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Research Article

Digestive proteolytic and amylolytic activities in *Helicoverpa armigera* (Lep.: Noctuidae) larvae fed on five host plants

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Abstract: The cotton bollworm, *Helicoverpa armigera* (Hübner), is a serious pest on a wide range of economic crops in many parts of the world. In this study, digestive proteolytic and amylolytic activities of *H. armigera* larvae (3rd to 5th instars) were evaluated on five main host plants including chickpea (cv Hashem), cowpea (cv Mashhad), soybean (cv 033), navybean (cv Dehghan), and corn (cv SC 704) at 25 ± 1 °C, relative humidity of 65 ± 5% and a photoperiod of 16: 8 (L: D) hours. The results indicated that the highest enzyme activity was in 5th instar. The highest general protease activity of 3rd, 4th and 5thinstar larvae was found on corn. The larvae fed on corn had the lowest trypsin activity. This leads to hyperproduction of chymotrypsin and elastase-like enzymes to compensate the inhibition of trypsin. The larvae of *H. armigera* reared on cowpea had the highest level of amylase activity, and the lowest activity was in the larvae fed on corn. The results revealed that among host plants tested, corn was more resistant (unsuitable) to *H. armigera*. Study on digestive proteolytic and amylolytic activities of *H. armigera* can be used in identifying the antidigestive or antifeedent compounds, which will help us to design appropriate management programs.

Keywords: Cotton bollworm, *Helicoverpa armigera*, digestive enzymes, host plants

Introduction

The cotton bollworm, *Helicoverpa armigera* (Hübner) is one of the major pests with global distribution (Fathipour and Naseri, 2011). This species is a typical polyphagous insect with a wide range of host plants including cultivated and wild plants (Liu *et al.,* 2004). Application of chemical pesticides is one of the traditional tools for insect control (Nimbalkar *et al.,* 2009). However, improper use of conventional insecticides has led to resistance in *H. armigera* (Kranthi *et al.,* 2002). Therefore, practical application of host plant resistance in combination with other control measures is the keystone of integrated pest management (IPM) programs of this pest (Fathipour and Sedaratian, 2013).

Plants have capacity to synthesize certain biologically active substances, which play a major role in plant defense against insect pests and wounding. Some of these substances include defense proteins like proteinase inhibitors (PIs), amylase inhibitors, and lectins (Ryan, 1990). When PIs enter the insect digestive tract along with the food they lead to block the gut proteases of insects. Hence, decreasing the amino acids and energy, resulting in retardation of growth and

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development (Giri *et al.,* 2004). Also, insects exhibit mechanisms to produce inhibitor-insensitive responses in their midgut to overcome the effect of PIs (Damle *et al.,* 2005).

Therefore, understanding the digestive enzymes function in midgut is important for development of host plant resistance as a pest management strategy. Naseri *et al.* (2010) studied digestive proteolytic and amylolytic activities of *H. armigera* in response to feeding on different soybean cultivars. Protease and α-amylase activities have been described in *H. armigera* (Kotkar *et al.,* 2009; Naseri *et al.,* 2010; Fallahnejad-Mojarrad *et al.,* 2013) and different lepidopteran species (Pritchett *et al.,* 1981; Zibaee *et al.,* 2008).

In the present study, we determined digestive protease and amylase activities in 3rd, 4th and 5thinstars of *H. armigera* when fed on different host plants incorporated into artificial diets. This information will help us to realize the insect-plant interactions and design a proper pest management program. Combination of information related to digestive enzymes studies accompanied with demographic parameters presents more reliable output for host plant resistance evaluation.

Materials and Methods

Plant seeds

Seeds of the five different host plants including cowpea (cv Mashhad), chickpea (cv Hashem), soybean (cv 033), navy bean (cv Dehghan) and corn (cv Sc704) were obtained from Seed and Plant Improvement Institute (Karaj, Iran) and used in the experiments.

