earlier but the buffer solution was Tris-HCl from 3–12.

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

A temperature range from 15–60°C was used to find the optimal temperature of general proteolytic activity in the midgut of *U. pulchella* by using two general substrates. The reaction mixtures were similar to those described earlier but the buffer solution was Tris-HCl buffer at pH 7.

Specific proteolytic activity

Serine proteolytic activity

Trypsin-, chymotrypsin- and elastase-like activities (as three subclasses of serine proteinases) were assayed using a concentration of 1mM of BA*p*NA (Nabenzoyl-L-arginine-*p*-nitroanilide), 1 mM of SAAPP*p*NA (N-succinyl-alanine-alanine-proline-phenylalanine-p-nitroanilide) and 1 mM of SAAA*p*NA (N-succinyl-alanineala-nine-alanine-*p*-nitroanilide) as substrates, respectively. The reaction mixture consisted of 40 µl of Tris-HCl buffer (pH 7, 20 mM), 10 µl of each mentioned substrate and 5 µl of enzyme solution. The absorbance of the resulting mixture was then measured spectrophotometrically at 410 nm by *p*-nitroaniline release.

Exopetidase activity

Activities of the two exopeptidases in the midgut of *U. pulchella* were obtained by using Hippuryl-L-Arginine and Hippuryl-L-Phenylalanine for carboxy- and aminopeptidases, respectively. The reaction mixture was 35 µl of Tris-HCL buffer (pH 7, 20 mM), 5 µl of each mentioned substrate and 5 µl of enzyme solution. The reaction mixture was incubated at 30°C for 0–10 min. before adding 30% TCA to terminate the reaction and read at 340 nm. To prove the specific proteolytic activity, negative controls were provided for each substrate separately containing all the mentioned components, except for the enzyme pre-boiled at 100°C for 30 min.

Optimal pH determination of specific proteases

Tris-HCl buffer (pH range 3–12, 20 mM) was used to obtain the optimal pH of each specific protease and find the possible pH dependency of each substrate. The reaction mixtures were similar to those above, but the pH of the used buffer varied from 3 to 12.

Specific inhibitors

The following compounds were used to find any alteration in the proteolytic activity of the midgut of *U. pulchella* regarding the specifically used substrates; SBTI (soybean trypsin inhibitor, 5 mM), PMSF (phenylmethylsulfonyl fluoride, 5 mM); trypsin inhibitor, TLCK (Na-p-tosyl-L-lysine chloromethyl ketone, 5 mM); chymotrypsin inhibitor, TPCK (N-tosyl-L-phenylalanine chloromethyl ketone, 5 mM); cysteine proteinase inhibitor E-64 [(L-trans-epoxysuccinyl-leucylamido-(4-guanidino)-butane, 5 mM)], cystatin (5 mM) and metalloprotease inhibitors, phenanthroline, also, DTT (dithiothreitol\5 mM) used as a cysteine activator.

Electrophoresis zymogram

Electrophoretic detection (Laemmli 1970) of proteolytic enzyme was performed by 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 Polyacryl Amaide Gel Electrophoresis (PAGE) was carried out at 4°C in a constant voltage of 110 mV, gelatin (0.5%) was added in resolving gel. When the dye reached the bottom of the glace, the gel was carefully separated and put in Tris-HCl buffer (pH 8) for 15 min. Then, the 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 Sodium Dodecyl Sulfate Polyacryl Amaide Gel Electrophoresis (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 a 5 mM concentration of SBTI, PMSF, TLCK, TPCK, E64, DTT, Cystatin, Phenanthroline and the control.

Protein determination

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

Statistical analysis

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

RESULTS AND DISCUSSION

In insects, the processing of precursors and the secretory mechanism of digestive enzymes differ from that of other animals (Terra and Ferreira 1994). Relevant studies are therefore, an important contribution to cell biology and may provide new targets for alternative control methods of pests. The first study of the mechanism of enzyme secretion in Lepidoptera was done using larvae of *Bombyx mori* L. (Lepidoptera: Bombycidae) and indicated that membrane-bound trypsin-like proteinases are transported from the tissues to the lumen of the gut where they are solubilized and converted into an active form. (Eguchi *et al*. 1982; Kuriyama and Eguchi 1985).

