PARTIAL CHARACTERIZATION OF DIGESTIVE A-AMYLASE IN COTTON BOLLWORM, *HELICOVERPA ARMIGERA* HÜBNER (LEPIDOPTERA: NOCTUIDAE)

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ABSTRACT: In this study α-amylase activity of different developmental stages of cotton bollworm were studied. Insects reared on chickpea based artificial diet in controlled condition. Alpha-Amylase activity was determined using 4, 6-ethylidene (G7)-p-nitrophenyl-(G1)-α-D-maltoheptaoside as substrate. The most enzyme activity was observed in 6th larval stage. Male and female adults and pupas showed a minor α-amylase activity. Lumen content of 6th larval midgut showed significantly more enzyme activity in comparison with midgut tissues during circadian cycle. Starvation period non-significantly affected α-amylase activity in whole body and gut extract of 6th larval stage alimentary canel assays. Enzyme activity during embryogenesis and pupa developing period were studied and results revealed that with aging of eggs amylase activity increased but just before hatching, it started to decrease. In pupal growth period, amylase activity showed a decline manner to last period of developing days. Also the results showed that, the optimal pH for α-amylase activity in 6th larval stage of cotton bollworm was alkaline (pH 12).

KEY WORDS: *Helicoverpa armigera*, α-amylase, developmental stages.

Helicoverpa armigera Hübner (Lep., Noctuidae) is a polyphagous pest which causes economic damages on different crops including cotton, tomato, corn, sunflower and etc. worldwide (Mathews, 1999). Results of digestive physiology research's about insects' leads to developing control methods based on digestive system disruption. It is documented that proteolytic enzymes especially trypsin and chymotrypsin like activity in lepidopteran midgut are predominant (Nation, 2002). Digestive enzymes were developed in alimentary canal of insects in relation to food ingested (Sarate et al., 2012). The lepidopteran midgut content is alkalin and serin proteinases are more active than systeins (Terra & Ferreira, 2005). Proteins are not the only source of energy, carbohydrates generally presents in plants, fruits and foliages that are consumed by insects hence related enzymes should be active in alimentary canal of insects. Amylase activity of cotton bollworm poorly was surveyed and almost all enzyme related researches were concentrated on proteolytic properties of midgut and proteinase inhibitors (Chougule et al., 2003; Giri et al., 2003).

Özgur et al. (2009) were detected α-amylase activity of cotton bollworm. Presence and activity of digestive enzymes in developmental stages of insects may be differ because of feeding habitat of them especially in holometabolus insects such as Lepidoptera (Babic et al., 2008). Alpha-amylses (EC 3.2.1.1) catalyse the hydrolysis of α-D-(1-4) glucan linkages in starch and glycogen components. Starch in plants and glycogen in animals are targeted by amylase (Strobli et al., 1998; Franco et al., 2002). It is documented that enzyme activity in different developmental stages of insects in male and females may show some variations

(Tanaka & Kusano, 1980; Papadopoulos et al., 2004; Mehrabadi & Bandani, 2009). But there is a lack of information about changes in amylase activity during a given developmental stage. In egg and pupal developmental period some important events including embryogenesis and metamorphosis occurs, and these process need more enzymatic activity such as them used in metabolism (Fink, 1925; Terra, 1987).

In this study α-amylase activity during developmental period of egg and pupal stages in addition with some properties of α-amylase in last larval stages were investigated. In preparing process of enzyme samples from insects midgut the time of sampling have a critical role in uniformity of enzyme samples, in this study α-amylase activity was surveyed in lumen and tissues of midgut separately during day and night. In some insects specially that one's which have mechanisms for storing ingested foods in alimentary canals, period of starvation could affect amylase activity by concentrating it in midgut. In this study attempt was made to test effect of starvation for 24 and 48 hours on haemolymph and midgut of 6th larval stage of cotton bollworm.

MATERIALS AND METHODS

Insects

Insects reared in controlled condition, 26±2˚C and 60% RH and 16:8 (L: D) h. photoperiod regime on artificial diet based on cowpea (Shorey & Hale, 1965). Enzyme samples prepared after mass rearing for 5 generations in mentioned condition.

