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Effect of artificial diets containing different maize hybrids powdered seeds on digestive proteolytic and amylolytic activities and nutritional responses of *Helicoverpa armigera* (Lep.: Noctuidae)

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Abstract

Digestive proteolytic and amylolytic activities and feeding responses of fifth instar *Helicoverpa armigera* on different maize hybrids (DC370, SC704, SC700, SC500 and SC260) incorporated into artificial diets were investigated under controlled conditions (25±1˚C, 65±5% R.H. and a photoperiod of 16:8 (L:D) h). The highest total proteolytic activity was in the larvae reared on DC370 (7.954±0.543 U mg^{-1}) and SC500 (7.965±0.171 U mg^{-1}), whereas the lowest one was in the larvae fed on SC704 (5.878±0.160 U mg^{-1}). The highest amylolytic activity was detected on DC370 (0.055±0.001 mU mg^{-1}) and SC500 (0.047±0.007 mU mg^{-1}) and the lowest activity was on SC704 (0.012±0.001 mU mg^{-1}). Larval weight of fifth instar *H. armigera* demonstrated significant difference, being heaviest on SC704 (45.77±6.16 mg) and lightest on DC370 (18.14±1.61 mg). The highest and lowest values of food consumption were on SC500 (219.56±11.4 mg/larva) and DC370 (155.05±10.3 mg/larva), respectively. The feces weight of *H. armigera* was the highest on SC704 (50.74±8.2 mg/larva) and lowest on SC500 (27.51±4.21 mg/larva). According to the results, hybrid DC370 is unfavorable host for feeding fifth instar larvae of *H. armigera*.

Key words: Digestive enzymes, Feeding response, *Helicoverpa armigera*, Maize hybrids.

تاثير غذاي مصنوعي حاوي بذر پودر شده هيبريدهاي مختلف ذرت روي فعاليت پروتئوليتيك و آميلوليتيك گوارشي و واكنش‌هاي تغذيه‌اي *Helicoverpa armigera* (Lep.: Noctuidae)

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چكيده

فعاليت پروتئوليتيك و آميلوليتيك و واكنش تغذيه‌اي لارو سن پنجم *Helicoverpa armigera* روي هيبريدهاي مختلف ذرت (DC370، SC704، SC700، SC500 و SC260) در قالب غذاي مصنوعي تحت شرايط كنترل شده (دماي ۱±۲۵ درجه سلسيوس، رطوبت نسبي ۵±٦۵ درصد و دوره نوري ١٦ ساعت روشنايي و ٨ ساعت تاريكي) بررسي شد. بيشترين فعاليت پروتئوليتيك كل در لاروهاي پرورش يافته روي DC370 (۷/۹٥٤±۰/٥٤۳ U mg^{-1}) و SC500 (۷/۹٦٥±۰/۱۷۱ U mg^{-1}) بود ولي كمترين فعاليت آنزيمي در لاروهاي تغذيه شده با SC704 (٥/۸۷۸±۰/۱٦۰ U mg^{-1}) بود. بيشترين فعاليت آميلوليتيك نيز روي DC370 (۰/۰٥٥±۰/۰۰۱ mU mg^{-1}) و SC500 (۰/۰٤۷±۰/۰۰۷ mU mg^{-1}) و كمترين فعاليت آنزيمي روي SC704 (۰/۰۱۲±۰/۰۰۱ mU mg^{-1}) بود. وزن لارو سن پنجم *H. armigera* اختلاف معني‌داري روي هيبريدهاي مختلف داشت به طوري كه روي هيبريد SC704 (٤٥/۷۷±٦/۱٦ ميلي‌گرم) بيشترين و روي هيبريد DC370 (۱۸/۱٤±۱/٦۱ ميلي‌گرم) كمترين وزن را داشت. بيشترين و كمترين وزن غذاي مصرفي به ترتيب روي SC500 (۲۱۹/٥٦±۱۱/٤ ميلي‌گرم بر لارو) و DC370 (۱٥٥/۰٥±۱۰/۳ ميلي‌گرم بر لارو) بود. وزن فضولات *H. armigera* روي SC704 (٥۰/۷٤±۸/۲ ميلي‌گرم بر لارو) بيشترين و روي SC500 (۲۷/٥۱±٤/۲۱ ميلي‌گرم بر لارو) كمترين مقدار بود. بر اساس يافته‌هاي حاصل از اين پژوهش، هيبريد DC370 ميزبان نامناسبي براي تغذيه لارو سن پنجم *H. armigera* است.