In the current study, two general substrates were used to determine the optimal pH of general proteolytic activity in the midgut of *U. pulchella*. The optimal pH was found to be pH 7–9 (Azocasein) (Fig. 1). In pH 3–7, the enzymatic activity increased, reaching its maximum at pH 7 then it continued to pH 9, after that it decreased, and the lowest enzymatic activity occurred in pH 12 (Fig. 1). By using hemoglobin as the substrates, the optimal pH was found at pH 7 (Fig. 1). The optimal temperature for the general proteolytic activity in the midgut of *U. pulchella* was 25 and 30°C by using azocasein and hemoglobin as substrates, respectively (Fig. 2). The enzyme activity

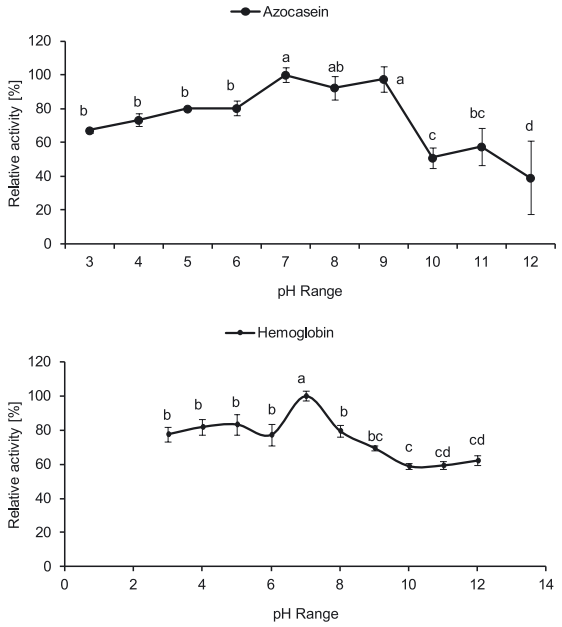

Fig. 1. Optimal pH determination for general proteolytic activity in the 4th instar larvae of *U. pulchella*. Azocasein (2%) and hemoglobin (20 mg/mol) were used as substrates. Statistical analysis was calculated by Tukey's test and showed by different letters ($p \leq 0.05$; n = 3)

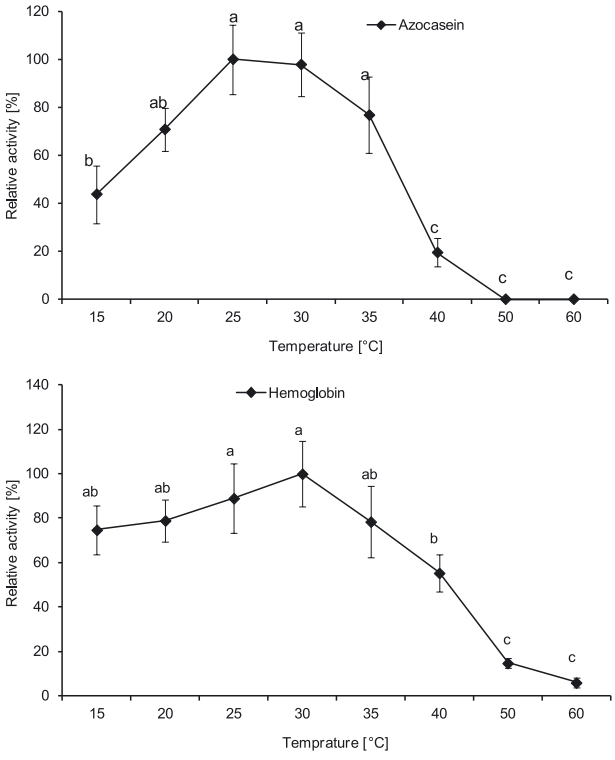

Fig. 2. Optimal temperature (°C) determination for general proteolytic activity in the 4th instar larvae of *U. pulchella*. Asocasein (2%) and hemoglobin (20 mg/ml) were used as substrates. Statistical analysis was calculated by Tukey's test and showed by different letters ($p \leq 0.05$; n = 3)

stopped at the highest temperature when azocasein were used as the substrate (Fig. 2).