Rearing methods and experimental conditions

The eggs of *H. armigera* were obtained from University of Tabriz, Iran and kept on a defined artificial diet. The insects were reared on artificial diets containing the seeds of host plants for two generations before carrying out the experiments. Rearing condition was set at 25 ± 1 °C, 65 ± 5% RH, and a photoperiod of 16:8 (L: D) h.

Each artificial diet contained powdered seeds of one of the five host plants (250 g), wheat germ (30 g), sorbic acid (1.1 g), ascorbic acid (3.5 g), sunflower oil (5 ml), agar (14 g), methyl-p-hydroxyl benzoate (2.2 g), formaldehyde 37% (2.5 ml) and distilled water (650 ml) (Teakle, 1991). Prepared artificial diets were kept in refrigerator for no longer than two weeks before use.

Enzyme sampling

Mdgut of 3rd, 4th and 5th instar larvae were carefully dissected under a stereomicroscope. The removed midgut was washed with precooled distilled water and transferred to 1.5 ml micro tubes containing 1 ml distilled water. Each micro tube contained 17 midguts for the third, 10 midguts for the fourth and 6 midguts for the fifth instar larvae. After homogenizing with a handheld glass grinder on ice, the suspension was centrifuged at 16000 g for 10 min at 4 °C. The supernatant was collected, frozen in aliquots and stored at -20 °C until required for protease and amylase assays.

Proteinase activity assay

General proteolytic activity was determined using azocasein 1.5% as a substrate at the optimum pH as follows: 80 µL of 1.5% azocasein solution in 50 mM universal buffer [50 mM sodium phosphate-borate, pH 12 (unpublished data)] was incubated with 50 µL of crude enzyme at 37 °C for 50 min. The reaction was stopped by adding 100µL of 30% trichloracetic acid (TCA) and the sample was centrifuged for 10 min at 16000 g. An equal volume of 2 M NaOH was added to the supernatant, and absorbance was read at 440 nm. Unit activity was expressed as an increase in optical density mg^{-1} protein of the tissue min^{-1} due to azocasein proteolysis (Vinokurov *et al.,* 2007).

Specific proteolytic activity assay

Enzyme-specific substrates, 1mM BA*p*NA (N-benzoyl-L-arg-p-nitroanilide), 1 mM SAAPF*p*NA (N-succinyl-ala-ala-pro-phe-p-nitroanilide) and 1 mM SAAA*p*NA (N-succinyl-ala-ala-ala-p-nitroanilide), were used for trypsin, chymotrypsin and elastase-like activities, respectively.

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The reaction mixture contained 20 µL of enzyme extract for trypsin and elastase-like activities but 10 µL of enzyme extract (midgut of fifth instar larvae) for chymotrypsin-like activity, 75 µL of universal buffer at the appropriate pH (pH 10.5 for trypsin and chymotrypsin-like enzymes and pH 11 for elastase-like enzyme) as well as 5 µL of the above mentioned substrate. Absorbance was read at 405 nm for 40 min (at 1, 2 and 4 min intervals, respectively). All assays were carried out in triplicate against appropriate blanks.

Amylase activity assay

Digestive amylolytic activity was assayed by the dinitrosalicylic acid (DNSA) method (Bernfeld, 1955) using 1% soluble starch as substrate. The reaction mixture consisted of 50 µL of crude enzyme 250 µL of universal buffer (pH 10) and 10 µL of soluble starch; the whole mixture was incubated at 37 °C for 30 min. The reaction was terminated by adding 50 µL DNSA and heating in boiling water for 10 min. The absorbance was then measured at 540 nm after cooling on ice. One unit of amylase activity was expressed as the amount of enzyme required to produce 1 mg of maltose in 30 min at 37 °C under the given assay conditions (Highley, 1997). All assays were carried out in triplicate against appropriate blanks.

Protein quantification

Total protein concentration in the samples was determined by the Bradford method using bovine serum albumin as a standard (0.125, 0.25, 0.5,1 and 2 mg mL^{-1}) (Bradford, 1976).

