PROTEASE INHIBITOR FROM THE CRUDE EXTRACT OF PLANT SEEDS AFFECTS THE DIGESTIVE PROTEASES IN *HYPHANTRIA CUNEA* (LEP.: ARCTIIDAE)

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Abstract: Proteases are one of the most important digestive enzymes in the midgut of *Hyphantria cunea* Drury. Proteases are responsible for protein digestion. In the present study, we evaluated the efficiency of some plant inhibitors on proteases in the gut of the *H. cunea*. Last instar larvae were collected from mulberry trees. The digestive system of the larvae was used as an enzyme source. The total proteolytic and trypsin activity were assessed by the hemoglobin and BApNA, respectively, as the substrate. The evaluation of the total proteolytic and trypsin activities in various pHs showed the highest relative activity at a pH of 11. Also, the inhibitory effect of inhibitors extracted from *Alhagi maurorum* Medik., *Lathyrus sativus* L., *Vicia faba* L., *Prosopis farcta* (Banks & Sol.) Eig., and *Panicum miliaceum* L. on the digestive protease of the fall webworm was measured. Protease inhibitors extracted from *A. maurorum, P. farcta* and *P. miliaceum* showed negligible inhibition but *L. sativus* was able to inhibit 34.72% and 100% of the total activity of proteolytic and trypsin, respectively. Also, the total proteolytic and trypsin activities were inhibited by the inhibitor from *V. faba,* at 22.27% and 100%, respectively. The zymogram pattern of trypsin with nitro-cellulose membranes showed 2 isoforms in the gut of *H. cunea*. The inhibitor from *L. sativus* completely inhibited both isoforms. Gel electrophoresis of proteolitytic activity revealed at least 6 isoforms the inhibitor extracted from *L. sativus*; completely inhibiting some of them. The inhibitor from *L. sativus* was purified by ammonium sulfate precipitation and gel-filtration. The molecular mass of the inhibitor was determined as 45 kDa. The highest inhibition of trypsin activity by the inhibitor from *L. sativus* occurred at a pH of 10. The stability of the inhibitor from *L. sativus* was evaluated at different pHs and temperatures. The results showed that the inhibitor from *L. sativus* was stable at a pH of 11.0, and showed 45% inhibition on trypsin activity at a pH of 11. Also, this inhibitor revealed stability up to 50°C.

Key words: *Lathyrus sativus*, *Vicia faba*, *Prosopis farcta, Panicum miliaceum,* fall web worm, protease inhibitors, trypsin inhibitor

INTRODUCTION

Protein is one of the main macromolecules in the diet of lepidopteran larvae. Herbivorous lepidopteran larva intensively feed on their hosts. In this way, the larvae obtain sufficient nutrients for their growth and reproduction. The proteases, which break down the proteins into absorbable elements (*i.e*. amino acids), are categorized as exopeptidase and endopeptidase. Exopeptidases are responsible for cleavage of one or a few amino acids from the N- or C-terminus of protein, while endopeptidases can cleave the internal peptide bonds of polypeptides. Digestion of proteins in the digestive systems of the lepidopteran larva is completed by a complex of proteases such as trypsin, chymotrypsin, elastase, aminopeptidase, and carboxypeptidase. Previous work on lepidopteran digestive protease has shown that serine proteases are the dominant protease in the midgut. Serine proteases are responsible for about 95% of total protease activities (Patankar *et al*. 2001; Srinivasan *et al*. 2006; Chougule *et al*. 2008).

Plant protease inhibitors (PPIs) are small proteins that interfere with digestive proteases. These inhibitors are defensive proteins that are in seeds or are produced in particular tissues. Plant protease inhibitors play a crucial role in a plant's defense against herbivores especially insects (Koiwa *et al*. 1997). The inhibition of protease activity by PPIs causes a reduction in the necessary amino acid contents that are fundamental for insect growth and development (De Leo *et al.* 2002; Nanasahe *et al.* 2008). Most PPIs interact with the active site of protease, and cause formation of the stable inhibitor-protease complex without any enzymatic activity (Norton 1991). Plant inhibitors show enormous diversity as well as efficiency against insect proteolytic activity (Leung *et al*. 2000). The majority of PPIs have been reported from seeds and tubers of Leguminosae, Gramineae, and Solanaceae (Connors *et al.* 2002). Previous investigations on the resistance of host plants to insects, revealed that plants containing serine protease inhibitors have the ability to defend against insects and pathogens (Ramos *et al.* 2009; Oliva and Sam-

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paio 2009; Oliva *et al.* 2010). Serine protease inhibitors have been reported from various plant species (Oliva *et al*. 2000; Haq and Khan 2003). The seeds of the Leguminosae family contain an enormous amount of proteins that can inhibit insect proteolytic activity in *in vivo* and *in vitro* conditions (Richardson 1991).