Experiments revealed the presence of both serine proteinase (trypsin, chymotrypsin and elastase) and exopeptidases (aminopeptydase and carboxypeptidase) in the insect. Prior investigations proved the higher activity of serine proteinases in lepidopteran larvae (Broadway 1995; Gatehouse *et al*. 1999; Hegedus *et al*. 2003; Terra and Ferriera 2005; Chougule *et al*. 2008). Meanwhile, an alkali condition was found in optimal activity of specific proteases in the midgut of *U. pulchella,* by using specific substrates (Fig. 3). The optimal pH for elastase, trypsin-like and chimotrypsin-like activity were 7, 8 and 9, respectively but it was 7 and 9 for amino- and carboxypeptidase (Fig. 3). Additionally, no activity was found for cysteine proteinases.

The two key factors which affect characterization of enzymes in biochemistry are pH and temperature. High affinity between the enzyme and substrate are what undergo these parameters (Zibaee *et al*. 2011). In this study, pH 7 of the midgut of *U. pulchella* was found to be optimal for both general substrates and elastase. But pH 8, 9 was optimal for proteolytic activity in the presence of trypsin, chimotrypsin and carboxypeptidase, respectively. The results indicate that the highest proteolytic activity is when pH is 7–9. Our results are similar to Terra and Ferreira's (1994) conclusions about high pH of the lepidopteran gut which feed on leaves and wood. Teo *et al*. 1990 have reported a pH of 7.6 for the midgut content of *Anticarsia gemmatalis* Hubner (Lepidoptera: Erebidae). Also, hydrolysis of the substrates influenced by the buffer systems on enzymatic assays have been reported previously for insect digestive enzymes (Purcell *et al*. 1992; Johanston *et al*. 1995; Harrison 2001). The alkaline optimal pH for azocasein hydrolysis strongly suggests the presence of serine proteinases in midgut extracts, confirming the occurrence of protein digestion in *U. pulchella*. Other reports regarding the influence of pH on proteases activity are: *Phtorimaea opercula* Zeller (Lepidoptera: Gelechidae), pH > 9.0 (Christeller *et al*. 1992); *Manduca sexta* L. (Lepidoptera: Sphingidae), pH 8.5 (Samuels *et al*. 1993); *Lacanobia oleracea* L. (Lepidoptera: Noctuidae) pH < 11 (Gatehouse *et al*. 1999) and *Mamestra brassicae* L. (Lepidoptera: Noctuidae), pH 11 (Chougule *et al*. 2008), *Ectomyelois ceratoniae* Zeller (Lepidoptera: Pyralidae) pH 9–10 (Ranjbar *et al*. 2011), *Pieris brassicae* L. (Lepidoptera: Pierridae) pH 8–9 (Zibaee

