

Biochemical characterization of digestive proteases of *Pieris brassicae* (Lepidoptera: Pieridae) feeding on cruciferous hosts

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Abstract

Pieris brassicae is one of the most serious pests of cultivated crucifers in the world. The present experiment is intended to determine the proteolytic digestion of fifth instar larvae and the effects of four cruciferous hosts to *P. brassicae* nutritional physiology. *Brassica oleracea* var. *botrytis*, *Brassica oleracea* var. *capitata*, *Brassica oleracea* var. *italica* and *Brassica oleracea* var. *viridis* were assessed under laboratory conditions at $25 \pm 2^\circ\text{C}$, $60 \pm 5\%$ RH and a photoperiod of 16:8 (L: D) h. The maximum total proteolytic activity in the midgut extract was observed at pH 11, suggesting the presence of serine protease. The lowest rate of tryptic activity and both the highest general proteolytic ($0.11 \pm 0.01 \text{ Umg}^{-1}$) and chymotryptic activities ($0.45 \pm 0.002 \text{ Umg}^{-1}$) were observed in *B. oleracea* var. *viridis*. The proteolytic activity of *P. brassicae* on this host plant indicates that *B. oleracea* var. *viridis* is not a suitable host for the pest likely due to the presence of some secondary chemicals or proteinaceous protease inhibitors.

Keywords: *Pieris brassicae*, Digestive enzymes, Proteolytic activity, Cole crops

ویژگی‌های بیوشیمیایی پروتئازهای گوارشی پروانه سفیده بزرگ کلم (*Pieris brassicae*)

(Lepidoptera: Pieridae) با تغذیه از میزبان‌های مختلف خردلیان

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چکیده

پروانه سفیده بزرگ کلم (*Pieris brassicae*) (Lepidoptera: Pieridae) یکی از مهمترین آفات مخرب خردلیان در اغلب کشورهای جهان می‌باشد. هدف از این تحقیق، تعیین آنزیم‌های پروتئولیتیک لارو سن پنجم سفیده بزرگ کلم و تأثیر چهار رقم مختلف کلم بر فیزیولوژی تغذیه آن می‌باشد. در این تحقیق ارقام مختلف کلم شامل کلم گل، کلم برگ سفید، بروکلی و کلم برگ قرمز استفاده شد. آزمایشات در آزمایشگاه، تحت شرایط دمایی $25 \pm 2^\circ\text{C}$ ، رطوبت نسبی $60 \pm 5\%$ درصد و دوره نوری ۱۶ ساعت روشنایی و ۸ ساعت تاریکی انجام گرفت. بیشترین میزان فعالیت پروتئولیتیک عصاره روده میانی لارو در ناحیه قلیایی (برابر ۱۱) اتفاق افتاد، که بیانگر فعالیت سرین پروتئاز می‌باشد. کمترین میزان فعالیت تریپتیک در کلم قرمز بود، در حالی که بیشترین میزان فعالیت ویژه پروتئولیتیک (0.11 ± 0.01 / واحد آنزیمی / میلی گرم) و کیموتریپتیک (0.45 ± 0.002 / واحد آنزیمی / میلی گرم) در لاروهای تغذیه شده از کلم قرمز مشاهده شد، فعالیت پروتئولیتیک پروانه سفیده کلم روی میزبان‌های مذکور بیانگر نامناسب بودن کلم قرمز در میان میزبان‌های مورد مطالعه بود که دلیل آن می‌تواند احتمالاً به وجود ترکیبات شیمیایی ثانویه و یا مهارکننده های پروتئاز موجود با این رقم مرتبط باشد. **واژگان کلیدی:** پروانه سفیده بزرگ کلم، آنزیم‌های گوارشی، فعالیت پروتئاز و خردلیان