واژه‌هاي كليدي: آنزيم‌هاي گوارشي، واكنش تغذيه‌اي، *Helicoverpa armigera*، هيبريدهاي ذرت.

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Introduction

Lepidoptera larvae, especially the larvae of family Noctuidae, are known as one of the most serious insect pests of field and horticultural crops in the world (Matthews, 1999; Valencia-Jiménez *et al.*, 2008). They can attack various parts of host plants and cause heavy losses in crop production (Valencia-Jiménez *et al.*, 2008). One of the species of the mentioned family, the cotton bollworm, *Helicoverpa armigera* (Hübner), has been known as a major agricultural pest in Africa, Asia, Australia and Europe (Reed, 1965; Hackett and Gatehouse, 1982; Zalucki *et al.*, 1986; Farrow and Daly, 1987). The larvae of this species are polyphagous and attack different crops including cotton, chickpea, maize, tomato, tobacco and many other host plants (Fitt, 1989; Naseri *et al.*, 2011). High fecundity and potential to develop resistance against insecticides make this insect to be an important pest of the widely grown and economically important crops worldwide (Fitt, 1989; Fathipour and Naseri, 2011). Although the application of chemical pesticides is often effective to control insect pests (Warner *et al.*, 1990), many populations of *H. armigera* have evolved resistance to most synthetic chemical pesticides (Wu *et al.*, 1997). Thereby, the necessity and demand for finding other safe and easy available options of pest control have incremented recently. As a tool of pest management, therefore, developing host plant resistance is not only advantageous to the environment, but also decreases management costs for farmers (Li *et al.*, 2004).

Nowadays, the knowledge of physiology and biochemistry of the insect midgut enzymes and feeding adaptation is essential for the development of new management techniques such as using digestive enzymes inhibitors and transgenic plants against herbivorous insects (Razavi Tabatabaei *et al.*, 2011). Insect midgut plays a main role in digestive enzymes secretion and the absorption of digested substances, and all of these processes are significantly influenced by its pH (Terra and Ferreira, 1994). Since there is a considerable difference among the properties of insect digestive enzymes, hence, it is necessary to gain more information about the gut enzymes activity of insects to devise a reasonable control method using plant-proteinaceous inhibitors of insect proteinases and amylases (Wilhite *et al.*, 2000).

Some proteins in seeds and vegetative organs of host plants may affect the key gut digestive enzymes of insects including amylases and proteinases (Biggs and McGregor, 1996). Since in insects, the abundance and activity of α-amylases (α-1, 4-glucan-4-glucanohydrolases, EC 3.2.1.1) are dependent on food sources, many of lepidopteran insects living on a polysaccharide rich diet, require digestive α-amylase for starch digestion (Valencia-Jiménez *et al.*, 2008). Also, phytophagous lepidopteran larvae use serine proteases (trypsins, chymotrypsins and elastases) as their main digestive enzymes (Terra *et al.*, 1996). It was known that these enzymes could be engineered into transgenic crops and used as control strategies against lepidopteran pests (Gatehouse and Gatehouse, 1998).

Several studies have recently been done about the effect of different host plants on the activity of digestive enzymes and feeding responses of *H. armigera* larvae (Kotkar *et al.*, 2009; Naseri *et al.*, 2010; Hemmati *et al.*, 2011a, b; Fallahnejad-Mojarrad *et al.*, 2011). Since the food quality and quantity, particularly protein/carbohydrate ratio, can influence the feeding rate of insects larvae (Bede *et al.*, 2007), and there isn't any information concerning the influence of maize hybrids on these characteristics of *H. armigera*, we considered nutritional response (including larval weight, food consumption and feces weight) of *H. armigera* fifth instar larvae on different maize hybrids. Furthermore, the midgut proteolytic and amylolytic activities of the fifth instar *H. armigera* larvae were assessed because appropriate quality and quantity of food compounds are essential to the reproductive prosperity of specialized phytophagous insects and it is only ensured by secretion of a set of specific enzymes (Broadway, 1989). Thus, the goal of this study was to compare the impact of five commercial maize hybrids on the activity of two key midgut enzymes as well as feeding responses of *H. armigera* in order to develop a comprehensive pest management program of this pest on maize.