Serine proteases are the dominant digestive proteases in the gut of the lepidopteran larva. Thus, serine protease inhibitors seem to be effective against lepidopteran species (McManus *et al*. 1994; Yeh *et al.* 1997). For example, trypsin inhibitors extracted from the seeds of *Peltophorum dubium* (Spreng.) have been reported to efficiently inhibit the tryptic activity of *Anagasta kuehniella* (Zeller) (Macedo *et al*. 2003). Similarly, the crude extract taken from *Cajanus indicus* (L.) seeds has been shown to present satisfactory inhibitory effects against protease activities in some insect species (Giri 2003).

The fall webworm, *Hyphantria cunea* Drury (Lep: Arctiidae) is an economically important pest causing serious damage to forests, ornamental trees, and mulberry trees. The wide application of chemical insecticides has been the main strategy for the control of *H. cunea*. Insecticides, though, can be seriously toxic to people and animals. Pesticides can also cause side effects on non-target organisms (Talebi *et al*. 2011). An integrated pest management (IPM) program, including resistant plant varieties, would be the best option. Disruption in an insect's ability to digest protein by transgenic plants expressing PPIs is an alternative approach. Therefore, in this paper we investigated the inhibitory effects of some plant protease inhibitors on the total protease and trypsin activities of *H. cunea* to obtain the appropriate way for controlling the fall webworm pest.

MATERIALS AND METHODS

The insect

The eggs of *H. cunea* were collected from infested mulberry trees in the province of Guilan, Iran. The eggs were maintained in the optimum rearing conditions of 25±2°C, 60±10% relative humidity (RH) with a photoperiod of 16:8 (L:D). The larvae were fed fresh mulberry leaves. The last instar larvae were randomly used as an enzymatic source.

Extraction of the digestive system protease of *H. cunea*

Last instar larvae were immobilized on ice and dissected under a stereo microscope. The digestive system was separated from those tissues and fat bodies that were sticking to the digestive system samples, and moved into micro-tubes. The samples were homogenized using a hand-held glass homogenizer on ice. Homogenates were centrifuged at 15 000 × g at 4°C for 15 min. The resulting supernatants were transformed into new microtubes and stored at –20°C for the total proteolytic and tryptic assays and inhibition assays.

Protein concentration

Protein concentration was determined using the method of Lowry *et al*. (1951). Bovine serum albumin was used as the standard.

The total proteolytic and tryptic assays

Proteolytic activity was measured using hemoglobin as a substrate, based on the method of Cohen (1993). Ten µl of enzyme and 30 µl of hemoglobin (2%) were added to 90 µl of phosphate-acetate-borate buffer (40 mM; at a pH of 5.0 to a pH of 12) and incubated at 30°C. After 2 h, 30 µl of 30% trichloroacetic acid (TCA) was added to stop the enzyme activity. The sample was stored at 4°C and then centrifuged. The resulting supernatant was mixed with the proper volume of Folin-Ciocalteu 1% reagent containing Na_2CO_3 2.9%. Absorbance was determined at 630 nm using a microplate reader (Stat Fax 3200®).

Tryptic activity was measured using the chromogenic substrate, BApNA (*N*-benzoyl DL-arginine *p*-nitroanilide). Five µl of substrate (1 mM) and 10 µl of enzyme were added to 85 µl of universal buffer at a pH of 5.0 to a pH of 12 (Gholamzadeh Chitgar *et al*. 2013). The universal buffer was composed of acetate, phosphate, and sodium borate 40 mM. The absorbance increase was continuously monitored for 10 min at 405 nm with an interval time of 0.5 min, to obtain ΔA/min values. The calibration curve was constructed using different concentrations of *p*-nitrophenol for expressing trypsin activity as µmol/min/mg protein.

Extraction of inhibitors from plant seeds

Inhibitor extraction was carried out according to the method of Ferrasson *et al*. (1997), with some modification. Plants seeds were ground, and the resulting flour was mixed into sodium-acetate buffer (0.05 M; pH 4.9) and stirred at 4°C for 3 h. The suspension was then centrifuged at 9000 × g at 4°C for 30 min. The resulting supernatant was subjected to ammonium sulfate 80% (w/v) and stirred at 4°C for 2 h. The protein was precipitated. The precipitate was collected by centrifugation at 9000 × g at 4°C for 15 min, re-dissolved in one ml of the phosphate buffer (20 mM; pH 7.0), and dialysed against the 0.05 M Tris-HCl buffer, pH 8.8 at 4°C for 24 h on a stirrer. After 24 h, the suspension was centrifuged at 12 000 × g at 4°C for 5 min. The resulting supernatant was stored at –20°C for further assays.