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Introduction

It has been estimated that damage from insect pests alone causes more than 40% of yield loss on vegetable crops annually (Hasan & Ansari, 2011). Pajmon (1999) listed 38

insect pests of Cole crops, of which *Pieris brassicae* L. remains one of the most destructive onethrough developing stages (seedling, vegetative, and flowering) (Bhalla & Pawar, 1977; Sachan & Gangwar, 1980; Siraj, 1999; Lal & Ram, 2004; Blatt *et al.*, 2008; Bhandari *et al.*, 2009; Zibae, 2012; Kumar *et al.*, 2015). This pest infests 83 species of food plants belonging to Cruciferae (Jainulabdeen & Prasad, 2004; Raqib, 2004). Garden nasturtium was used to feed the larvae of *P. brassicae* prolifically (Kumar *et al.*, 2015) on which a larva of *P. brassicae* consumed 74 - 80 cm² leaf area (Younas *et al.*, 2004).

Resistance to insecticides has been developed in *P. brassicae* (Sharma and Gupta, 2009). Modification of economically important crops by adding insecticidal proteins is one of the alternative methods, which can be used to control pests. The first step in designing inhibitor-transgenic crops is the understanding of biochemical characterization of the enzyme activities in the midgut of the target pest (Oppert, 2000; Oppert *et al.*, 2000; Wilhite *et al.*, 2000).

The herbivorous lepidopteran larvae feed actively on plants to gather nutritional components for their development and progression into the reproductive adult phase. Proteins serve as primary constituents in the development, growth and fecundity of insects and are digested by protease. Any disorder to digestion causes impair in insect development and fecundity of the adults. Srinivasan *et al.* (2005, 2006) reported that among proteins, serine proteases play an important role in digestion processes in lepidopteran larvae. These enzymes have been shown to have a high pH optimum of 8–11, which is consistent with the alkaline conditions in the midgut (Zibae, 2012).

The biological (Hasan & Ansari, 2010a, b; Mehrkhoh & Taheri-Sarhozaki, 2014) and nutritional indices (Mehrkhoh *et al.*, 2013) of large white butterfly, on Cole crops have been previously studied. Zibae (2012) investigated the enzyme diversity of *P. brassicae* on cabbage. Kumar *et al.* (2015) evaluated the midgut serine proteases and alternative host plant utilization in *P. brassicae*, to design sustainable pest management strategies, including transgenic approaches using genes encoding plant protease inhibitors.

The present study was conducted to identify types of proteases in the midgut of *P. brassicae* and their responses to different host plants to use the data in IPM program in order to improve the control strategies against this pest.

Material and methods

Host plants

Seeds of four Cole crops namely, *Brassica oleracea* var. *botrytis*, *Brassica oleracea* var. *capitata*, *Brassica oleracea* var. *viridis* and *Brassica oleracea* var. *italica* were grown in a research field at the Department of Crop Protection of Urmia University (Urmia, Iran).

Insect rearing and dissections

The eggs of *P. brassicae* were collected from the leaves of each host plant and kept in plastic rearing containers (14 cm width×20 cm length×8 cm height) covered with fine nylon mesh containing a piece of moist cotton for providing the ventilation and moisture, respectively. The neonate larvae were taken out and reared to their fifth instar. The experiments were conducted in growth chambers at temperature of $25\pm 2^{\circ}\text{C}$, $60 \pm 5\%$ R.H. and a photoperiod of 16: 8 h (L:D). For dissection, fifth instar larvae of *P. brassicae* were anaesthetized on ice and their midguts homogenized immediately in distilled water. The crude gut homogenate was transferred to 1.5 ml tubes and centrifuged at 13,000 rpm for 15 min at 4°C . The clear supernatant was transferred to a pre-chilled Eppendorf tube. The samples were stored at -20°C until further use.

Protein quantification

The protein concentrations of gut digestive enzymes were determined according to the Bradford's method (1976). Bovine serum albumin (BSA; Bio-Rad, biorad.com) was used as a standard (0.1, 0.4, 0.7, 1.0, 1.2, 1.6 and 1.8 mg/ml).