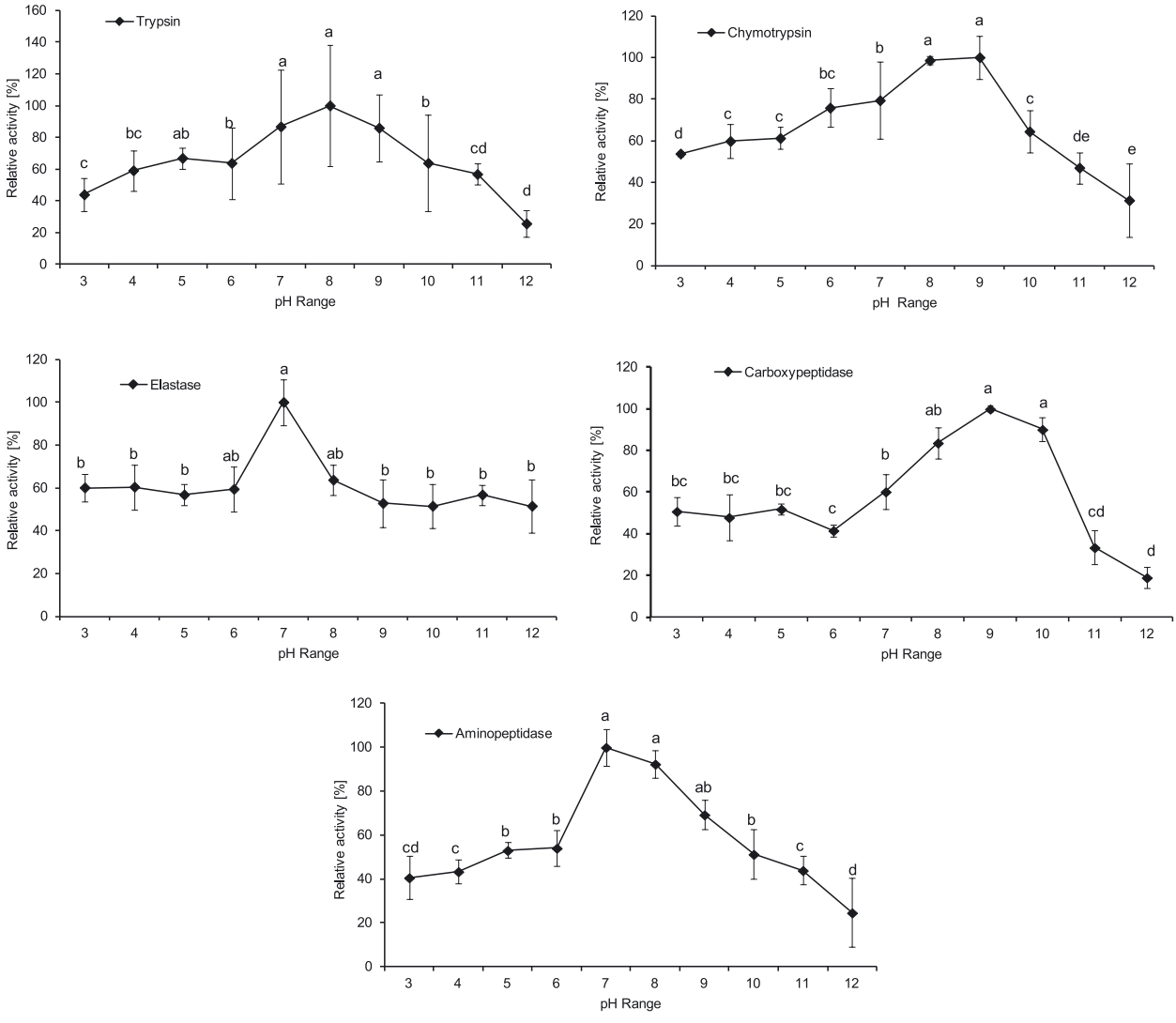

Fig. 3. Optimal pH determination of the specific proteolytic activity in the midgut of *U. pulchella* by using specific substrates. One way analysis Tukey's test was used to determine statistical differences by various letters ($p \leq 0.05$; $n = 3$)

2012a) and *Chilo suppressalis* Walker (Lepidoptera: Crambidae) pH 9–10 (Zibaee 2012b).

Temperatures of 25–30°C were found to be the optimal temperatures for activity of proteases in the midgut of *U. pulchella* by using two general substrates. Proteolytic activity was strongly temperature-dependent and was similar to that reported for other Lepidoptera larvae (Lee *et al*. 1995; Bernardi *et al*. 1996; Marchetti *et al*. 1998) On the other hand, raising the temperature will increase the rate of enzyme-catalyzing reactions, this will occur by increasing the kinetic energy and collision frequency of the reacting molecules (Mohammadi *et al*. 2010). The effect of temperature on the metabolism of these insects and consequently on their life cycle, is well known (Gazzoni *et al*. 1998). Most enzymes were not extremely heat stable, and in most cases were partially or totally destroyed after short exposures to temperature above 50 or 60°C (Zibaee *et al*. 2011; Zibaee *et al*. 2012b). The current results showed that proteolytic activity stopped when the temperature was 50–60°C.