Effect of plant inhibitors on total proteolytic and tryptic activities

To determine the inhibitory effects of plant inhibitors extracted from *Latinus sativus* L., *Vicia faba* L., *Prosopis farcta* (Banks & Sol.) Eig., *Panicum miliaceum* L., and *Alhagi maurorum* Medik. on total proteolytic activity of *H. cunea*, crude extract obtained from the digestive system of the last instar larvae was added to the inhibitor and incubated for 30 min. Then, 30 µl of hemoglobin was added and incubated at 30°C for 2 h and the activity was determined as described above. In the inhibition assay of trypsin, before starting the enzymatic reaction, 10 µl of crude extract was incubated along with 70 µl of plant inhibitors. After 30 min, 20 µl of universal buffer and 5 µl of BApNA were added. The absorbance was then determined for 10 min at 405 nm with a time interval of 0.5 min, to obtain ΔA/min values.

Effect of plant inhibitors on proteolytic and tryptic activities in gel

Electrophoretic detection of proteolytic activity was performed using 10% resolving and 4% stacking polyacrylamide gels (Garcia-Carreno 1993). To maintain the enzyme activity, non-denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used. Electrophoresis was carried out in a refrigerator at 4°C. After electrophoresis, the gel soaked in 50 mM glycine at a pH of 9 and 2.5% Triton X-100 and, was shaken for one hour. The gel was then washed with distilled water and transferred to the casein solution (1%) as the substrate. Then the gel was washed with distilled water again and stained in 0.1% Coomassie brilliant blue R-250 in a solution of 50% methanol, 10% acetic acid, and 40% water. Subsequently, the gel was destained in 20% ethanol and 10% acetic acid solution until the bands appeared. In the inhibition assays on the gel, the sample was mixed with inhibitor and incubated at 30°C for 30 min. The other process was performed as described above.

Activity staining of trypsin was carried out using an overlay technique according to the methods of Vinokorov *et al.* (2005), with some modifications. After native-PAGE electrophoresis, the gel was immersed in 50 mM Tris-HCl buffer at a pH of 9. Then, the gel was washed with distilled water and gently subjected to a nitrocellulose membrane, which had been soaked in BApNA for 40 min. The gel and nitrocellulose membrane were incubated at 37°C until faint yellow bands became visible on the membrane. The membrane was then removed, and placed for 5 min in 0.1% sodium nitrite, 0.5% ammonium sulfamate, and 0.05% N-(1-naphthyl) ethylenediamine solutions, so that the bands would appear. The effect of plant inhibitors on the tryptic activity was evaluated based on the method of Razavi Tabatabaei *et al*. (2011). The enzymes and inhibitors were briefly mixed together and incubated for 30 min. Subsequently, 20 µl of this solution and 5 µl of the sample buffer were applied on the polyacrylamide gel. The staining activity of trypsin was done as described above.

Partial purification of inhibitor from *L. sativus*

After extraction of the inhibitor, the sample was precipitated with ammonium sulfate at 80% saturation and the precipitate was segregated and dialyzed against 0.05 M Tris-HCl buffer, pH 8.8. After dialysis, the sample was loaded to a Sephadex G-100 gel filtration column equilibrated with 0.02 M phosphate buffer, pH 7.0. Elution was completed at a flow rate of 0.3 ml/min and those fractions with high absorbance at 256 nm were collected.

Effect of the purified inhibitor on tryptic activity

The inhibitory effect of purified inhibitor, taken from *L. sativus*, was assayed against *H. cunea* tryptic activity as described above.

Determination of the molecular mass of the purified inhibitor

Molecular mass of the purified trypsin inhibitor taken from *L. sativus*, was determined using SDS-PAGE.

Determination of the optimum pH of the inhibitor

Activity of the inhibitor against trypsin activity was measured at different pHs. In this assay, 15 µl of the inhibitor and 10 µl of the enzyme were mixed with 75 µl of the universal buffer (pH 3.0 to 11.0) at 37°C for 40 min. Five µl of BApNA was then added to the complex as the substrate. Absorbance was continuously recorded at 405 nm.

Effect of pH and temperature on inhibitory activity

Stability of the inhibitor activity was determined in different pHs (2–12). Twenty µl of the inhibitor and 20 µl of universal buffer with different pHs were incubated at 4°C for 120 min. After that, 10 µl of enzyme, 50 µl of buffer with an optimum pH of trypsin activity in the gut of *H. cunea*, and 5 µl BApNA were added to the complex. The absorbance was continuously recorded at 405 nm.

The stability of inhibitor activity was also determined at different temperatures (20–70°C). In this assay, 20 µl of the inhibitor was mixed with 20 µl of the universal buffer and stored at the aforementioned temperatures for 30 min. After that, 10 µl of the enzyme, 50 µl of universal buffer, and 5 µl of BApNA were added to the solution. The increase in absorbance was recorded.