Midgut's pH and caseinolytic activity

General proteolytic activity in the midgut of *P. brassicae* was measured by using 2% azocasein (Sigma-Aldrich, A2765) as a substrate at broad pH range (pH 5-12) of universal buffer (50 mM sodium acetate-phosphate-borate), according to Elpidina *et al.* (2001) with slight modification. The universal buffer (90 μl), 2% azocasein solution (30 μl) and enzyme solution (15 μl) were used in reaction, which incubated in Benmary (at 37°C for 60 min), and trichloroacetic acid (TCA) 30% (30 μl) was used to stop the reaction. Precipitation was achieved by cooling at 4°C for 60 min and the reaction mixture centrifuged at 12000 ($\times g$) for 15 min. We added 50 μl of 2 M NaOH to the supernatant (100 μl) and the absorbance was recorded 440 nm. All assays were carried out in triplicate. The blank consisted of all mentioned portions except for the enzyme solution.

Specific proteolytic activity

Determining the optimum endoproteolytic activity was conducted at extent pH range of universal buffer on susceptible cultivar (*Brassica oleracea var. capitata*). The optimum pH was selected for determining of endoproteolytic activity on other host plants. Trypsin-like and chymotrypsin-like activities (as two sub-classes of serine proteases) were assayed using the concentration 1 mM of Na-benzoyl-L-arginine-p-nitroanilide hydrochloride (Sigma-Aldrich, 3279) and 1 mM of N-succinyl-alanine-alanine-proline-phenylalanine-p-nitroanilide (Sigma-Adrich, S7388) as substrates, respectively. The reaction mixture

included 85 μ l of universal buffer (50 mM, pH 5-12), 5 μ l of each mentioned substrate, and 10 μ l of the enzyme solution. All assays were carried out in triplicate along with appropriate blanks (Sarboland *et al.*, 2017).

Protease inhibitors assay

The effect of different inhibitors on proteolytic activities of the midgut was determined. The used inhibitors and their concentrations are as follows: Serine protease inhibitors, PMSF (phenylmethyl sulfonyl fluoride, 1 mM; Sigma-Aldrich, P7626); trypsin inhibitor, TLCK (N α -p-tosyl-L-lysine chloromethyl ketone, 0.1 mM; Sigma-Aldrich, T5012); chymotrypsin inhibitor, TPCK (N-tosyl-L-phenyl-alanine chloromethyl ketone, 0.1 mM; Sigma-Aldrich, T7254). To determine the effect of these compounds on enzyme activities, the enzymes were pre-incubated with the inhibitors at room temperature for 15 min, then the substrate was added before performing assays (Hosseiniaveh *et al.*, 2007).

Statistical analysis

All data were obtained from a complete randomized design and compared with one-way analysis of variance (ANOVA) followed by Tukey's test once significant differences were determined at $P \leq 0.05$ (SPSS, 2010).

Results

Midguts' pH

Optimum pH of general proteolytic activity was obtained for each host plant separately. General proteolytic activity of fifth instar larval gut extracts reared on four different Cole crops are shown in Fig. 1. Results of the study demonstrated the presence of serine proteases in the midgut of *P. brassicae* after feeding on different host plants. The optimal pH for general proteolytic activity in the midgut of *P. brassicae* was obtained over a broad alkaline pH range (pH 8 to pH 11), with maximum activity at pH 11 using azocasein (2%) as a substrate (Fig.1). In highly alkaline conditions (pH 11), over 80% of maximum activity was recorded.