Table 1 shows the effect of some compounds on the general proteolytic activity in the midgut of *U. pulchella*. There were no significant effects from DTT and Cystein on the proteolytic activity, but SBTI, PMSF, TLCK, TPCK and phenanthroline significantly decreased the enzymatic activity so that most inhibition was observed in the case of TPCK (Table 1). It seems that DTT and Cystein was rejected in the midgut of *U. pulchella*. Instead, SBTI, PMSF, TLCK, TPCK and phenanthroline significantly decreased the proteolytic activity, so it was confirmed that serine proteinases were the major proteases in the midgut of *U. pulchella* (Table 1). In more details, inhibition experiments against proteases showed that SBTI, TPCK and Phenanthroline were very potent inhibitors for trypsin and chymotrypsin-like activity causing around 70% inhibition on general proteases activity. The serine proteinase inhibitor, PMSF, also inhibited the protease activity of *U. pulchella.* TLCK, a trypsin inhibitor, decreased L-BA*p*NA hydrolysis in the present study, probably by alkylating thr histidine residue of the catalytic triade of these enzymes (Shaw *et al*. 1965). Electrophoresis revealed six proteolytic bands in the control as well. Adding different inhibitors caused a decrease in band sharpness or the disappearing of bands (Fig. 4). These results indicate that serine proteinases or more particularly, trypsin-like, are present as proteases, since they are inhibited by PMSF and TLCK.

Table 1. Effect of some general inhibitors on the proteolytic activity in the midgut of *U. pulchella*

Component	Inhibition [%]
SBTI	29.72 c
PMSF	44.03 bc
TLCK	66.89 b
TPCK	27.92 c
E64	135.92a
Phenanthroline	31.75 c
The control	100 ab

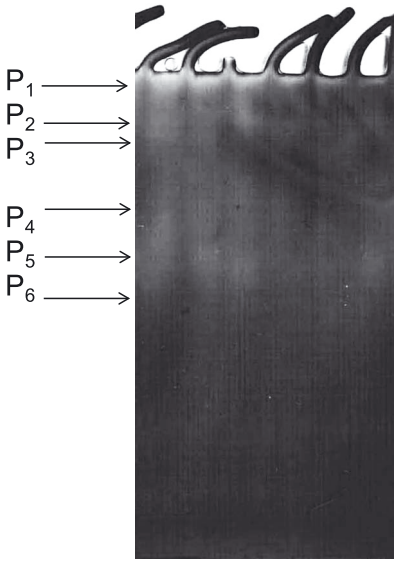

Fig. 4. Non-denaturing SDS-PAGE electrophoresis of proteolytic activity in the presence of different inhibitors. The control was just midgut homogenates without any compounds

The induction of host plant proteases inhibitors is a defensive reaction to insects and pathogens (Farmer and Ryan 1992). This response can inhibit or forbid the proteolytic activity and reduces availability of essential amino acids for insect growth and development (Broadway and Duffey 1986; Broadway 1995). Plant induced protease inhibitors can inhibit insect proteases leading to death, or a control method may be use against pests like *U. pulchella*.

REFERENCES

Applebaum S.W. 1985. Biochemistry of digestion. p. 279–312. In: "Comparative Insect Physiology, Biochemistry and Pharmacology" (G.A. Kerkut, L.I. Gilbert, eds.). Pergamon, Toronto, Canada, 592 pp.

Bernardi B., Tedeschi G., Ronchi S. 1996. Isolation and some molecular properties of a trypsin-like enzyme from larvae of European corn borer *Ostrinia nubilalis* Hübner (Lepidoptera: Pyralidae). Insect. Biochem. Mol. Biol. 26 (9): 883–889.

Bradford M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt. Biochem. 72 (2): 248–254.

Broadway R.M. 1995. Are insects resistant to plant proteinase inhibitors? J. Insect. Physiol. 41 (2): 107–116.