Enzyme extraction

In this study two different extraction procedures were used. First, the midguts' of larvae dissected from last larval stages in the aim of amylase property assays, and in second procedure whole body of different developmental stages were homogenized in phosphate buffer pH 7, after carefully crushing by handy mortar. Enzyme samples prepared at 24 hours old of each developmental stage. Fifty eggs per 1ml phosphate buffer, 20, 8 and 5 first, second and third larval stages respectively, and one individual for rest of developmental stages including 4-6 larval stages, pre-pupa, pupa and adult insects per 1ml phosphate buffer were used. For evaluating the enzyme activity during egg developmental period samples including newly oviposited, 12, 24, 48 and 72 hours old eggs prepared. In pupal stage, samples including newly developed, 6hours, 1, 2, 4, 6, 8, 10 and 12 days olds prepared. Enzyme activity also in male and female pupa and adults were compared. Each sample containing one pupa per 1ml phosphate buffer. Samples after homogenization were centrifuged in 12000 rpm (4˚C) for 10 minutes. Supernatants were stored at -20˚C for further assays.

Alpha-amylase activity in different pH values

Activity of enzyme samples were detected in 6th larval stages in pH sets of 4, 5, 6, 7, 8, 9, 10, 11 and 12. Optimal pH for amylase activity was determined using phosphate buffer and pH gradient justified using NaOH and HCl. amylase activity detected after incubation for 30 minutes in each pH values.

Alpha-amylase activity during day and night in lumen and tissues of midgut

For studying dial periodicity of alpha-amylase activity, 24 hours old last larval instars were used. Sampling was carried out every 3 hours during day and night cycles. In the aim of comparing amylase activity in lumen and tissue of midgut during day and night, alimentary canal of 6th larval stages in each sampling times, after dissection were cut longitudinally, content of lumen and tissue of each gut

separately transferred to 1.5 ml micro tubes containing cold phosphate buffers pH 7, homogenized and centrifuged such as above and assayed for amylase activity.

Effect of starvation on alpha-amylase activity in cotton bollworm

Midgut and whole body of 6th larval stage of cotton bollworm were compared after 24 and 48 hours starvation and the results compared with controls that feed normally on artificial diets.

Alpha-amylase activity assay

Alpha-Amylase activity was determined using 4, 6-ethylidene (G7)-p-nitrophenyl-(G1)-α-D-maltoheptaoside (EPS-G7) as substrate using an autoanalyzer (Alcyon 300) system. The reactions were carried out at 37˚C and the absorbance which is directly related to the enzyme activity was measured at 405 nm.

Total protein determination

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.

Statistical analysis

Statistical analysis: completely randomized design (CRD) was used and data comparing carried out by one-way analysis of variance. Duncan multiple range test (p=0.05) was used for comparing of means. Colmogorov-smirnov test were used for homogeneity tests. All experiments were studied in four replications.

RESULTS AND DISCUSSION

Alpha-amylase activity in different developmental stages

Statistical analysis of α-amylase activity in different developmental stages of cotton bollworm showed significant differences among developmental stages. The most and the least activity were detected in 6th larval stage and pupa of cotton bollworm, respectively (Fig. 1). Amylase activity decreased from eggs to 4th larval stage, and then started to increase in 5th and 6th larval stages. Amylase in pre-pupa, pupa and adults significantly were less active in comparing with other developmental stages. Male and female adult and pupas have the same amylolytic activity.

Alpha-amylase activity during day and night in lumen and tissues of midgut

Alpha-amylase activity during day and night showed some variations in different sampling times (Fig. 2). Two distinct activity picks especially in lumen content of 6th larval stage of cotton bollworm were detected in 12 and 3 o'clock. In midgut tissues, alpha-amylase activity was significantly less than lumen and variation in activity was also detected.