RESULTS

Effect of pHs on the total proteolytic and tryptic activities

The effect of different pHs on the total activity of *H. cunea* was measured with hemoglobin as the substrate. Proteases were active in pH from 7.0 to 12.0, and the proteases reached the highest activity at pH 11.0 (Fig. 1). Also, the effect of the pHs on trypsin activity was assessed using BApNA as the substrate. Results revealed the highest activity was with alkalin pHs, and there was negligible activity in acidic conditions. Trypsin showed high activity when the pH was from 8.0 to 11.0, with optimal pH being 11.0 (Fig. 1).

Effect of inhibitors on proteolytic and tryptic activities

The inhibition of trypsin activity by inhibitors extracted from *L. sativus* and *V. faba* was studied. As shown in figure 2, *L. sativus* and *V. faba* have the ability to inhibit trypsin activity. These inhibitors can inhibit 100% of trypsin activity. The inhibitory effects of *P. farcta* and *P. miliaceum* on trypsin activity were also determined. These inhibitors showed negligible inhibitory effects on the trypsin activity in *H. cunea* compared to the inhibitors extracted from *L. sativus* and *V. faba*. So, inhibitor extracted from *L. sativus* showed the highest inhibitory effect on trypsin, whereas inhibitor taken from *P. farcta* and *P. miliaceum* had the lowest inhibition.

Evaluation of the effect of PPIs on *H. cunea* proteolytic activity showed that 34.72% and 22.27% of proteolytic activity was inhibited by inhibitors extracted from *L. sativus* and *V. faba*, respectively. Other inhibitors, extracted from *P. farcta* and *P. miliaceum* did not show any significant inhibitory activities (Fig. 3). The inhibitory activity differences between crude extract (~100%) and the purified inhibitor from *L. sativus* (34.72%) and *V. faba* (22.27%) were due to the elimination of some inhibitors during the purification procedures.

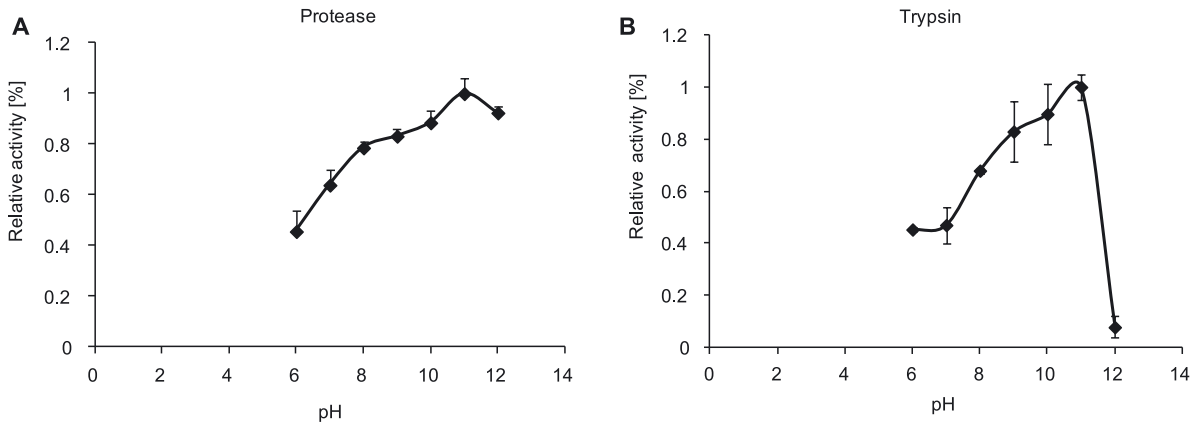

Fig. 1. Effects of varoius pHs on proteolytic (A) and trypsin (B) activities in the digestive system of *H. cunea*

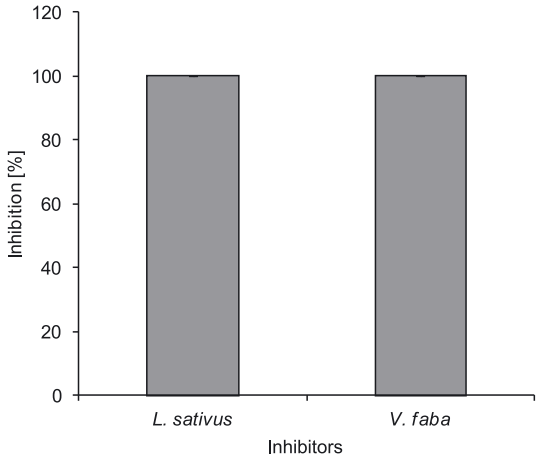

Fig. 2. Effect of plant inhibitors on the trypsin activity of *H. cunea*

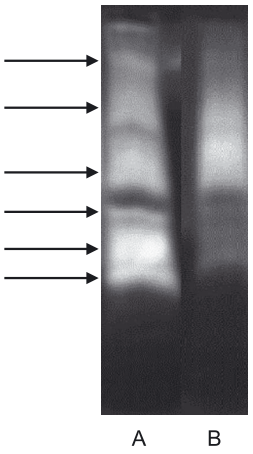

Fig. 4. The zymogram pattern of proteolytic activity (A) and the inhibitory effect of inhibitor extracted from *L. sativus* on proteolytic activity (B) in *H. cunea*