Materials and methods

Chemicals: All enzyme substrates, Bradford reagent, maltose and dinitrosalicylic acid (DNS) were obtained from Sigma Chemical Co., St Louis, USA. Bovine serum albumin (BSA) was obtained from Roche Co., Germany, and

ascorbic acid, sorbic acid, methyl-p-hydroxy benzoate and formaldehyde were purchased from Merck Co., Germany.

Host plants: Seeds of different maize hybrids including SC260, SC500, SC700, SC704 and DC370 were provided from the Plant and Seed Improvement Research Institute (Karaj, Iran).

Artificial diet preparation: There were five treatment diets, each with different maize hybrids. Hybrid seeds were used to prepare artificial diets according to the methods described by Teakle (1991). The artificial diet was prepared in two parts. Part 1: powdered maize seed (250 g), wheat germ (30 g), yeast (35 g), sorbic acid (1.1 g), ascorbic acid (3.5 g), sunflower oil (5 mL), methyl-p-hydroxy benzoate (2.2 g), formaldehyde 37% (2.5 mL) and distilled water (350 mL). Part 2: agar (14 g) and distilled water (300 mL). Briefly, agar was dissolved in boiling water, cooled to about 70°C, and then mixed with part 1 ingredients. Diets were dispensed to plastic containers (10 cm diameter × 5 cm height) and refrigerated until use.

Rearing of *H. armigera*: The eggs of *H. armigera* were acquired from a laboratory colony kept on a cowpea-based artificial diet at Tarbiat Modares University (Tehran, Iran). Five separate colonies were maintained for two generations on artificial diets prepared by the seeds of five maize hybrids before being used in the experiments. The young larvae until third instar were simultaneously kept and grown in plastic containers (16.5 cm diameter × 7.5 cm height) with outlets covered by a proper mesh net for ventilation. In order to avoid cannibalism, the fourth instars to the end of larval stage were reared individually in Petri dishes (Ø 8 cm). All experiments at this stage were carried out at 25±1°C, 65±5% R.H. and a photoperiod of 16:8 (L: D) h.

Preparation of digestive enzymes: Last instar larvae (fifth instar) of *H. armigera* fed on artificial diets for 24 h were anesthetized on ice and immediately dissected under a stereoscopic microscope. Then, their midguts were cleaned from adhering unwanted tissues, and were collected into a known volume of distilled water. The homogenates were centrifuged at 16000 × g for 10 min at 4 °C and the resulting supernatants were collected into new micro tubes and stored at -20°C in aliquots for further use (Hosseininaveh *et al.*, 2007).

Proteolytic activity assay: Total protease activity present in the midgut of *H. armigera* larvae fed on different maize hybrids (for 24 h feeding) was determined using azocasein substrate in a broad pH range (pH 7-13). The universal buffer system (50 mM sodium phosphate-borate) was used to assay the optimum pH of proteolytic activity in the midgut (Elpidina *et al.*, 2001). To assess the azocaseinolytic activity, the reaction mixture containing 80 µL of 1.5% azocasein solution in 50 mM universal buffer (pH 7-13) and 50 µL of crude enzyme was incubated at 37 °C for 50 min. The reaction was terminated by the adding 100 µL of 30% trichloroacetic acid (TCA), continued by cooling at 4 °C for 30 min and centrifuging at 16000 × g for 10 min. The supernatant (100 µL) was added to 100 µL of 2M NaOH and the absorbance was read at 440 nm. Appropriate blanks, which TCA had been added prior to the substrate, were prepared for each treatment. One unit of protease activity was defined as an increase in optical density mg^{-1} protein of the tissue min^{-1} due to azocasein proteolysis (Elpidina *et al.*, 2001). All experiments were repeated 3 times.