Effect of starvation on alpha-amylase activity

Starvation for 24 hrs did not affect amylase activity significantly in whole body assays. Although a minor increase in activity detected. In midgut, amylase activity increased with developing starvation, but it was not significant. Amylase activity in 48 hrs.starved larvae was more than 24 hrs and control.

Amylase activity during pupal and egg developmental periods

There were significant differences in sampling times, the most activity measured in 48 hours old eggs and the least activity in newly oviposited eggs followed by 12 hours old eggs. Figure 4 shows that enzyme activity increased with aging of eggs for 48 hours and decreased in 72 hours old eggs.

In pupas of cotton bollworm alpha-amylase activity did not changed significantly during developmental time (Fig. 5). However the change in enzyme activity was non-significant, a decreasing rate with developing pupas could be observed. With a minor difference in both male and female pupas, this decreasing manner was detected. Alpha-amylase activity in final developing period (after 12 days) started to increase in both male and females.

Alpha- amylase activity in different pH values

Alpha-amylase activity in pH ranges from 4-11 was approximately stable with two minor pikes in pH 5 and 8, but the optimal enzyme activity was in alkaline condition (pH 12). In pH 12 alpha-amylase activities dramatically increased (Fig. 6).

DISCUSSION

Digestive enzyme activity in different developmental stage of insects well documented. Especially in holometabolus insects because of different feeding habitat digestive enzymes show some variations in larval and adults. Alpha-amylase as an important digestive enzyme is active in cotton bollworm (Ozgur et al., 2009). Blahovec et al. (2006) showed that amino peptidase, trypsin, chymotrypsin and elastase activity in house fly decreased with developing larval instars. To some extent these results are true for primary larval stage of cotton bollworm that amylase activity decreased with developing larvae to 4th instar. But in 5th and 6th larval stage amylase activity started to increase and reached to highest activity in 6th larval stage. Glutathion s-transferase are active in all developmental stages of *Apis mellifera* L. (1758), also the highest and lowest activity are found in the adult and egg stages respectively (Papadopoulos et al., 2004). Mehrabadi & Bandani (2009) have detected amylase activity in all nymphs of *Eurygaster maura* (L., 1758). They showed that with developing nymphs alpha-amylase activity increased but there were non-significant differences in amylase activity of 3rd, 4th and 5th instars. In lepidopteran insects because of feeding activity of larval stage digestive enzyme activity are more than adults. Results of our previous study revealed that in larval stages of cotton bollworm proteolytic activity was more than other developmental stages, but with developing insects there midgut proteolytic activity decreased. Also proteolytic activity of adult and pupas was significantly less than larval stages, that the same results in amylolytic activity of cotton bollworm were detected in this study. The same proteolytic activity in male and female adults and pupas of cotton bollworm were detected in cotton bollworm, which is same about amylase activity (Mohammadi et al., 2010).

The current study showed that alpha-amylase activity in tissues of midgut was significantly less than lumen contents. Figure 2 shows that during day and night amylase activity in lumen changed in a circadian rhythmic procedure and two distinct major and minor pikes of amylase activity could be distinguished in 12 and 3 o'clock respectively. Circadian rhythmic activity and behavior of insects well studied (Bebas et al., 2001; Sato, 2003; Steel & Vafopoulou, 2006; Mizutani et al., 2008) but digestive activity during circadian cycles poorly investigated in researches. Regulating of enzyme activity was related to different factors including; release of neuropeptides, humoral regulation or intrinsic properties of the insect (Dadd, 1960; Audsley & Weaver, 2009).

Starvation could affect digestive enzyme secretion especially in insects that have non-continues feeding habitat. In Lepidoptera because of non-storage mechanisms involved in alimentary canal, digestion and feeding are continues

processes (Nation, 2002). Hori (1973) showed that starvation period affected digestive enzymes of *Lygus disponsi* Linnavuori. He reported that with increasing starvation period to 12 hrs, amylase and protease activity increased but it tended to decrease after 24 hrs starvation (but the changes was non-significant). Also amylase activity of *Bombyx mori* L. was affected a little with developing starvation to 24 hrs (Hori, 1973). It is probable that starvation tends to secrete alpha-amylase within lumen without interface of food presence. Starvation could cause to increase concentration of digestive enzyme in lumen and absence of food will increase specific activity of enzyme in this study.