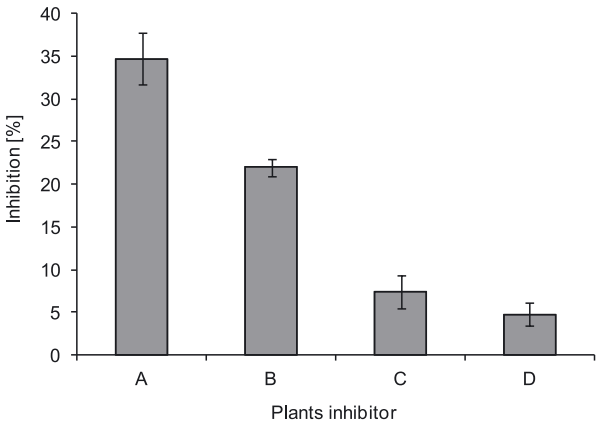

Fig. 3. Effect of inhibitors extracted from *L. sativus* (A), *V. faba* (B), *P. miliaceum* (C), and *P. farcta* (D) on the trypsin activity of *H. cunea*

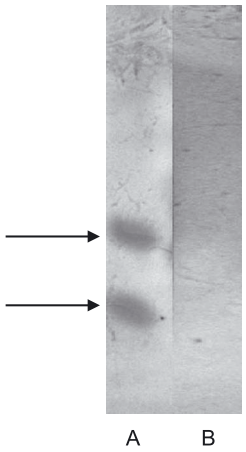

Fig. 5. The zymogram pattern of trypsin activity (A) and the inhibitory effect of inhibitor extracted from *L. sativus* on proteolytic activity (B) in *H. cunea*

Electrophoretic analysis

Electrophoretic analysis of total protease using casein as a general substrate showed at least 6 isoforms of proteases in the gut of *H. cunea* (Fig. 4A). Also, electrophoretic analysis of the inhibitory potency of inhibitors extracted from *L. sativus* on SDS-PAGE showed that some isoforms of protease disappeared due to this inhibitor (Fig. 4B). Zymogram analysis showed that trypsin has two isoforms in the digestive system of *H. cunea* (Fig. 5A). Additionally, *L. sativus* extract completely eliminated two trypsin isoforms (Fig. 5B).

Purification of inhibitor

Our results showed that fractions numbered 10 and 11 showed the highest inhibitory effect against proteases (Figs. 6–7) where they inhibited 48% and 75% of the trypsin activity of *H. cunea*, respectively (Fig. 7). So, the highest inhibitory activity was obtained in fraction number 11.

Electrophoresis of the purified inhibitor

Electrophoretic analysis of the crude and purified inhibitors on SDS-PAGE revealed 7 (Fig. 8A) and 1 bands (Fig. 8C), respectively.

Determination of molecular mass

The molecular mass of the inhibitor was determined through SDS-PAGE markers. As shown in figure 8B, the molecular weight of the trypsin inhibitor purified from *L. sativus* was determined as 45 kDa.

Optimum pH for inhibitory effect

The assay for optimum pH for inhibitory activity revealed an optimal pH of 10.0 (Fig. 9).

Stability of the inhibitor at different pHs and temperatures

Stability of the inhibitor at different pHs (2–11) was determined. The inhibitor taken from *L. sativus* has stability at a pH of 9.0 to 11.0, with maximal activity at a pH of 11. Thus, this inhibitor is stable at an extensive range of alkaline pHs (Fig. 10). Temperature stability at a broad range of temperatures (20–70°C) was also measured. Results showed that inhibitor extracted from *L. sativus* was stable up to 50°C. The inhibitory potency was decreased significantly at 70 and 80°C (Fig. 10).

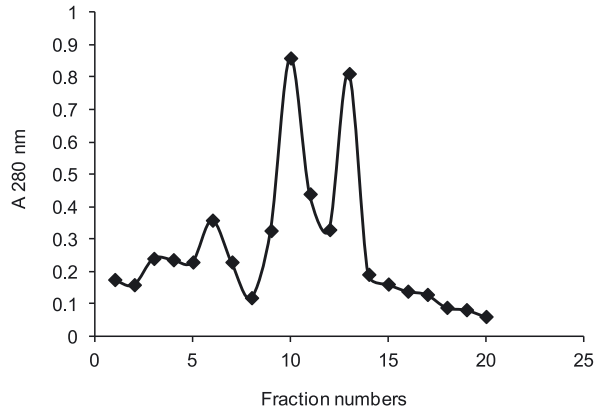

Fig. 6. Chromatogram of Sephadex G-100 gel filtration chromatography of ammonium sulfate precipitation of inhibitor from *L. sativus*