Caseinolytic and endoproteolytic activity

Specific protease activity was assayed with synthetic substrates for trypsin-like (BAPNA) and chymotrypsin-like (SAAPFpNA) enzymes at different pH (5-12) on *Brassica oleracea var. capitata*. Results showed a continuous increase from pH 5 to 12. The substrates were hydrolyzed in alkaline conditions, with an activity peak recorded at pH 11, indicating presence of trypsin- and chymotrypsin-like proteinases (Fig. 2). Among the

different Cole crops ($F=17.22$; $df=3,8$; $P<0.01$), protease activity was the highest in the larvae fed on *B. oleracea* var. *viridis*, although it was not significantly different among other three host plants. A midgut serine proteinase value was estimated 0.057, 0.068, 0.11 and 0.045 $U\text{mg}^{-1}$ on *B. oleracea* var. *botrytis*, *capitata*, *viridis* and *italica*, respectively (Fig. 3a).

There was a significant difference among different host plants, in terms of trypsin ($F=20.35$; $df= 3,8$; $P<0.01$) and chymotrypsin ($F= 40.82$; $df= 3,8$; $P<0.01$) activity. Trypsin activity of larvae, which fed on *B. oleracea* var. *capitata* and *viridis*, was significantly higher than other host plants. A gut trypsin proteinase specific activity value was estimated 0.27, 0.42, 0.45 and 0.28 ($U\text{mg}^{-1}$) substrate hydrolyzed on *B. oleracea* var. *botrytis*, *capitata*, *viridis* and *italica*, respectively (Fig. 3b). Chymotrypsin activity (SAAPFpNA as substrate) was high on *B. oleracea* var. *botrytis*-fed larvae but not significantly different from larvae reared on the rest of Cole crops (Fig.3b). The chymotrypsin- like activity was estimated 0.77, 0.41, 0.62 and 0.30 ($U\text{mg}^{-1}$) on *B. oleracea* var. *botrytis*, *capitata*, *viridis* and *italica*, respectively.

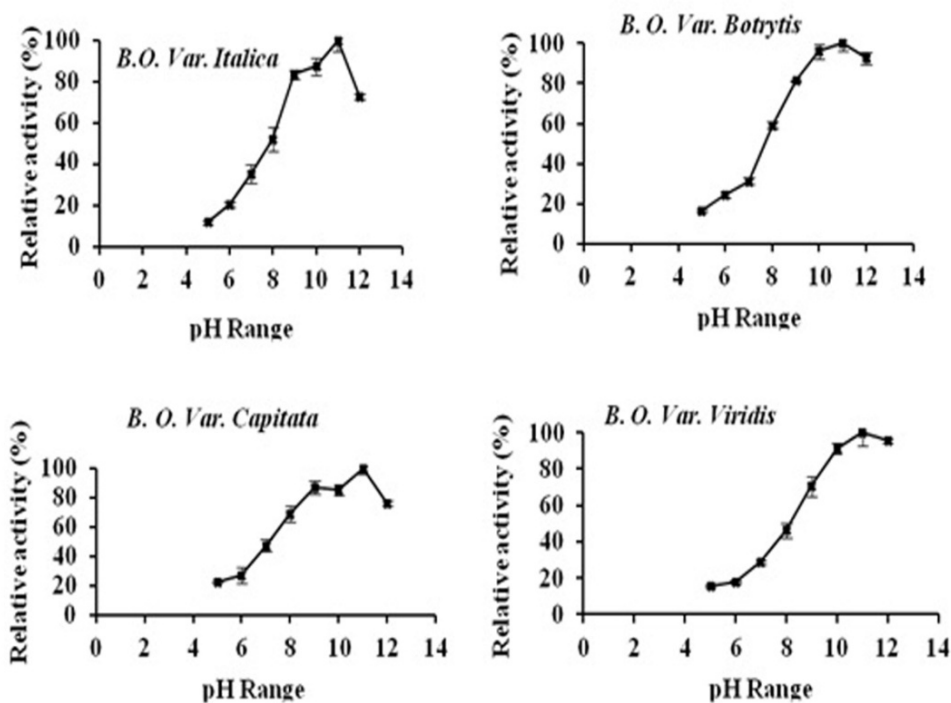


Fig. 1. Effect of pH on *P. brassicae* larval gut protease activity. Midgut extracts were assayed for total proteolytic activity by using Azocasein substrate on different of host plants. Activities are expressed as a change in Elisa reader at 440 nM/min due to hydrolysis of substrate. Each point represents mean of three independent estimations associated with standard errors.