It is documented that within the same instars, enzyme activity increases with aging (Hori, 1973) but there is a lack of information about changes in digestive enzyme activity in eggs and pupal developmental period. Recent study revealed that in cotton bollworm midgut, with aging the eggs to 48 hrs, amylase activity increased and started to decline toward final embryogenesis. In pupal stage enzyme activity declined with aging in both male and females. Near the emerging of adults in both sexes amylase activity showed a non-significant increased manner. Sanjayan et al. (1988) reported that total protein and carbohydrates of *Atractomorpha crenulata* (Fabricius, 1793) during embryogenesis increased until just prior to hatching then started to decrease. In camel tick (*Hyalomma dromedarii* Koch, 1844) alpha-amylase activity during embryogenesis showed a sharp decline and then a gradual increase few days before hatching (Mohamed, 2000). Changes in enzyme activity during the same developmental stages with aging are related to the metabolism of carbohydrates. Pant et al. (1979) reported that carbohydrates declined during embryogenesis developing of *Antheraea mylitta* (Drury, 1773). They also reported that glycogen content of eggs decreased during embryogenesis, this decline in carbohydrates and glycogen shows activity of amylase and other enzymes. Utilization of glycogen as metabolic and physiological functions such as energy source and role in chitin synthesis has been well recognized. Levels of trehalose decreased with developing pupa of *manduca sexta* (Linnaeus, 1763), but glucose content of them increased with aging of pupas (Phalaraksh et al., 2008), increasing the glucose is a result of amylase hydrolysis activity on glycogen. Tanaka & Kusano (1980) reported that in silkworm, during pupal stage, alpha-amylase activity of males was less than females and a decrease rate during pupal developmental period also was observed. In recent study the mean amylase activity in female pupas was more than males.

The optimum pH of alpha-amylase activity varies in different orders of insects (Zeng & Cohen, 2000; Bandani et al., 2010), but in Lepidoptera midgut is generally alkaline, thus digestive enzymes should be active in this condition (Dow, 1992). The optimal pH of *Tecia solanivora* (Povolny, 1973) (Valencia-Jimenez et al., 2008), *Chilo suppressalis* Walker, 1863 (Zibaei et al., 2008), and *B. mori* (Abraham et al., 1992) were 9, 11 and 9.2 respectively. In this study also optimal pH for cotton bollworm larvae was alkalin that is in order with mentioned researches.