Broadway R.M., Duffey S.S. 1986. Plant proteinase inhibitors: Mechanism of action and effect on the growth and digestive physiology of larval *Heliothis zea* and *Spodoptera exigua*. J. Insect. Physiol. 32 (10): 827–833.

Chougule N.P., Doyle E., Fitches E., Gatehouse J.A. 2008. 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 (3): 563–572.

Christeller J.T., Liang W.A., Markwick N.P., Burgess E.P.J. 1992. Midgut protease activities in 12 phytophagous lepidopteran larvae: dietary and proteases inhibitory interactions. Insect. Biochem. Mol. Biol. 22 (7): 248–254.

Cohen A.C. 1993. Organization of digestion and preliminary characterization of salivary trypsin like enzymes in a predaceous Heteropteran, *Zelus renadii*. J. Insect. Physiol. 39 (10): 823–829.

Eguchi M., Iwamoto A., Yamauchi K. 1982. Interrelation of proteases from the midgut lumen, epithelia and peritrophic membrane of the silkworm, *Bombyx mori* L. Comp. Biochem. Physiol. (A) 72 (2): 359–363.

Elpidina E.N., Vinokurov K.S., Gromenko V.A., Rudenskaya Y.A., Dunaevsky Y.E., Zhuzhikov D.P. 2001. Compartmentalization of proteinases and amylases in Nauphoeta cinerea midgut. Arch. Insect. Biochem. Physiol. 48 (4): 206–216.

Farmer E.E., Ryan C.A. 1992. Octadecanoid precursors of jasmonic acid activate the syntesis of wound-inducible proteinase inhibitors. Plant. Cell. 4 (2): 129–134.

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

Garcia-Carreno F.L., Dimes L.E., Haard N.F. 1993. Substrate-gel electrophoresis for composition and molecular weight of proteinases or proteinaceous protease inhibitors. Analyt. Biochem. 214 (1): 61–69.

Gatehouse A.M.R., Gatehouse J.A. 1998. Identifying proteins with insecticidal activity: use of encoding genes to produce insect-resistant transgenic crops. Pest. Sci. 52 (2): 165–175.

Gatehouse A.M.R., Norton E., Davison G.M., Babbe S.M., Newell C.A., Gatehouse J.A. 1999. Digestive proteolytic activity in larvae of tomato moth, *Lacanobia oleracea*; effects of plant proteinase inhibitors *in vitro* and *in vivo*. J. Insect Physiol. 45 (6): 545–558.

Gazzoni D.L., Pedroso Junior M., Garagorry F. 1998. Mathematical simulation model of the velvetbean caterpillar. Pesquisa Agropecuária Brasileira 33: 385–396.

Gorman M.J., Andreeva O.V., Paskewitz S.M. 2000a. Molecular characterization of five serine protease genes cloned from *Anopheles gambiae* hemolymph Insect Biochem. Mol. Biol. 30 (1): 35–46.

Gorman M.J., Andreeva O.V., Paskewitz S.M. 2000b. Sp22D: a multidomain serine protease with a putative role in insect immunity. Gene 251 (1): 9–17.

Harrison J.F. 2001. Insect acid-base physiology. Ann. Rev. Entomol. 46: 221–250.

Hegedus D.D., Baldwin M., O'Grady L., Braun S., Gleddie A., Sharpe D., Lydiate M. 2003. Midgut proteases from *Mamestra configurata* (Lepidoptera: Noctuidae) larvae: characterization, cDNA cloning and expressed sequence tag analysis. Arch. Insect Biochem. Physiol. 53 (1): 30–47.

Hilder V.A., Gatehouse A.M.R., Sheerman S.E., Barker R.F., Boulter D. 1987. A novel mechanism of insect resistance engineered into tobacco. Nature 330: 160–163.

Kuriyama K., Eguchi M. 1985. Conversion of the molecular form alkaline treatment of gut protease from the silkworm *Bombyx mori*. Comp. Biochem. Physiol. (B) 82 (4): 575–579.

Laemmli U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685.

