

## ACTIVITY AND SOME PROPERTIES OF *HELICOVERPA ARMIGERA* HÜBNER AND *SPODOPTERA EXIGUA* HÜBNER (LEP.: NOCTUIDAE) MIDGUT PROTEASE

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**ABSTRACT:** In this study some properties of Cotton bollworm and Beet army worm midgut protease activity was investigated. Insects were reared on special artificial diets in controlled conditions. Different developmental stages including, larval stages, 5-6 inter-moult period, pre-pupa, male and female pupa and adult's protease activity measured using azocaseinolytic assay method. Optimum pH and temperature of protease activity determined by testing different pH and temperature gradients. Also protease stability in two different storage conditions (4 and -20 °C) for 15 weeks were studied. Results showed that the optimum temperature for protease activity was 35°C in both insects. Studying the effects of different pH values on protease activity in both insects showed that except pH 13, enzyme activities were not affected by acid or alkaline conditions. Specific protease activity in 4<sup>th</sup> larval stages of both studied insects was more than other developmental stages. Protease activity in male and female pupa and adults of *S. exigua* was not significantly different, but in *H. armigera* male pupa and female adults have significantly more protease activity than other related sexes.

**KEY WORDS:** Midgut protease, *Helicoverpa armigera*, *Spodoptera exigua*, Noctuidae, Lepidoptera.

Insects of noctuidae family are the most important insect pests in the world (Matthews, 1999). Cotton bollworm exists in Africa, Asia, Australia and Europe that attacks different crops such as cotton, chickpeas, tomatoes, tobacco, corn, sesame, hemp, sunflower, peanuts, okra and etc. (Fitt, 1989; Smith et al., 1992). Cotton bollworm is able to adapt to different cropping systems. High polyphagy and mobility, wide geographical distribution, migration, facultative diapause, high fecundity and potential to develop resistance against insecticides make this insect one of the most important pests of the agricultural crops (Fitt, 1989; Tuttle & Ferro, 1988). *Spodoptera exigua* is a noctuid species with a wide range of hosts and broad distribution that causes economic damage on several crops including maize, cotton, soybean, alfalfa and tomato (Sertkaya et al., 2004; Greenberg et al., 2001).

Recently studying and reaching useful information about digestive system of insects tended researchers to find new control methods that in addition with their safety to environment and biocontrol agents also are effective in IPM programs (Lawrence & Koundal, 2002). Knowledge about enzyme properties is first and main step in physiologically based control methods. Enzymes are affected by changes in pH. The most favorable pH value the point where the enzyme is most

active is known as the optimum pH. Extremely high or low pH values generally result in complete loss of activity for most enzymes. pH is also a factor in the stability of enzymes (Murray et al., 2004; Eisenthal & Danson, 2002). In any enzyme studies, it is essential to assay its activity at regular intervals under standard conditions to check that it remains constant. It is a much better procedure however, to try to find conditions under which the stock enzyme solutions may be stored without appreciable loss of activity over the time involved (Eisenthal & Danson, 2002).

Different studies indicated that digestive protease and specially serine protease are main enzymes in midgut digestive complex of Lepidoptera (Chapman, 1985; Nation, 2002). In this study some properties of midgut protease activity of two important pests of crops were investigated. And we hope this will provide useful information about protease of these insects to use them in complementary studies for goal of finding new control method and managing these important pests.

## MATERIAL AND METHODS

### Insects

Insects used for assays prepared from a colony presents in plant protection department of Tabriz University. Cotton bollworms were reared on artificial diets based on cowpea (Shorey & Hall, 1965) in controlled condition of  $26\pm 2^{\circ}\text{C}$ ,  $50\pm 5\%$  relative humidity and a photoperiod of 16:8 (L: D) h. Beet armyworms also were reared on artificial diet based on Mung bean (*Vigna radiate* L.) (Singh, 1977) in above mentioned controlled condition. Last larval stages (24 h old) of both insects were used for evaluating properties of total protease enzyme. For studying protease activity in different developmental stages 24 h old 4-6 instars, pre-pupa, 5-6 inter-moult, male and female pupa and adults were used.