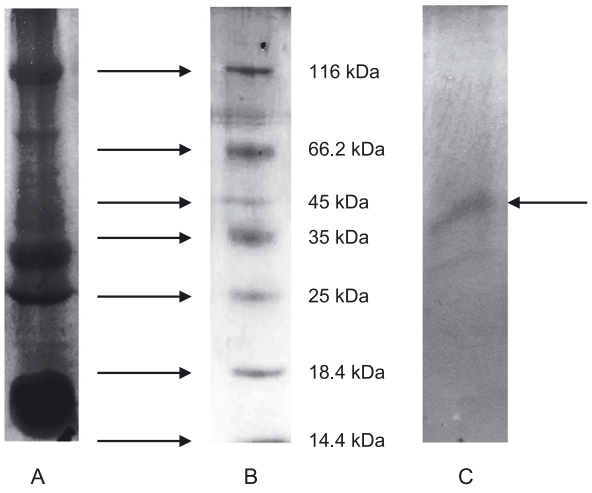

Fig. 8. Electrophoresis of inhibitor extracted from *L. sativus*: after dialyse (A), marker (B), and after gel filtration (C)

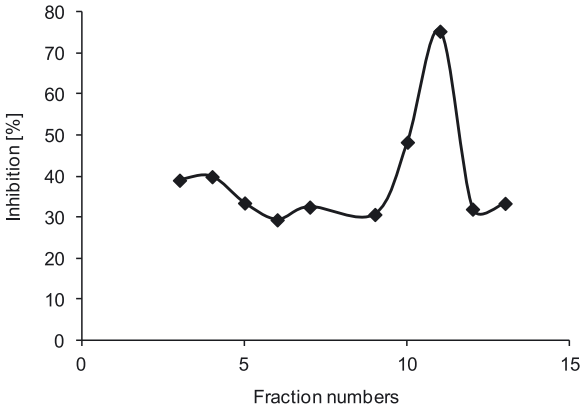

Fig. 7. Effect of purified inhibitor on the trypsin activity in *H. cunea*

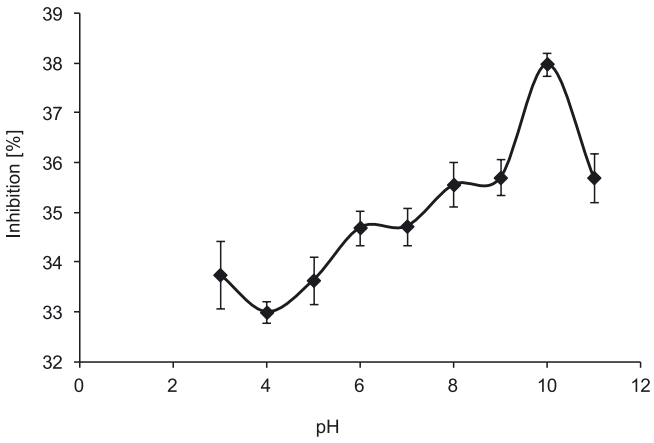

Fig. 9. Effect of pHs on inhibitory activity of inhibitor purified from *L. sativus* on trypsin in *H. cunea*

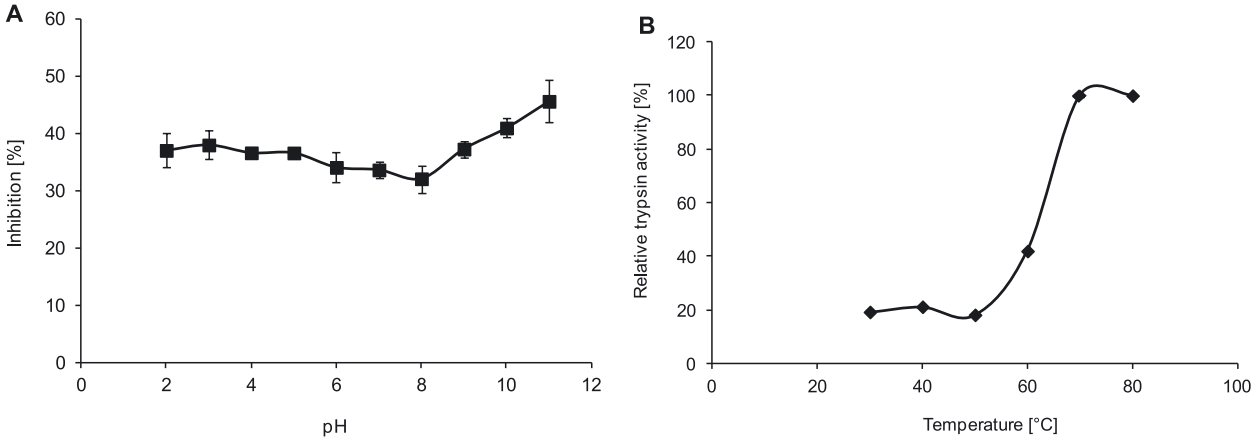

Fig. 10. pH (A) and thermal stability (B) of trypsin inhibitor purified from *L. sativus*