Amylolytic activity assay: Dinitrosalicylic acid (DNS) method (Bernfeld, 1955), with 1% soluble starch as substrate at the optimum pH was used to assay the digestive amylolytic activity of *H. armigera* larvae fed on different maize hybrids (for 24 h feeding). The universal buffer system (10 mM succinate-glycine-2, morpholinoethan sulfunic acid) was used to evaluate the optimum pH of amylolytic activity in a broad pH range (pH 2–12). A quantity of 20 µL of the enzyme was incubated with 500 µL of universal buffer and 40 µL of soluble starch for 30 min at 37 °C. The reaction was stopped by addition of 100 µL of DNS and heating in boiling water for 10 min. The absorbance was read at 540 nm after cooling on ice. Unit activity was characterized as the amount of enzyme required to produce 1 mg of maltose in 30 min at 37°C under the given assay conditions. All experiments were repeated 3 times.

Protein quantification: Total protein concentrations were determined using bovine serum albumin as a standard according to Bradford (1976).

Nutritional responses of *H. armigera*: For evaluation of nutritional responses of *H. armigera*, the amount of the larval weight, food consumption, and feces produced by fifth instar larvae fed on different maize hybrids were measured

daily. The results were presented as the percentage of dry weight. To obtain the dry weights, twenty specimens of newly molted fifth instars, feces and food were collected and weighed daily, and dried at 60°C for 48 hours and then re-weighed (Waldbauer, 1968).

Statistical analysis: One-way analysis of variance (ANOVA) was applied for the data analysis using the Minitab ver. 15 software, and the means were compared by LSD.

Results and Discussion

Proteolytic and amylolytic activities of *H. armigera* at different pHs: The effect of different pH on protease and amylase activities of *H. armigera* is shown in Figures 1 and 2. Protease activity of *H. armigera* on most tested hybrids increased gradually up to pH 11. The highest value of protease activity occurred at pH 12 and the lowest value was at pH 13. Also, amylase activity was the lowest at pH 2 and increased progressively up to pH 10. The results showed maximum proteolytic and amylolytic activity of *H. armigera* in higher alkaline pH.

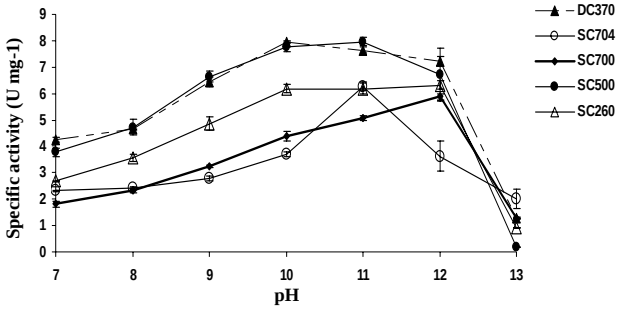

Fig. 1- Effect of different pHs on total proteolytic activity of *H. armigera* feeding on artificial diets containing powdered seeds of different maize hybrids using azocasein 1.5% as substrate

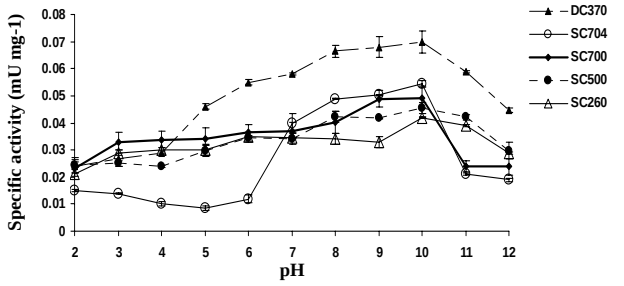

Fig. 2- Effect of different pHs on amylolytic activity of *H. armigera* feeding on artificial diets containing powdered seeds of different maize hybrids using starch 1% as substrate

Total proteolytic activity: Figure 3 represents total proteolytic activity in *H. armigera* larval midgut extracts fed on different maize hybrids until fifth instar (for 24 h feeding). The highest total proteolytic activity was in the larvae reared on DC370 (7.954±0.543 U mg^{-1}) and SC500 (7.965±0.171 U mg^{-1}), whereas the lowest one was in the larvae fed on SC704 (5.878±0.160 U mg^{-1}) (P<0.01).