Lee M.J., Anstee J.H. 1995. Endoproteases from the midgut of larval *Spodoptera littoralis* includes a chymotrypsin-like enzyme with an extended binding site. Insect. Biochem. Mol. Biol. 25 (1): 49–61.

Ma C.C., Kanost M.R. 2000. A beta 1,3-glucan recognition protein from an insect, *Manduca sexta* agglutinates microorganisms and activates the phenoloxidase cascade. J. Biol. Chem. 275: 7505–7514.

Marchetti S., Chiaba C., Chisa F., Bandiera A., Pitotti A. 1998. Isolation and partial characterization of two trypsins from the larval midgut of *Spodoptera littoralis* (Boisduval). Insect Biochem. Mol. Biol. 28 (11): 449–458.

Mohammadi D., Farshbaf Pour Abad R., Rashidi M.R., Mohammadi S.A. 2010. Activity and some properties of *Helicoverpa armigera* Hubner and *Spodoptera exigua* Hubner (Lep.: Noctuidae) midgut protease. Munis. Entomol. Zool. 5 (2): 697–706.

Nakajima Y., Tsuji Y., Homma K., Natori S. 1997. A novel protease in the pupal yellow body of *Sarcophaga peregrine* (flesh fly). J. Biol. Chem. 272 (38): 23805–23810.

Ozgur E., Yucel M., Oktem H.A. 2009. Identification and characterization of hydrolytic enzymes from the midgut of Sunn Pest of wheat (*Eurygaster integriceps*). Int. J. Pest Manage. 55 (4): 359–364.

Purcell J.P., Greenplate J.T, Sammons R.D. 1992. Examination of midgut luminal proteinase activities in six economically important insects. Insect Biochem. Mol. Biol. 22 (1): 41–47.

Ranjbar M. Sendi J.J., Zibaee A. 2011. Proteolytic activity in the midgut of *Ectomyelois ceratoniae* Zeller (Lepidoptera: Pyralidae), Pomegranate carob moth. Res. Rep. 8 (2): 132–142.

Ryan C.A. 1990. Proteinase inhibitors in plants: genes improving defenses against insects and pathogens. Ann. Rev. Phytopathol. 28: 425–449.

Samuels R.I. Charnley A.K., Reynolds S.E.A. 1993. cuticle degrading proteinase from the moulting fluid of the tobacco hornworm, *Manduca sexta*. Insect Biochem. Mol. Biol. 23 (5): 607–614.

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

Shaw E., Mares M., Cohen W. 1965. Evidence for an active-center hystidine in trypsin through use of a specific reagent 1-chloro-3-tosylamido-7-amino-2-heptanona, the chloromethyl ketone derived from *N*-tosyl-L-lysine. Biochem. 4 (10): 2219–2224.

Teo L.H., Hammond A.M., Woodring J.P. et al. 1990. Digestive enzymes of the velvetbean caterpillar (Lepidoptera: Noctuidae). Ann. Entomol. Soc. Am. 88: 820–826.

Terra W.R., Ferreira C. 1994. Insect digestive enzymes: properties, compartmentalization and function. Com. Biochem. Physiol. (B) 109 (1): 1–62.

Terra W.R., Ferreira C. 2005. Biochemistry of digestion. p. 171–224. In: "Comprehensive Molecular Insect Science" (L.I. Gilbert, K. Iatrou, S.S. Gill, eds.). San Diego, Elsevier, 3300 pp.

Zibaee A., Bandani A.R., Malagoli D. 2011. Purification and characterization of phenoloxidase from the hemocytes of *Eurygaster integriceps* (Hemiptera: Scutelleridae). Comp. Biochem. Physiol. B 158 (1): 117–123.

Zibaee A. 2012a. Digestive enzymes of large cabbage white butterfly, *Pieris brassicae* L. (Lepidoptera: Pieridae) from developmental and site of activity perspectives. Italian. J. Zool. 79 (1): 13–26.

Zibaee A. 2012b. Proteolytic profile in the larval midgut of *Chilo suppressalis* Walker (Lepidoptera: Crambidae). Entomol. Res. 42 (1): 142–150.