### Enzymes sampling

For preparing midgut enzyme samples, 4-6 larval stages dissected in cold glycine-NaOH pH 10 buffer under stereomicroscope and before dissection insects anaesthetized with laying them on ice. The midgut removed and transferred to 1.5 ml micro tubes containing 1 ml cold glycine-NaOH pH 10 buffer. After homogenizing the tubes left for 2 hours in  $4^{\circ}\text{C}$ , after then centrifuged in 10'000 rpm for 30 minutes in  $4^{\circ}\text{C}$ . The supernatants used as enzyme sources for assays. Whole body of pupa and adults were used for enzyme preparing and a suitable hand mortar used for this goal. Homogenizing and centrifuging processes carried out such as above mentioned order. Enzyme activity in 5-6<sup>th</sup> inter moult period also was studied.

### Enzyme properties

Temperature gradients from  $20-65^{\circ}\text{C}$  with 5 degree intervals carried out for studying optimum temperature activity using water bath, activity measured after 60 minutes incubation in different temperatures. For studying enzyme activity in different pH values, pH gradients from 4-13 prepared using phosphate buffer, NaOH and HCl was used for adjusting pH values. Enzyme stability studied in two refrigerator ( $4^{\circ}\text{C}$ ) and freezer ( $-20^{\circ}\text{C}$ ) conditions for at least 3 months.

### Enzyme assays

Total protease activity carried out using azocaseinolytic assay as follows: azocasein (at final concentration of 1% w/v) was incubated with the enzyme

fraction in Glycine-NaOH 200 mM buffer, pH 10, containing 5 mM CaCl<sub>2</sub>, at 37°C for 60 min. The reaction was stopped by the addition of 300 µl of TCA (10% v/v) and the sample was centrifuged for 10 min at 10000 rpm. The supernatant (600 ml) was added to 600 ml 1M NaOH and the absorbance of the supernatant was read at 450 nm in a UV/Visible spectrophotometer (CECIL, CE 1021, England). One protease unit was defined as the amount of enzyme that increased the absorbance by 1.0 OD under the given assay condition.

Total protein concentrations of samples were determined by the Bradford protein assay using bovine serum albumin as a standard (Bradford, 1976). Specific activity of protease was calculated by dividing enzyme activity to protein concentration.

### Data analysis

All experiments carried out in three replications and one-way ANOVA used for data analyzing. The differences were compared by Duncan's multiple range test ( $\alpha = 0.05$ ).

## RESULTS

### Enzyme properties

Protease activity in temperature range from 30 -55°C without significant differences was more than other studied temperatures but the most and optimum temperature for Protease activity was 35 °C in both studied insects (Fig. 1). With increasing temperature more than 55°C enzyme activity dramatically decreased and the least activity was observed in 65°C.

Result of effects of different pH values on protease activity of Cotton bollworm and Beet armyworm showed that except pH 13, enzyme activities were not affected by acid or alkaline conditions (Fig. 2). In Beet armyworm three minor peaks in pH of 4, 9 and 12 was observed and the optimum (the most) enzyme activity in pH 12 was detected and about Cotton bollworm the optimum activity was detected in pH of 10, although the differences were not statistically significant.

Protease enzyme stability in two storage condition showed significant differences in both studied insects. Fig. 3 Shows that proteolytic activity of Cotton bollworm midgut protease in freezing condition (-20 °C) was stable after 15 weeks. Although with increasing storage times, enzyme activity decreased in non significant rate. But in other storage condition (4°C) protease activity for 13 weeks was stable although a decreasing trend could be observed. After 13 weeks, enzyme activity significantly decreased and the least enzyme activity was detected after 15 weeks (Fig. 3-A).

In *S. exigua* the same trend was observed (Fig. 3-B) in freezing storage condition (-20°C), enzyme activity without significant differences was stable in storage period. While a decreasing slop could be observed. But in 4°C, enzyme activity was not stable in storage period. After 2 weeks enzyme activity was started to decrease significantly with prompt slop to 5<sup>th</sup> week. After then the slop of decrease was not sharp and without significant differences enzyme activity decreased till 14<sup>th</sup> week. The least enzyme activity was detected after 14 weeks.