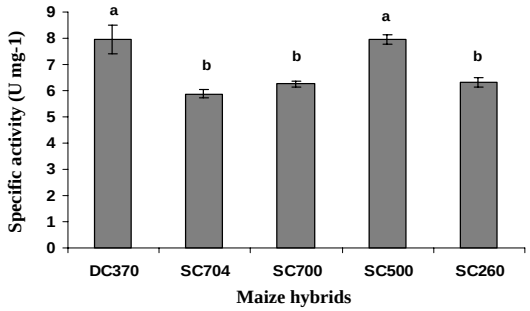

Fig. 3- Total proteolytic activity of midgut extracts from *H. armigera* fifth instar feeding on artificial diets containing powdered seeds of different maize hybrids using azocasein 1.5% as substrate. Bars represent standard error of the means. The means followed by different letters are significantly different (P<0.01, LSD).

Amylolytic activity: Amylolytic activity values of *H. armigera* larval midgut extracts fed on different maize hybrids (for 24 after feeding) are indicated in figure 4. The larvae reared on DC370 (0.055±0.001 mU mg^{-1}) and SC500 (0.047±0.007 mU mg^{-1}) showed the highest levels of amylolytic activity, the same as proteolytic activity, whereas the lowest activity was in the larvae reared on hybrid SC704 (0.012±0.001 mU mg^{-1}) (P<0.01).

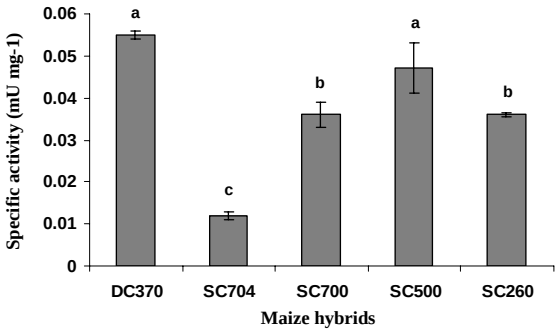

Fig. 4- Amylolytic activity of midgut extracts from *H. armigera* fifth instar feeding on artificial diets containing powdered seeds of different maize hybrids using starch 1% as substrate. Bars represent standard error of the means. The means followed by different letters are significantly different (P < 0.01, LSD).

Nutritional responses of *H. armigera*: The findings revealed significant differences regarding the weight of *H. armigera* 5th instar larvae fed on different maize hybrids diet, being the heaviest on SC704 (45.77±6.16 mg) and the lightest on DC370 (18.14±1.61 mg). Moreover, the highest and the lowest values of food consumption ($P<0.01$) were on SC500 (219.56±11.4 mg/larva) and DC370 (155.05±10.3 mg/larva), respectively. Furthermore, the highest and lowest weights of feces produced ($P<0.05$) were on SC704 (50.74±8.2 mg/larva) and SC500 (27.51±4.21 mg/larva), respectively (Figure 5).