DISCUSSION

The results of the current study showed the presence of protease, especially trypsin proteinase, in the gut of *H. cunea*. Many studies have highlighted the serine protease as the dominant protease in the guts of the lepidopteran insect (Gatehouse *et al*. 1997; Chougule *et al*. 2008). Proteolytic activity in *H. cunea* showed the highest activity at an alkalin pH. Johnston *et al*. (1991) demonstrated that the optimal pH for digestive protease in most lepidopteran larvae occurred at an alkalin condition with a maximal pH of 10 and 11. Also, in the digestive system of *Cameraria ohridella* Deschka & Dimič (Lep.: Gracillariidae), the highest proteolytic activity was observed at a pH of 8–9.5 (Stygar *et al*. 2010). George *et al*. (2008) showed that proteases from *Busseola fusca* Fuller (Lep.: Noctuidae) were active in alkalin pH conditions with the highest activity at 9.5. Also, Chougule *et al*. (2008) demonstrated that proteases in *Mamestra brassicae* (Linnaeus) (Lep.: Noctuidae) have activity at the pH range of 7 to 12.5, with maximal activity at 11.5.

Serine proteases, like trypsin and chymotrypsin, play a main role in primary protein digestion (Srinivasan *et al*. 2006). The maximal activity of trypsin in the gut of *H. cunea* was obtained at a pH of 11.0 using BApNA as the substrate. The optimum trypsin activity in *Spodoptera littoralis* (Boisduval) was reported as 11 (Dorrah 2004). Also, trypsin from *B. fusca* showed high activity when the pH was from 11 to 11.5 (George *et al*. 2008). The most activity of trypsin in *Lacanobia oleracea* (Linnaeus) was at a pH of 10.5 (Gatehouse *et al*. 1999).

Protease inhibitors available in Leguminosae are among the most important protease inhibitors responsible for plant resistance against herbivorous pests (Kansal *et al*. 2008). Protease inhibitors have been considered as a safe alternative method against herbivorous pests, because they cause interruption in proteolytic activity and retard larval growth and development (Gatehouse 1999). The effect of inhibitor extracted from *L. sativus* and *V. faba* on the proteolytic activity of *H. cunea* showed a 34.72% and 22.27% inhibitory effect, respectively. On the other hand, inhibitors extracted from *P. farcta* and *P. miliaceum* showed a negligible inhibitory effect on the proteolytic activity of *H. cunea*. Inhibitor extracted from *L. sativus* and *V. faba* also demonstrated a significant inhibitory effect on trypsin activity. Inhibitors extracted from different chickpea varieties showed that P-256 inhibited 66% of the trypsin activity in *Helicoverpa armigera* (Hübner). Also, Prasad *et al.* (2010) revealed that 100% of the trypsin activity in *Achaea janata* (Linnaeus) was inhibited by inhibitors present in red gram (RgPI) and black gram (BgPI). Inhibitors extracted from the seeds of *Prosophis juliflora* (Sw.) showed a 45 and 83% inhibitory effect on trypsin-like activity in the digestive system of *A. janata* and *H. armigera,* respectively. Prasad *et al*. (2010) revealed that protease inhibitors extracted from *Cajanus cajan* (L.), inhibited midgut trypsin-like proteases by 21%, 14%, 12%, 10%, and 10% in lepidopteran larvae of *H. armigera*, *Spodoptera litura* (Hübner), *Papilio demoleus*, *Alabama argillacea* and *Corcyra cephalonica* (Stainton), respectively. Also, protease inhibitors taken from *Vigna mungo* (L.) inhibited 66%, 48%, 45%, 28%, and 22% of trypsin-like proteinase activities in the midgut of *P. demoleus*, *H. armigera*, *A. argillacea*, *S. litura,* and *C. cephalonica,* respectively. Plant protease inhibitors have been reported as a main control strategy for many lepidopteran insects (Yeh *et al*. 1997; Reed *et al*. 1999). Previous research showed that transgenic plants that express proteinase inhibitors can retard the growth and development of *Heliothis virescens* (Fabricius) (Hilder *et al*. 1987), *Pieris rapae* (Linnaeus) (Fang *et al.* 1997), *H. armigera* (Li *et al*. 1998), *Manduca sexta* (Linnaeus) (Johnson *et al*. 1989), and *Plutella xylostella* (Linnaeus) (De Leo *et al.* 2001).