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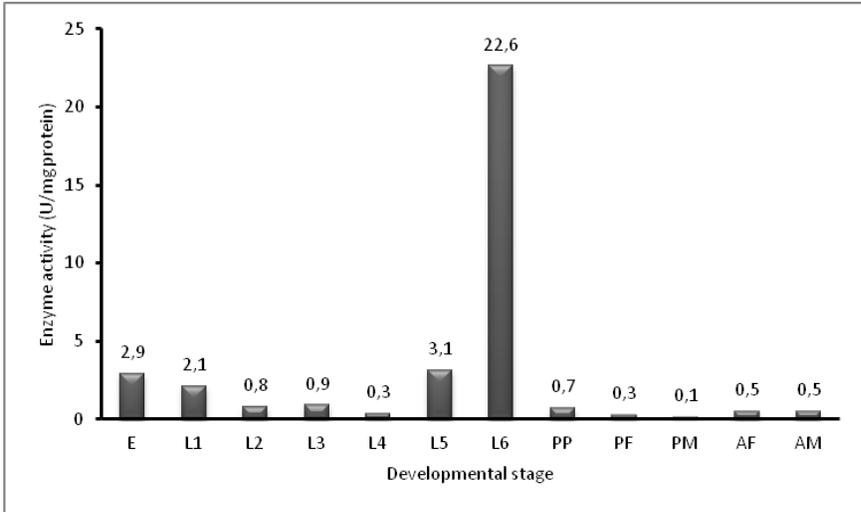
Figure 1. Alpha-amylase activity in different developmental stages of cotton bollworm (E: eggs; L1-L6: first to 6th larval stages; PP: Pre-pupa; PF and PM: male and female pupa; AF and AM: male and female adults).
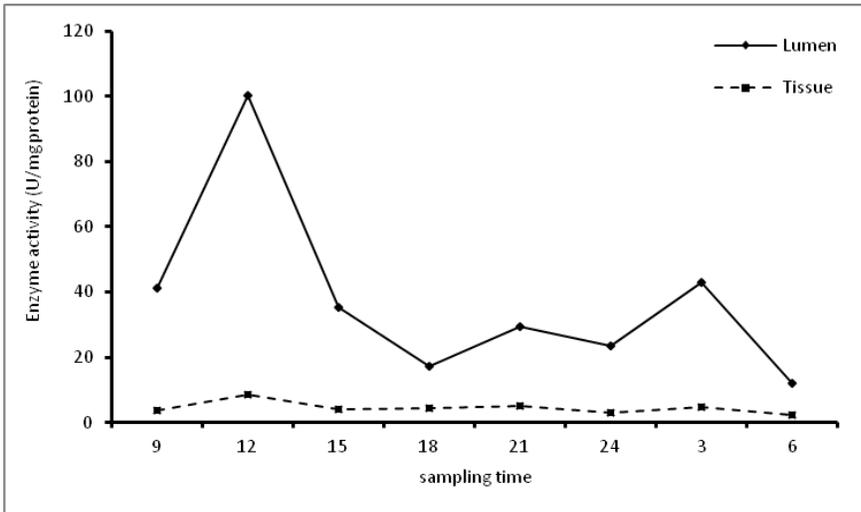

Figure 2. Comparing alpha-amylase activity in midgut lumen and tissue of 6th larval stage of cotton bollworm.
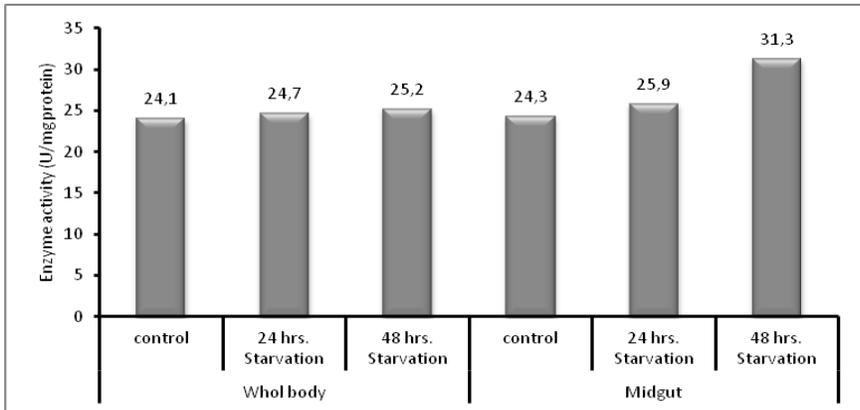
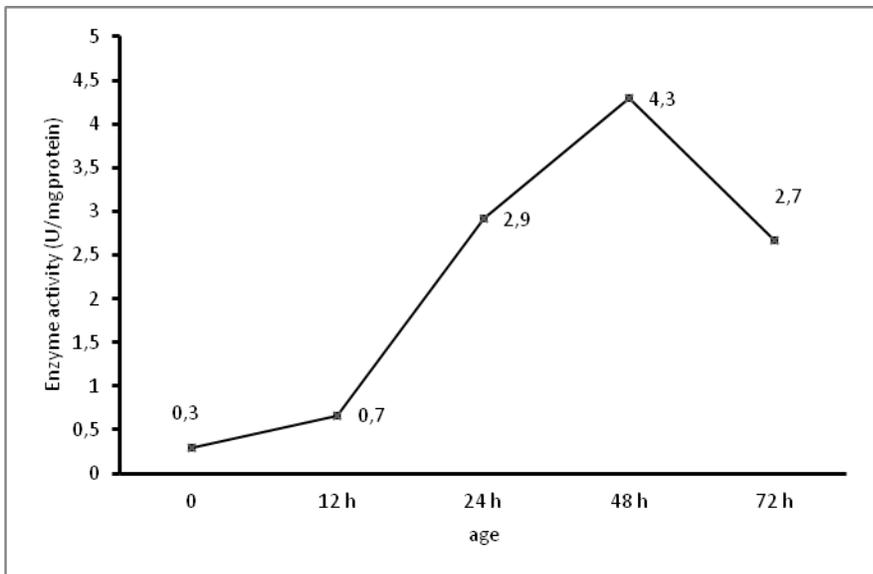Figure 3. Effect of starvation in two different times on whole body and midgut amylase activity.