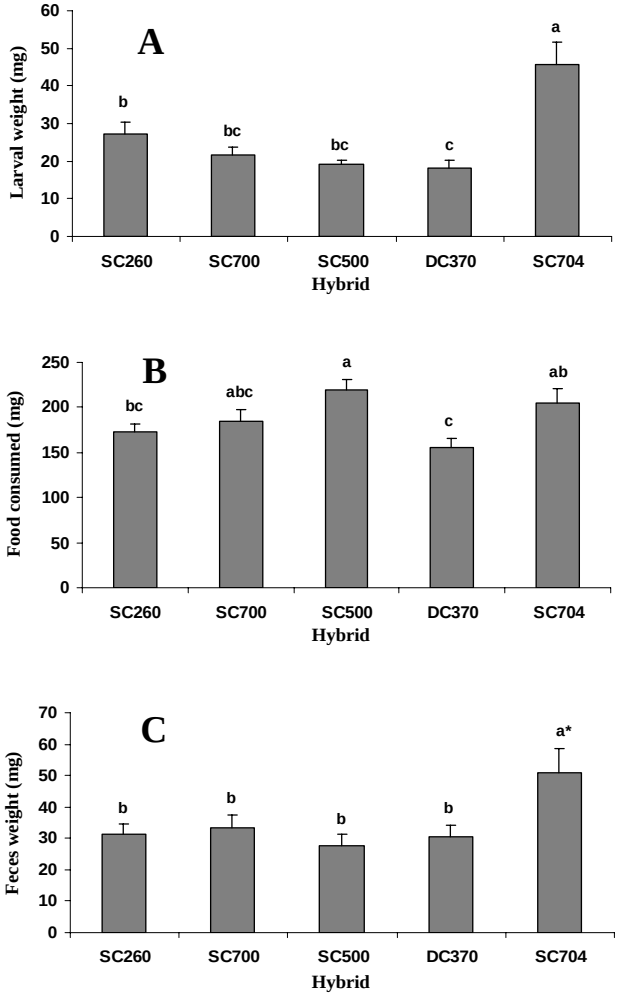

Fig. 5- Mean weight of larvae (A), food consumed (B) and feces produced (C) in *H. armigera* fifth instar feeding on artificial diets containing powdered seeds of different maize hybrids. Bars represent standard error of the means. The means followed by different letters are significantly different ($P < 0.01$, $P < 0.05^*$, LSD).

Identifying of the digestive protease and amylase activities is essential for selecting PIs[1] to expand host plant resistance against insects (Patankar *et al.*, 2001). The chronic ingestion of protease and amylase inhibitors by insects leads to inactivation of digestive enzymes consequently interfering with the bioavailability of essential amino acids. It was reported by several authors that plant PIs when delivered in artificial diets or when expressed in transgenic crops, prevent larval growth and development. Poor nutrient consumption may delay insects' development and famine can be the main reason resulting in death of these insects (Gatehouse and Gatehouse, 1999; Zhu-Salzman *et al.*, 2003).

In insects, the amount of food which is consumed and its pH have been known as a few main factors having a direct effect on the digestive enzymes activities. As the rate of all enzymes reveals a considerable dependency on specific pH, thus all enzymes have their optimum pH ranges (Murray *et al.*, 2003). This is due to the particular three-dimensional structure of enzymes which are stable at diverse pH conditions (Price and Stevens, 2000; Eisenthal and Danson, 2002; Murray *et al.*, 2003; Mohammadi *et al.*, 2010). In the present study, the optimum pH for digestive proteolytic and amylolytic activities in the fifth instar larvae of *H. armigera* was approximately similar on five maize hybrids. The optimum pH of protease activity in the midgut extracts from *H. armigera* larvae was at pH 10 to 12, which is congruent with the results previously reported for *H. armigera* larvae fed on different host plants (Naseri *et al.*, 2010; Mohammadi *et al.*, 2010; Hemmati *et al.*, 2011b). The optimum pH value of protease activity in several lepidopteran species was reported in alkaline pHs; e.g. pH 12 in *Spodoptera exigua* (Hübner) (Mohammadi *et al.*, 2010), and pH 10 in *E. ceratoniae* (Zeller) (Razavi Tabatabaei *et al.*, 2011). Therefore, it was detected that alkaline pH is optimal in lepidopteran species because of serine proteases that are dominant and more active in alkaline conditions (Nation, 2002). Also, amylase in *H. armigera* larvae reared on maize hybrids had the highest activity at pH 10. Hemmati *et al.* (2011b) and Fallahnejad-Mojarrad *et al.* (2011) have individually reported that the optimum pH of amylase in *H. armigera* larvae was in pH 9. The results recommend that

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there are no major differences in the expression levels among the digestive protease and amylase isoforms in the fifth instar larvae of *H. armigera* fed on different maize hybrids.

It could be suggested that there are some materials in some maize hybrids (DC370 and SC500) which can increase enzymes activities or can influence the production of enzymes. Also, due to additional load on the insect for energy and essential amino acids, the activity of digestive enzymes in response to ingested PIs leads to retardation of growth (Broadway and Duffy, 1986). On the other hand, variations in digestive proteolytic and amylolytic activities of *H. armigera* in response to feeding on different maize hybrids can be due to the presence of isoenzymes. The gut of insects as well as complexity of different protease specificities has a set of diverse protease isoforms. For example, the gut of *H. armigera* approximately includes twenty different types of active serine protease isoforms (Purcell *et al*., 1992).