Zymogram analysis of proteolytic activity with casein as the substrate, determined at least 6 isoforms for *H. cunea,* in which *L. sativus* eliminated some of these bands completely and the intensity of some isoforms were reduced slightly. The zymogram pattern of the SDS-PAGE showed that inhibitor purified from red gram (*C. cajan*) eliminated 2 bands from 4 bands of proteolytic activity in *A. janata* and one band from 5 bands of proteolytic activity in *H. armigera* (Prasad *et al.* 2010). Native-PAGE analysis of trypsin activity in polyacrylamide gel with nitrocellulose membrane and BApNA as the substrate, disclosed 2 isoforms of tryptic activity. Zymogram analysis of inhibitor on trypsin revealed that *L. sativus* completely eliminated 2 isoforms of trypsin activity. Previous

research on zymogram of trypsin in the digestive system of lepidopteran larva showed that there were diverse isoforms. For example, a zymogram study of *Ectomyelois ceratoniae* (Zeller) revealed that this insect has 4 isoforms of trypsin (Razavi Tabatabaei *et al.* 2011). Whereas, the zymogram pattern of *H. armigera* showed 20 isoforms of trypsin activity (Srinivasan *et al.* 2006).

Studying the effects of temperature and pH on the inhibitory potency of inhibitors was done to check the stability of the inhibitor in transgenic plants. The other target was to check the stability of the inhibitors in the insect midgut after the inhibitor is eaten by insects, and to see which pH has the biggest impact on the inhibitors. Inhibitory activity at different pHs revealed that *L. sativus* as the trypsin inhibitor was stable at alkalin conditions (pH 9.0–11). Also, trypsin inhibitor taken from pigeon pea seeds has been reported active at pHs of 7.0–10. In acidic conditions (pH 3.0–5.0), 20% of the inhibitor's activity against trypsin was lost (Godbole *et al.* 1994). Macedo *et al*. (2003) showed that inhibitory activity of inhibitor extracted from *P. dubium* was stable at different pHs against protease present in *A. kuehniella* (Lep.: Pyralidae).

The thermal stability of *L. sativus* on the inhibitory effect on trypsin activity was measured at different temperatures between 20–80°C. The results revealed that inhibitory effect on trypsin activity was stable up to 60°C, but afterwards the inhibitory activity decreased. Kansal *et al.* (2008) measured the thermal stability of the inhibitor extracted from *Cicer arietinum* L. at different temperatures. They observed that trypsin inhibitor was stable up to 80°C. Studies on the thermal stability of *P. dubium* demonstrated that 80% of inhibitory activity remained up to 80°C. The most stability of this inhibitor occurred at 50 and 60°C. Mikola and Mikkonen (1999) showed that trypsin inhibitors extracted from oat had stability in a wide range of temperatures; from 0 to 100°C. The relative stability of trypsin inhibitors was possible due to intramolecular disulfide bridges. These bridges can retain the functional stability of trypsin inhibitors in the presence of physical and chemical denaturants such as temperature, pH and reducing agents (Gomes *et al*. 2005). Our results indicated that *L. sativus* inhibitor has a high intrinsic stability due to the presence of possible disulfide bridges. The high degree of thermal stability in *L. sativus* inhibitor corresponded to other trypsin inhibitors (Souza 2000; Macedo *et al*. 2000, 2003).

The molecular mass of inhibitor purified from *L. sativus* was determined as 45 kDa. Hung *et al.* (2003) reported that the molecular mass of the inhibitor purified from seeds of *Brassica campestris* L. was 8 kDa. Also, the molecular mass of the inhibitor purified from pigeon pea was reported as 16.5 kDa (Lamate and Hivrale 2012). In addition, Giri *et al.* (2003) showed that the molecular mass of the inhibitor purified from winged bean (WBTI-1) with a high trypsin inhibitory activity, was 28 kDa. Molecular masses of trypsin inhibitor have been reported as 30 kDa in *C. arietinum*, 18.1 kDa in *Pithecellobium dumosum* (Benth), and 20 kDa in *P. dubium* (Macedo *et al.* 2003; Oliveira *et al.* 2007; Kansal *et al.* 2008).

In conclusion, our results revealed the presence of proteases, particularly trypsin proteases, in the digestive system of *H. cunea*. Results showed the optimum pH for proteases activity in *H. cunea* at alkaline conditions. Protease inhibitors extracted from seeds of *L. sativus* and *V. faba* showed a high potential for inhibiting the protease and trypsin activities in *H. cunea*. Also, an inhibitor with a molecular mass of 45 kDa showed the highest inhibitory effect on *H. cunea* tryptic activity. Contamination of the environment and an unbalance between pests and natural enemy populations are the results of the application of pesticides for controlling pests. So, the gene expressing inhibitor in *L. sativus* can be considered when making transgenic plants resistant to *H. cunea*.

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