Effect of protease inhibitors on proteolytic activity

General proteolytic activity of larval midgut extract was further characterized using general (PMSF) and specific (TLCK, TPCK) proteinase inhibitors (Table 1). Significant inhibition of protease activity by PMSF, TLCK, and TPCK suggested that serine proteinases were mainly responsible for protein digestion in the midgut. The highest inhibition by PMSF and TLCK were observed on *B. oleracea var. viridis* (Table 1).

Table 1. The effects of some protease inhibitors, on the general proteolytic activity from midgut extract of *Pieris brassicae* larvae using azocasein as substrate, on different Cole crops.

Cole crops	% Inhibition		
	PMSF (1mM)	TLCK (0.1mM)	TPCK (0.1mM)
<i>B. O. Var. Viridis</i>	29.13 ± 1.30 ^a	57.58 ± 1.39 ^a	22.38 ± 4.32 ^b
<i>B. O. Var. Capitata</i>	24.83 ± 1.29 ^{ab}	46.18 ± 1.23 ^{ab}	38.51 ± 2.20 ^b
<i>B. O. Var. Botrytis</i>	20.23 ± 1.46 ^b	23.33 ± 0.06 ^b	49.26 ± 4.55 ^a
<i>B. O. Var. Italica</i>	21.88 ± 1.76 ^b	34.41 ± 1.75 ^b	43.65 ± 3.22 ^{ab}

Means with different letters in each column are significantly different at $P < 0.01$ based on Tukey's HSD multiple range test.

Discussion

Some biochemical process including regulating enzymatic reactions related to digestion, control the solubility and toxicity of stomach poisons, and determine gut flora caused by gut pH (House, 1974; Berenbaum, 1980; Terra & Ferreira, 1994).

Screening of the pH-activity profile of digestive peptidases in the midgut homogenate of the larval *P. brassicae* was accomplished under different pH conditions by azocasein, at a wide range of pH (5-12). The results show one peak of activity, at alkaline (pH 11). It is likely due to the presence of serine protease activity. Further biochemical characterization of *P. brassicae* larval digestive proteases using general, specific substrates and synthetic inhibitors reveal that serine proteinases are dominant proteases, as is typical in other lepidopteran larvae (Johnston *et al.*, 1991; Gatehouse *et al.*, 1997; Chougule *et al.*, 2008). Similar findings have been reported for other lepidopteran species: 8-11 in *Ectomyelois ceratoniae* Zeller (Pyrilidae) (Razavi-Tabatabaei *et al.*, 2011), 12 in *Helicoverpa armigera* Hübner (Naseri *et al.*, 2010) although, Rahimi-Namin *et al.* (2011) found that, the optimal proteolytic activity in the midgut of *P. brassicae* occurs at pH 8.

Larvae of *P. brassicae* exhibit complex and diverse forms of proteolytic digestion that is influenced by their host plants. Within different Cole crops, the highest general proteolytic activity was in the larvae reared on *B. oleracea var. viridis*, likely due to the hyperproduction of proteases by midgut cells of *P. brassicae* in response to protease

inhibition by PIs. Zibae (2012) stated higher proteolytic activity in the midgut of fifth instar larvae of *P. brassicae* (36.62 U mg^{-1}) by Hemoglobine. This differences can be related to the type of general substrate (Sarboland *et al.*, 2017).