The digestive protease activity of *H. armigera* larvae fed on SC500 and DC370 showed 1.5 to 2- fold higher activities than the larvae fed on soybean cultivars reported by Naseri *et al.* (2010). However, it was approximately similar to the proteolytic activity reported for *H. armigera* fifth instar larvae fed on white kidney bean (cultivar Dehghan) (Hemmati *et al.*, 2011b). Typically, higher protease and amylase activities in *H. armigera* larvae fed on artificial diets may be due to the high protein substance of the diet or to response of the insect to the dietary activators. Since artificial diets are complete foods designed for high insect performance and usually considered to be healthier than natural diets, several studies have shown that artificial-diet-fed *H. armigera* had higher digestive amylase activities than those fed on natural diet, and these larvae completed development rather faster than natural diet-fed larvae (Kotkar *et al.*, 2009). Artificial diet provides a favorable condition to continue rearing and preservation of insect pests (Cohen, 2001; Castane and Zapata, 2005). Determining the potential resistance in seeds of various maize hybrids (as representatives of the gene pool) by using digestive enzymatic activity and feeding responses of *H. armigera* and finding the decisive importance of the host selection and breeding would be useful in management programs of the pest.

It is reported that the nutritional necessities of an insect are absolutely associated with body biomass and the duration of immature stages (Phillipson, 1981). Therefore, as the amount of food ingested decreases, the insect becomes smaller and lighter because of development retardation (Lazarevic and Peric-Mataruga, 2003). Considering of nutritional indices of *H. armigera* on five maize hybrids (DC370, SC704, SC700, SC500, and SC260), Arghand (2011) showed that the longest larval period and development time of *H. armigera* were on SC700 and DC370. Also, they reported lower relative growth rate (RGR) value of fifth instar *H. armigera* on DC370 because of decreased food consumption or increased larval period. Therefore, the less nutritive of DC370 due to lower nutritional performance and higher digestive enzymes activity than the other hybrids tested suggested unsuitability of this hybrid for feeding *H. armigera* larvae.

An important fitness indicator of insect population dynamics is the body weight (Liu *et al*., 2004). In this research, the highest larval weight in the fifth instar larvae of *H. armigera* was on SC704 probably because of having highly nutritious food. However, the lowest larval weight of fifth instar *H. armigera* was on DC370 (18.14 ± 1.61 mg). However, Fathipour and Naseri (2011) reported that the lowest larval weight of *H. armigera* was 47.42 ± 4.18 mg on Sahar (as a resistant soybean cultivar), indicating that maize is more unsuitable host plant than soybean for feeding of *H. armigera*. Studying on nutritional indices of *H. armigera* fed on artificial diet prepared by seeds of different host plants, Baghery *et al.* (2012) reported that the corn was less nutritive than the other hosts tested, suggesting that it is unsuitable host plant for growth and development of *H. armigera*.

The results, from previous study on nutritional indices and biology of *H. armigera* (Arghand, 2011) and the findings of the present study on the digestive enzymes activities and feeding responses of the larvae fed on DC370, show that some digestive enzymes inhibitors, as antibiosis agents, probably are present in this hybrid. In conclusion, DC370 can be recommended as an unsuitable host which can affect the growth and development of *H. armigera*. Identifying the antidigestive or antifeedant compounds is essential in host plant resistance (Lewis *et al*., 1997). Therefore, the results of this study can be useful for the development of new pest management strategies such as using digestive enzymes

inhibitors and transgenic plants against *H. armigera*.

For the future, variations in the protease and amylase activities detected in *H. armigera* midgut and the flexibility of expression related to feed on different host plants/cultivars must be considered. In addition, we need to understand if all inhibitors are resistant to the attack by the insect midgut proteases and amylases. Moreover, the insect's response to the PIs should be necessarily considered for selection of the proper PIs that could be used in the transgenic expression for insect resistance. Likewise, a comprehensive molecular analysis of midgut protease and amylase isoforms upon exposure of the insect to a particular food will highlight their specific properties. It would be interesting to concentrate on protease and amylase inhibitors from wider range of maize hybrids and other host plants to design an effective defense procedure against this major economic pest. For a better understanding of the insect-plant interaction to manage *H. armigera* on various maize hybrids, more attention should be allocated to study nutritional performance and digestive enzymes activity of this pest on maize hybrids under semi-field and field conditions.

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