### Enzyme activity in different developmental stages

Specific protease activity in developmental stages of Cotton bollworm and Beet armyworm has summarized in table 1. In both studied insects enzyme activity in larval stages was more than other developmental stages. Among larval

stages, with developing larval instars, specific enzyme activity decreased. The most enzyme activity measured in 4<sup>th</sup> larval instar in Cotton bollworm and Beet armyworm. Total protease activity in 5-6 inter-moult period was more than pre-pupa, pupa and adult stages in both insects. There were significant differences between male and female pupa and adults in *H. armigera*. Male pupa and female adults have more protease activity than other sexes. But about *S. exigua*, male and female pupa and adults haven't significant differences in protease activity (Table 2).

## DISCUSSION

Using Azocasein as a substrate for total protease, optimum pH and temperature of *S. exigua* and *H. armigera* was determined. It is clear that raising the temperature will increase the rate of enzyme-catalyzed reactions, this will occur by increasing the kinetic energy and collision frequency of the reacting molecules. However, heat can also increase the kinetic energy of the enzyme to a point that exceeds the energy barrier for disrupting the maintain condition of the enzyme and its three-dimensional structure after that enzyme begins to denature, results in loss of its catalytic activity. In our study more than 60°C specific protease activity sharply decreases in both insects. But enzyme activity between 35-55°C was more than other studied temperatures in both insects. The optimal temperature for the action of each enzyme varies among different insects (Hori, 1973) and other studies with different enzyme systems confirm that. But the temperature in which enzyme starts to decrease its activity approximately was in same manner. For example, Incubation of *Prostephanus truncates* alpha-amylase for 15 min in different temperatures showed that more than 40°C amyolytic activity rapidly decreased (Mendiola-Olaya et al., 2000). And Hasan khan et al. (2003), showed that at higher temperatures (more than 60°C), there is a sharp decrease in the protease activity. In *Anticarsia gemmatalis* larvae, BAPNA hydrolysis (trypsin activity) was temperature dependent and maximum activity was obtained at 50°C (Pereira et al., 2005). Also Blahovec et al. (2006) showed that larval stage peptidases of housefly are not so stable in high temperatures and after 50°C enzyme activity began to decrease sharply. Muyan et al. (2006) showed that optimal temperature for activity of protease digestive enzymes was 50°C. Also they showed that enzyme activity gradually increased with rising temperature to a peak at 50°C then rapidly declined as temperature increased. Cho et al., 2003 using azocasein as a substrate showed that the protease of *Aeromonas hydrophila* was active over the temperatures range of 50 to 65°C with an optimum activity at 60°C.

The optimum temperature of protease activity is positively or negatively in relation with pH values (Muyan et al., 2006). The rate of almost all enzyme-catalyzed reactions exhibits a significant dependence on specific pH. And all enzymes will have their optimum pH ranges (Murray et al., 2003). Most enzymes are stable in variety of pH conditions because of their special three-dimensional structure but a change in pH, will affect the state of amino-acid ionization that are one of maintenance factors of folded structure of enzyme (Murray et al., 2003; Eisenthal & Danson, 2002; Price & Stevens, 2000). Because of specific pH condition in each enzyme groups for maintaining this three-dimensional structure and catalytic activity of them, wide pH ranges should be a reason for activity of different protease enzymes in our study on *H. armigera* and *S. exigua*. Serine proteases including trypsin, chymotrypsin and elastases are more active in alkaline conditions while cysteine proteases optimum activity is in acidic

conditions (Nation, 2002), this wide range of pH could be related to occurrence and activity of these two important groups of proteases in Cotton bollworm and Beet army worm midgut. Protease of *A. hydrophila* was active over the pH range of 8 to 10, but exhibited its maximum activity at pH 9 (Cho et al., 2003).