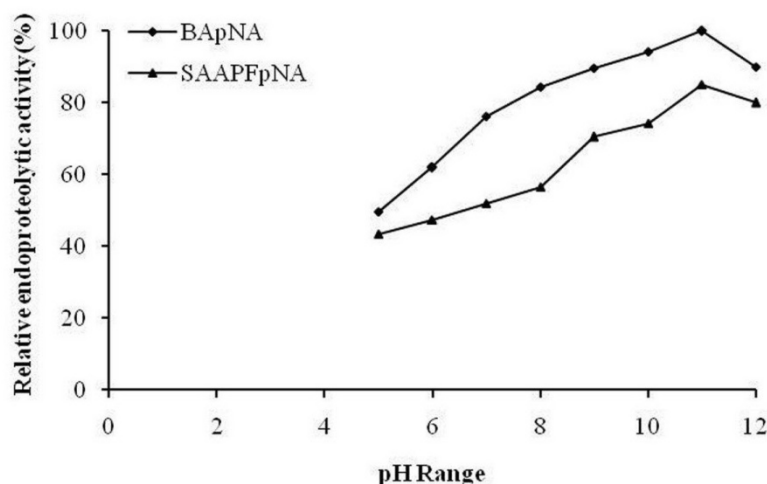


Fig. 2. Effect of pH on *P. brassicae* larval gut, which were assayed for endoproteolytic activity by using BApNA and SAAPFpNA substrates on *Brassica Oleracea. var. botrytis*. Activities are expressed as a change in Elisa reader at 405 nm/min due to hydrolysis of substrates on cauliflower. Each point represents mean of three independent estimations associated with standard errors.

The presence of trypsin-like serine proteases in the larval midgut of *P. brassicae* was clearly demonstrated by the hydrolysis of the trypsin-specific substrate BApNA and inhibition of general proteolytic activity by TLCK. Similarly, chymotrypsin-like were also demonstrated by the hydrolysis of SAAPFpNA and by the general proteolytic inhibition by the inhibitor TPCK. Within Cole crops, the trypsin activity in the larvae reared on *B. oleracea var. botrytis* and *italica* was the lowest. It can be resulted from the inhibition of tryptic activity by PIs of these two host plants that decrease the activity of trypsin-like enzymes in midgut extracts of the larvae fed on these crops. However, the larvae fed on *B. oleracea var. botrytis* had the highest chymotryptic activity compared with the other Cole crops. The biochemical analysis of *P. brassicae* larval digestive enzymes using specific substrates showed that serine proteinases (trypsin and chymotrypsin) had the highest activity (Gatehouse *et al.*, 1999; Hegedus *et al.*, 2003; Chougule *et al.*, 2008). Broadway (1989) found the highest activity of trypsin and chymotrypsin-like proteases in the midgut of *P. rapae* L.. Zibae (2012) observed higher tryptic activity compared with chymotryptic in *P. brassicae*, which fed on cabbage leaves. It has been proven that insects that are reared on different host plants, haved different digestive enzymatic responses (Saikia *et al.*, 2011).

Some protease inhibitor compounds were used for further proof of the proteolytic profile in the midgut of *P. brassicae*, which affecting serine proteases, significantly changed proteolytic activity, confirming the presence of serine proteases in the midgut extract of *P. brassicae*. PMSF, as a serine protease inhibitor, significantly decreased proteolytic activity. Although the highest general proteolytic activity was observed on *B. oleracea var. viridis*, but the lowest chymotryptic activity was possibly related to the presence of some serine protease inhibitors in this plant, resulting in hyperproduction of trypsin-like enzymes in response to the inhibition of these enzymes.