Statistical analysis

Data were analyzed by two-way factorial design through the PROC GLM procedure of SAS (SAS Institute) and the differences among means were compared by Duncan's multiple range test ($\alpha = 0.05$).

Results

General proteolytic activity

General proteolytic activity of *H. armigera* reared on five host plants is shown in Table 1. The enzyme activity increased with increasing the instar. The highest enzyme activity was recorded in 5th instar larvae on all host plants tested. Furthermore, the highest general protease activity of 3rd, 4th and 5th instars was found on corn. However, the lowest level of protease activity was observed in 3rd instar feeding on cowpea compared with other host plants.

Specific proteolytic activity

The results of specific proteolytic activity of *H. armigera* larvae fed on different host plants are summarized in Table 2. The highest and lowest level of trypsin activity were in the larvae fed on cowpea and corn, respectively. Chymotrypsin activity of midgut extracts from the larvae reared on corn was higher than those reared on the other host plants tested. However, the larvae that fed on cowpea had the lowest chymotrypsin activity. The results indicated that the larvae fed on cowpea had the lowest activity of elastase-like enzyme, while those fed on corn showed the highest level of activity.

Amylase activity

Amylase activity in 5th instar larvae was higher compared with the other instars examined in this study (Table 3). The third, fourth and fifth instars reared on cowpea had the highest level of amylase activity. The lowest amylase activity was obtained in 3rd, 4th and 5th instars fed on corn diet as compared to similar instars fed on the other host plants tested.

Discussion

Proteinases are the major digestive enzymes in insects' gut. They are responsible for a continuous supply of essential amino acids and energy from the food source for development (Telang *et al.,* 2005). Also, these proteinases are influenced by different host plants on which insects feed (Patankar *et al.,* 2001; Chougule *et al.,* 2005). The objective of this research was to reveal how different host plants affect midgut proteinases activity during larval period of *H. armigera*. The highest enzyme activities were observed in the 5th

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instar larvae. It is clear that each instar has different enzyme activity that these differences may be reflected on feeding behavior and developing stage of them (Mohammadi *et al.*, 2010). The high level of enzyme activity in fifth instar suggests greater rate of ingestion and maximum food intake during this developmental stage. The larval stages of *H. armigera* are responsible for accumulation of nutrients to complete its life cycle. Kotkar *et al.* (2009) and Patankar *et al.* (2001) showed that the maximum of proteinase levels was obtained in 5th instar. Our results are in conformity with the results of the mentioned research.

Table 1 Proteolytic activity of midgut extracts from *Helicoverpa armigera* larvae reared on five host plants.

Host plants	Protease general activity (OD/min)		
	Third instar	**Fourth instar**	**Fifth instar**
Chickpea	1.60 ± 0.134Cc	2.16 ± 0.317Bc	2.47 ± 0.562Ac
Corn	3.48 ± 0.228Ca	4.26 ± 0.344Ba	5.58 ± 0.266Aa
Cowpea	1.37 ± 0.196Cc	2.45 ± 0.185Bc	3.25 ± 0.442Ac
Navy bean	1.94 ± 0.595Cc	2.05 ± 0.267Bc	2.76 ± 0.435Ac
Soybean	2.24 ± 0.096Cb	2.91 ± 0.175Bb	4.06 ± 0.333Ab

The mean values followed by different uppercase letters within each row and lowercase letters within each column are significantly different ($P < 0.05$, Duncan).

Table 2 Specific proteolytic activity of midgut extracts from 5th instar larvae of *Helicoverpa armigera* reared on five host plants.

Host plants	Trypsin (OD/min)	Chymotrypsin (OD/min)	Elastase (OD/min)
Chickpea	0.20 ± 0.024b	0.075 ± 0.015b	0.035 ± 0.0005b
Corn	0.043 ± 0.006c	0.22 ± 0.015a	0.069 ± 0.009a
Cowpea	0.26 ± 0.024a	0.041 ± 0.002b	0.031 ± 0.003b
Navy bean	0.073 ± 0.023c	0.19 ± 0.028a	0.047 ± 0.003b
Soybean	0.14 ± 0.007b	0.05 ± 0.004b	0.039 ± 0.003b

The means followed by different letters in the same columns are significantly different ($P < 0.05$, Duncan).