The purified *Prostephanus truncates* alpha-amylase has the optimum pH values of 6, and in two extremes of studied pH values, enzyme activity decreased (Mendiola-Olaya et al., 2000). Other studies also showed that there is a distinct range of pH for enzyme activity in different insects (Pavasovic et al., 2004; Pereira et al., 2005; Hosseininaveh et al., 2009). In some cases more than one peaks of enzyme activity detected and researchers relates this with isozymes (Zeng & Cohen, 2000), also in different biological and physiological conditions, insects may not have same optimum pH ranges of enzyme activity (Abraham et al., 1992). Then we can see that different conditions will alter enzyme optimum conditions of activity, about our study the evaluated enzyme system was total protease (not purified special enzymes) which are complex of different groups of proteases, but biological, feeding, sampling and rearing condition were same for all experiments, we think the only reason for wide range of pH values is in relation with different protease groups in both insects midgut. Enzymatic studies on *H. armigera* midgut and other lepidopteran insects was indicated that alkaline pH is optimal and serine protease activity is dominant (Ozgur et al., 2009; Nation, 2002). Trypsin-like, chymotrypsin-like, leucine aminopeptidase-like, and minor elastase-like serine proteases were major serine proteases detected in the *H. armigera* midgut (Ozgur et al., 2009).

It is clear that each developmental stage of insects because of different habitats and feeding hosts could have different enzymatic activities especially in holometabolous insects. In this study protease activity of larval stages were more than other developmental stages. Lepidopteran larvae have a long midgut and food immediately pass from mouth to midgut, the digestive enzymes works on it and after digestion absorbance also occurs in midgut (Nation, 2002). In periods between two instar larvae enters an immobilized stage and its mouthparts loss their activity and larva is ready for moulting (our observations, data not available). In this period midgut is empty of food. Because the feeding and enzyme secretion occurs in same time (Nation, 2002; Chapman, 1985), we think low protease activity of inter-moult and pre-pupal stage is in relation with non-feeding behavior of them. Mendiola-Olaya *et al.* (2000) showed that alpha-amylase activity reached its highest value in the second larval instar of *P. truncates*, also enzyme activity in second larval stage was more than 3<sup>rd</sup> instar and only a little activity was detected in pupal stage, they suggest that no digestive processes are taking place during pupal stage. In *Culex pipiens* proteolytic enzymes reached a peak towards the end of larval life, but then decreased a few days before pupation (Chapman, 1985). In different larval instars of *M. domestica* with developing larva to 3<sup>rd</sup> instar, amino peptidase, trypsin, chymotrypsin and elastase activity decreased (Blahovec et al., 2006). Thus high specific protease activity in larval stage and especially decreasing activity with developing insects may be depends on feeding behavior and developing stage of them.

But about differences in male and female pupa and adults in some studied cases there were significant differences between sexes and in some cases the activity was same, but in all cases a minor enzyme activity in pupal stages reported. For example in *Dysdercus* spp. digestives enzyme activity in adult females was more than males (Chapman, 1985) and also protease activity in females of *B. mori* and *Locusta migratoria* was more than males (Hori, 1973). In other study Hori, (1973) showed that amylase and protease activities of the

salivary gland were particularly the same for both sexes in *Lygus disponi*, and also digestive amylase of *B. mori*, protease of stable fly were same in male and female insects. Another problem in comparing protease activity of developmental stages is that in pupa and adult insects whole body were used for enzyme assays, because of probability of activity of protease in hemolymph of this stages will not completely comparable with other developmental stages. Table 2 shows that in female adults protease activity was more than males, maybe it could be reasoned by egg and yolk formation process in females. But other digestive and protolytic systems should be studied to judge about that.

One of the important factors that in enzyme assays will affect severity of studies is enzyme stability in storage condition. Because of preparing enzyme sources and working on its properties and other assays will occur in long times, knowledge of enzyme stability in working and storage condition will be useful. In this study freezing condition (-20°C) kept protease stable in both insects for nearly long time. But another storage condition 4°C especially about *S. exigua* was not suitable and enzyme activity began to decrease after a short time of storage. It is recommendable that for storing this insect's digestive enzyme freezing condition works better than 4°C.