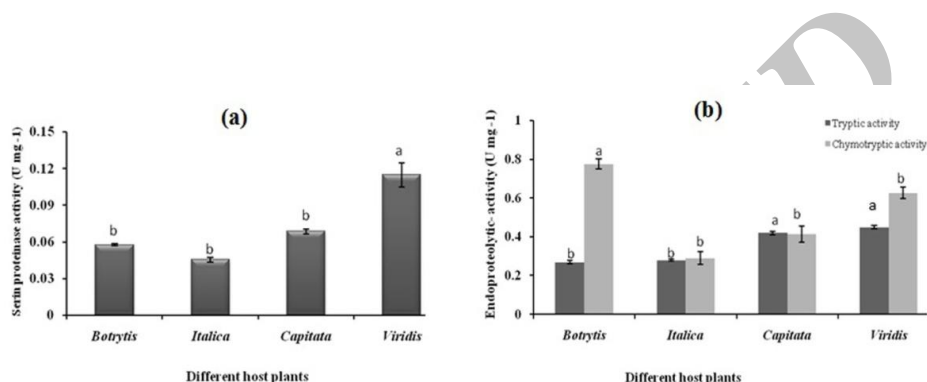


Fig. 3. Specific serin protease (a), Trypsin- (b) and chymotrypsin- -like (b) activities of midgut extracts from *Pieris brassicae* larvae reared on different brassicaceous Cole crops using Azocasin, BA_pNA and SAAPF_pNA (pH 11) as substrates respectively. Bars represent means of three independent estimations associated with standard error.

The effects of examined different Cole crops on biological, life table and nutritional indices of large white butterfly were studied by Mehrkhoh *et al.* (2013) and Mehrkhoh and Taheri- Sarhouzaki (2014). The larvae which were fed on cabbage (*B. oleracea var. capitata*) had the lowest rate of mortality and the shortest developmental time, which was consistent with Hasan & Ansari (2010 a,b) who had found the cabbage the most suitable host plant comparing to mustard, radish and cauliflower. *B. oleracea var. viridis* was found to be unsuitable because of the highest rate of mortality and the longest developmental time.

The eco-physiological parameters show that the highest enzymatic activity was observed in the larvae which were fed on *B. oleracea var. viridis*, although their biological and life table parameters were at the lowest rate. Some insects exhibit an interesting flexibility in adapting to various host plants by altering the specificities of their gut proteases in response to qualitative changes in dietary protein content, esp. when the existing proteases are ineffective and/or inefficient for digestion (Gatehouse *et al.*, 1997). Mechanisms of insect resistance to PIs include the upregulation of enzymes that degrade the PIs (Yanget *al.*, 2009), the induction of enzymes that resist inactivation by PIs

(Broadway, 1996), and overproduction of enzymes to maintain normal levels of gut proteolysis (Brioschi *et al.*, 2007).

B. oleracea var. *viridis* was the least suitable species for inflicting longer development time, high rates of mortality, and the lowest chymotryptic activity. The larvae which fed on *B. oleracea* var. *capitata* were heavier and showed higher relative growth rate, shorter developmental time, as well as higher survival rate and tryptic activity. Naseri *et al.* (2010) studied the ecophysiological aspects of *H. armigera*, on different soybean varieties and mentioned that the larvae which were reared on L17 variety of soybean had both the longest and the lowest biological and demographic parameters while their enzymatic activities were at the highest levels. They argued that the larvae had to increase enzymatic activity by hyperproduction of chymotrypsin- and elastase-like enzymes in response to the inhibition of these enzymes.

Generally, digestive enzymatic studies of insects in response to feeding on different host plants have different applications such as assessment of resistance and susceptibility of host plants (Naseri *et al.*, 2010; Saadat *et al.*, 2014; Sarboland *et al.*, 2017), identifying of resistance factor and antifeedant compounds (Lewis *et al.*, 1997), production of crops with elevated levels of endogenous resistance by plant proteinase inhibitors and glucosinolates (Ryan, 1990; Hosseiniaveh *et al.*, 2007; Velasco *et al.*, 2008).

Host plants have critical roles in the fitness of herbivorous insects; it is due to the fact that, the host components could directly affect their performance via influence on the digestive process (Chown and Nicolson, 2004). Changes of proteolytic activity of different host plants and genetically modified varieties have been reported in different insects (Chen, 2008). Ecological parameters especially population growth rate, food quality, presence of secondary components (e.g. glucosinolates) and possible inhibitors from a wide range of Cole crops are substantial for designing stable planting system.

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