Table 3 Amylolytic activity of midgut extracts from *Helicoverpa armigera* larvae reared on five host plants.

Host plants	Amylase activity (OD/min)		
	Third instar	**Fourth instar**	**Fifth instar**
Chickpea	0.905 ± 0.043Bb	0.766 ± 0.018Bc	0.940 ± 0.024Ac
Corn	0.388 ± 0.074Bd	0.485 ± 0.075Bd	0.513 ± 0.093Ad
Cowpea	1.04 ± 0.011Ba	1.03 ± 0.108Ba	1.05 ± 0.045Aa
Navy bean	0.930 ± 0.019Bb	1.01 ± 0.013Bb	1.03 ± 0.023Ab
Soybean	0.886 ± 0.058Bc	0.963 ± 0.008Bb	1.00 ± 0.013Ab

The mean values followed by different uppercase letters within each row and lowercase letters within each column are significantly different ($P < 0.05$, Duncan).

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The larvae of *H. armigera* reared on corn had the highest general protease activity. *H. armigera* is known as a polyphagous pest and the larvae of this pest attack more than 180 plant species (Giri *et al.,* 2003). So, this has wide array of proteolytic enzymes to adapt to broad range of inhibitors. In fact, when *H. armigera* is exposed to different PIs from different host plants it produces new proteinases, which are insensitive to the expressed PIs (Giri *et al.,* 2003). In this process, the insect uses essential amino acids and energy for secretion of new PIs-insensitive proteinases which may limit the availability of amino acids and lead to retardation of insect growth (Broadway and Duffey, 1986). Tamayo *et al.* (2000) showed that corn produces the maize proteinase inhibitor (MPI) in response to larval feeding. The results of demographic analysis indicated that developmental time of *H. armigera* reared on corn was significantly longer than those fed on other host plants (Baghery *et al.*, 2011). Maybe the presence of PIs in the mentioned host plant causes deleterious effects on larvae and pupae of this pest. The accumulation of proteins during the larval stages is critical to vitellogenesis (Telang *et al.,* 2001). The findings of the earlier studies on reproductive parameters of *H. armigera* (Baghery *et al.,* 2011) showed that females reared on corn had the lowest fecundity. Probably the PIs adversely affected protein uptake at the larval stage, which reduced fertility and fecundity of the adults. Naseri *et al.* (2010) demonstrated that larvae of *H. armigera* fed on soybean (cultivars L17 and Sahar) showed hyperproduction of proteases in response to protease inhibition by PIs and leading to weak potential to increase its population on these cultivars. Fallahnejad-Mojarrad *et al.* (2013) reported that the larvae of *H. armigera* fed on chickpea (cultivar Arman) showed reduction in protease activity which might be due to protease inhibition by PIs. Different host plants contain a variety of PIs. When insects ingest PIs from plants, the interaction of their proteinases with PIs determines the success or failure of PIs as antidigestive factors. However, PIs of some host plants could act synergistically and lead to hyperproduction of proteinases. Also, some PIs are able to decrease proteinase activity and in some cases, they are not strong inhibitors, which result in growing normally on the related host plant (Telang *et al.,* 2005). The data from our earlier study (Baghery *et al.,* 2013) on nutritional indices of *H. armigera* larvae on corn showed the lowest values of efficiency of conversion of ingested food (ECI) and efficiency of conversion of digested food (ECD) of the larvae fed on corn. The values of ECD and ECI depend on the activity of digestive enzymes (Lazarevic *et al.,* 2004). Probably the presence of some PIs in these cultivars can slow down the activity of digestive enzymes and lead to reduced values of ECD and ECI (Baghery *et al.,* 2013). Our results are in conformity with the results of the mentioned study on the proteinase activity of the larvae fed on these host plants.