Figure 4. Amylase activity during developmental period of cotton bollworm eggs.
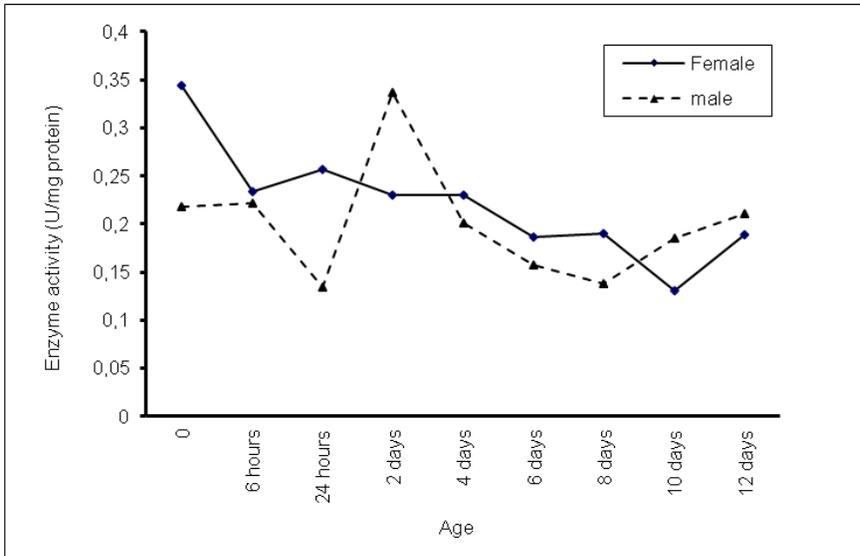
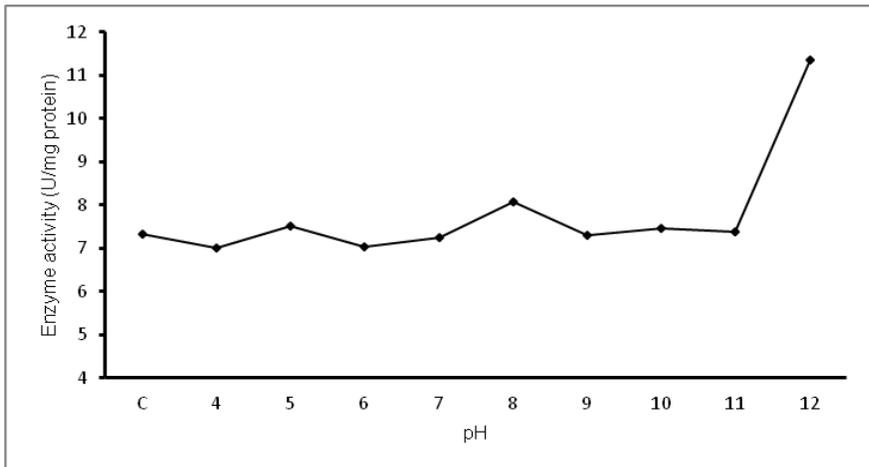Figure 5. Alpha-amylase activity during developmental period of male and female cotton bollworm pupas.

Figure 6. Cotton bollworm midgut alpha-amylase activity in different pH values.