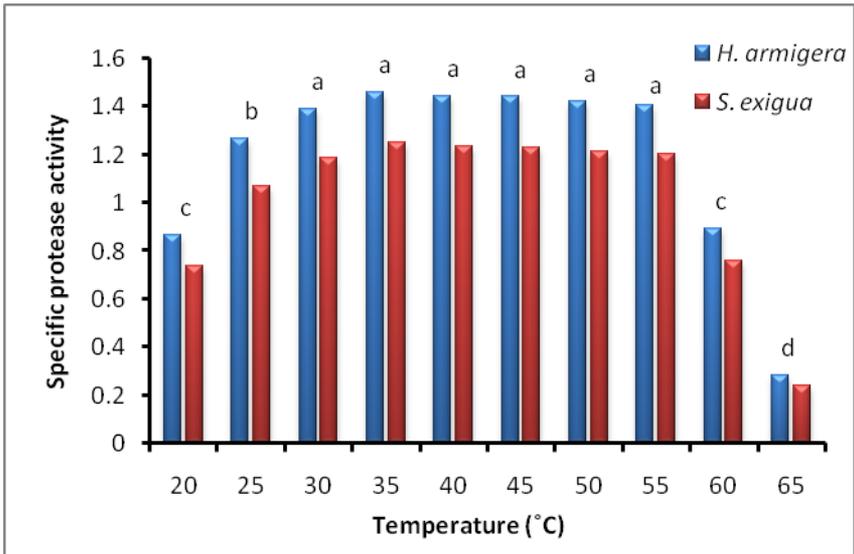
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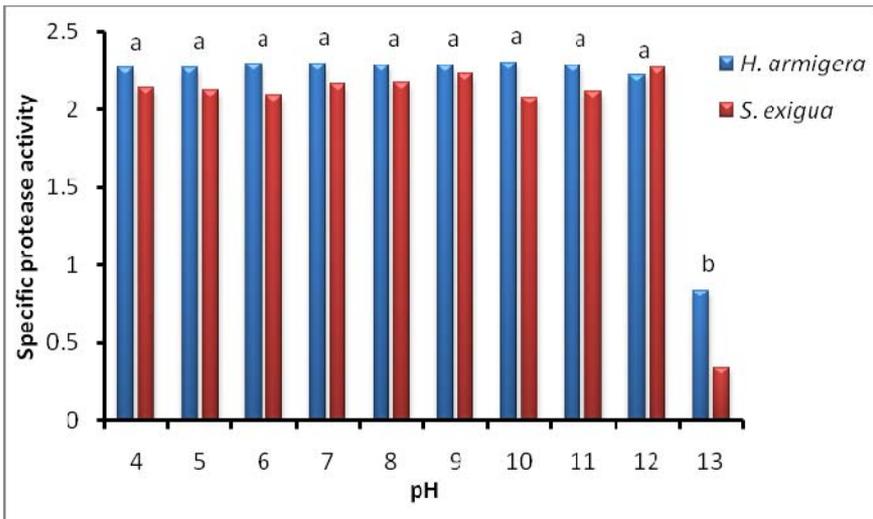
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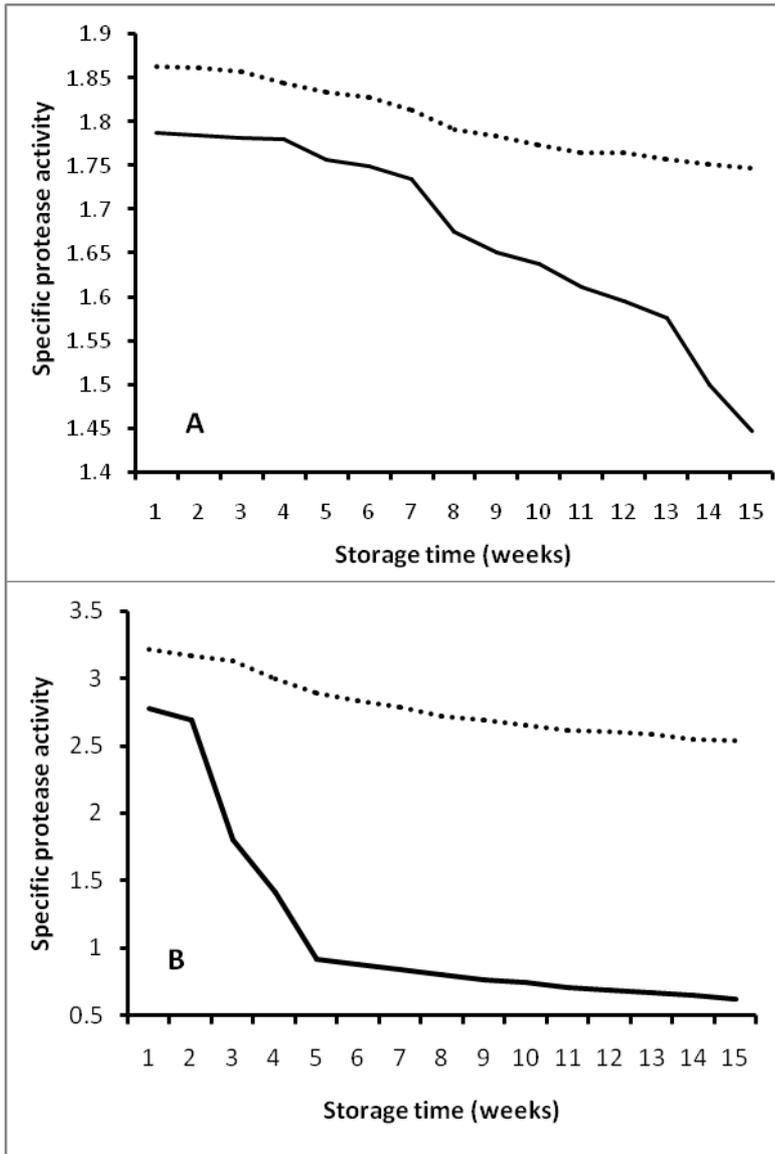
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**Figure 1.** Cotton bollworm and Beet armyworm protease activity in different temperatures. a: The means with similar words have no significant difference (Duncan's multiple range test,  $\alpha=0.05$ ).