The lowest level of trypsin activity was observed on corn. It may be due to the presence of PIs in this host plant. However, the larvae reared on corn had the highest chymotrypsin and elastase-like activity compared with other host plants, this may be due to the increasing of chymotrypsin and elastase-like enzymes to compensate the inhibitory effect of trypsin inhibitor of this host plant. According to the reports of Naseri *et al.* (2010) on soybean (cultivars L17 and Sahar), the inhibition of trypsin activity by PIs of these two cultivars causes the hyperproduction of chymotrypsin-like enzymes. Wu *et al.* (1997) have reported the secretion of chymotrypsin-like and elastase-like in *H. armigera* gut in response to giant taro trypsin inhibitor.

The larvae fed on cowpea showed the highest level of amylase activity and the lowest level of protease activity. Also, the highest level of protease activity and the lowest amylase activity were observed on corn diet. The larvae of *H. armigera* have mechanisms to precisely detect the diet contents and regulate the levels of digestive enzymes (Kotkar *et al.,* 2009). The latter researchers have reported that corn-fed

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larvae of *H. armigera* showed higher level of proteinases but did not produce high level of amylase. Our results are in conformity with the results of the mentioned study. Our study showed that corn as compared with other tested host plants had more resistance to *H. armigera* which may be due to the presence of some secondary metabolites that act as proteinase inhibitors (PIs). It is known that the type of nutrients affect digestive enzymes activity. Therefore, knowledge of interactions between plant PIs and insect gut proteases can be a beneficial guidance in development of modified plants with enhanced resistance to *H. armigera.*

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فعالیت پروتئولیتیک و آمیلولیتیک لاروهای *Helicoverpa armigera* (Lep.: Noctuidae) با تغذیه از پنج میزبان گیاهی

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چکیده: کرم غوزه پنبه *Helicoverpa armigera* (Hübner) یکی از آفات مهم روی بسیاری از محصولات کشاورزی در بسیاری از بخش‌های جهان از جمله ایران می‌باشد. در این پژوهش، فعالیت پروتئولیتیک و آمیلولیتیک لاروهای کرم غوزه پنبه (سن سوم تا پنجم) روی پنج میزبان گیاهی شامل نخود (رقم هاشم)، لوبیا چشم بلبلی (رقم مشهد)، سویا (رقم ۰۳۳)، لوبیا سفید (رقم دهقان) و ذرت (رقم Sc۷۰۴) در دمای ۱ ± ۲۵ درجه سلسیوس، رطوبت نسبی ۵ ± ۶۵ درصد ودوره نوری ۱۶ ساعت روشنایی و ۸ ساعت تاریکی مورد مطالعه قرار گرفت. این نتایج نشان داد که بیشترین فعالیت آنزیمی در لاروهای سن پنجم می‌باشد. بالاترین فعالیت پروتئازی کل در لاروهای سن سوم، چهارم و پنجم روی ذرت به‌دست آمد. لاروهایی که روی ذرت تغذیه کرده بودند کمترین فعالیت آنزیم تریپسین را داشتند. لاروهای کرم غوزه پنبه که روی لوبیا چشم بلبلی پرورش یافتند بالاترین فعالیت آمیلازی را داشتند وکمترین فعالیت در لاروهای تغذیه شده با ذرت بود. این نتایج نشان داد که در میان میزبان‌های گیاهی مورد مطالعه، ذرت مقاومت بالاتری نسبت به لاروهای کرم غوزه پنبه دارد. مطالعه فعالیت‌های پروتئولیتیک و آمیلولیتیک کرم غوزه پنبه پرورش یافته روی میزبان‌های گیاهی مختلف می‌تواند در شناسایی ترکیبات ضدتغذیه‌ای استفاده شود که به برنامه مدیریت تلفیقی این آفت کمک خواهد کرد.

واژگان کلیدی: کرم غوزه پنبه، *Helicoverpa armigera*، آنزیم‌های گوارشی، میزبان گیاهی

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