**Figure 2.** Protease activity in different pH values in Cotton bollworm and Beet armyworm. a: The means with similar words have no significant difference (Duncan's multiple range test,  $\alpha=0.05$ ).



**Figure 3.** Stability of Cotton bollworm (A) and Beet armyworm (B) midgut protease activity in two storage conditions (..... -20°C and \_\_\_\_\_ 4°C).

**Table 1.** Total protease activity in different developmental stages of Cotton bollworm and Beet armyworm.

Developmental stages	Larval stage			5-6 Inter moult	Pre-pupa	Pupa	Adults
	4 <sup>th</sup>	5 <sup>th</sup>	6 <sup>th</sup>				
<i>H. armigera</i>	8.764 <sup>a</sup>	5.855 <sup>b</sup>	1.903 <sup>c</sup>	0.943 <sup>cd</sup>	0.027 <sup>d</sup>	0.033 <sup>d</sup>	0.060 <sup>d</sup>
<i>S. exigua</i>	16.948 <sup>a</sup>	8.209 <sup>b</sup>	6.043 <sup>c</sup>	0.829 <sup>d</sup>	0.049 <sup>d</sup>	0.030 <sup>d</sup>	0.037 <sup>d</sup>

a: The means with similar words have no significant difference (Duncan's multiple range test,  $\alpha=0.05$ ).

**Table 2.** Protease activity in male and female pupa and adults of Cotton bollworm and Beet armyworm.

Insect	Sex	Pupa	Adult
<i>H. armigera</i>	Male	0.041 <sup>a</sup>	0.022 <sup>b</sup>
	Female	0.025 <sup>b</sup>	0.099 <sup>a</sup>
<i>S. exigua</i>	Male	0.034 <sup>A</sup>	0.038 <sup>A</sup>
	Female	0.025 <sup>A</sup>	0.046 <sup>A</sup>

a: The means with similar words have no significant difference (Duncan's multiple range test,  $\alpha=0.05$ ).