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Effect of Inhibitors from Plant Seeds on Digestive Proteolytic Activities in Larvae of the Date Palm Fruit Stalk Borer, *Oryctes elegans* Prell (Coleoptera: Scarabaeidae)

N. Saberi Riseh[1], M. Ghadamyari[1*], V. Hosseinnaveh[2], B. Moetamedinia[3], and N. Aghaali[1]

ABSTRACT

The date palm fruit stalk borer is one of the most important pests of date palm in the world. Biochemical properties of digestive proteases in *Oryctes elegans* Prell larvae were investigated in this research and optimal total proteolytic and trypsin activities were obtained at pH 9.0 and 11.0, respectively. Activity staining of protease on SDS-PAGE showed one isoform. Also, zymogram pattern of trypsin using nitro-cellulose membrane revealed two isoforms. The inhibitory effect of PMSF, TLCK, TPCK, EDTA, iodoacetate and iodoacetamide were determined on *O. elegans* proteolytic activity. The iodoacetamide showed the highest inhibition on total proteolytic activity. Therefore, cysteine protease accounted for the major proteases in the gut of *O. elegans.* Total proteolytic activity was inhibited 22.3 and ١٢.15% by inhibitors extracted from *Vicia faba* and *Lathyrus sativus*, respectively. However, the inhibitors extracted from seeds of *Prosopis farcta*, *Panecum miliaceum,* and *Alhagi maurorum* showed negligible inhibitory effects on proteolytic activities. Trypsin activity was inhibited 91.5 and 82.3% by inhibitors extracted from *V. faba* and *L. sativus*, respectively. Electrophoretic analysis showed that inhibitors extracted from *V. faba* reduced the intensity of total proteolytic and trypsin activities. The inhibitor from *V. faba* was purified by ammonium sulfate precipitation and gel-filtration, also the molecular mass of inhibitor was determined 35 kDa. This purified inhibitor was able to inhibit trypsin activity by 72.7%. In addition, the highest inhibition of trypsin activity by inhibitor from *V. faba* occurred at pH 11.0. Also, the stability of inhibitor from *V. faba* was evaluated at different pHs and temperatures. This inhibitor was stable at pH 11.0 and 30 °C.

Keywords: Digestive protease type, Plant protease inhibitors, Trypsin.

INTRODUCTION

Palm tree is one of the most popular and widely planted crops in arid regions of North Africa and Middle East, including Iran (Murphy and Briscoe, 1999). The petiole and buds of palm trees are severely damaged by the larval stage of the date palm fruit stalk borer, *Oryctes elegans* (Coleoptera: Scarabaeidae) (Mohammadpour and Avandfaghih, 2007). The use of chemical insecticides has been traditionally developed as the only strategy for control of *O. elegans*. However, chemical control strategies seem to be impractical because the larvae feed internally and, therefore, it is not usually exposed to the applied insecticides. Additionally, with the increasing concerns related to pesticide residues in agricultural products and environment, insect resistance to

[1] Department of Plant Protection, Faculty of Agricultural Sciences, University of Guilan, Rasht, Islamic Republic of Iran.
[*] Corresponding author; e-mail: ghadamyari@guilan.ac.ir
[2] Department of Plant Protection, College of Agriculture and Natural Resources, University of Tehran, Karaj, Islamic Republic of Iran.
[3] Agricultural and Natural Resources Research Center of Southern Khorasan, Birjand, Islamic Republic of Iran.

insecticides, and adverse effects of chemical pesticides on beneficial organisms (Talebi *et al*., 2011), searching for alternative strategies have assumed greater attention in recent decades.

Plants have developed a wide range of strategies in response to invasion by their natural enemies. It has been proposed that 1 to 10% of total protein storages in plant seeds are involved in resistance against pests and pathogens (Ussuf *et al*., 2001). These proteins, known as protease inhibitors, are able to interfere with the normal growth and development of insects by inhibiting the proteolytic enzyme activity in their digestive gut (Volpicella *et al*., 2003) and, therefore, play a key role in plant defense against phytophagous insects.

As a recently developed method alternative to chemical strategies, natural or synthetic inhibitors can be artificially utilized for control of insect pests. The transgenic plants expressing protease inhibitors are believed to overcome invasion by phytophagous insects. To achieve this goal, it is essential to select appropriate inhibitors with satisfactory inhibition against the target pest, the least negative effects on non-target organisms including human, birds, and natural enemies, and the lowest rate of resistance development in target pest against the expressed inhibitor. Already, protease inhibitors extracted from seeds of a wide range of plant taxa such as Solanaceae, Gramineae, and Leguminosae have been approved to show inhibitory effects on serine proteases (Connors *et al*., 2002). In this study, the inhibitory effects of some plant protease inhibitors on total protease and trypsin activities of *O. elegans* was evaluated in order to obtain an appropriate strategy for control of this pest.

MATERIALS AND METHODS

Insects

O. elegans was collected from date palm in Saravan, Sistan and Balouchestan, Iran. Last instars larvae were selected for assay.

Larval Enzymatic Extract Preparation

Last instars larvae were immobilized by putting them on the ice, then, they were dissected under stereo microscope. Digestive systems were removed and cleaned from content, fat bodies, and malpigian tubes. Theses tissues were homogenized with hand-glass homogenizer on ice and then centrifuged at 15,000×g at 4°C for 15 minutes. The resulting supernatants was passed through the filter paper and stored at -20°C for subsequent analysis. Protein concentration in samples was measured using the method of Lowry *et al*. (1951) with bovine serum albumin as a standard.

Measurement of Total Proteolytic and Trypsin Activities

Proteolytic assay was performed by the method of Hosseininaveh *et al.* (2007) with slight modification. Hemoglobin was used as substrate. Ten µL enzyme with 30 µL of 2% hemoglobin were added to 90 µL phosphate-acetate-borate buffer (40 mM; at pH 5 to 12) and incubated at 30°C for 2 hours. After the period of incubation, 30 µL trichloroacetic acid (TCA 30%) was added to the reaction. The reaction mixture was stored at 4°C for 1 hour and then centrifuged. The supernatant was mixed with suitable volume of Folin-Ciocalteu 1% (50 µL) reagent that contained Na_2CO_3 2.9%. Absorbance was recorded at 630 nm using microplate reader (Awareness, USA, Model Stat Fax 3200®). Also, activities of proteases were measured with azocasein according to George *et al*. (2008) with some modifications by Sharifi *et al.* (2012).

Trypsin activity in *O. elegans* was measured using BApNA (*N*-benzoyl DL-arginine *p*-nitroanilide) as specific substrate. Five µL of substrate (1 mM, in DMSO) with 10 µL enzyme were added to 85 µL universal buffer (pH 5 to pH 12) in 30C° for 10 minutes. Then, absorbance was determined at 405 nm continuously for 1, 2, 5, 10, 20 and 30 minutes using microplate

reader (Awareness, USA, Model Stat Fax 3200®). The aforementioned assays were carried out in triplicate; appropriate blanks (without enzyme) were run for all assays. The relative activities were expressed as the ratio of the activity obtained at a certain pH to the maximum activity obtained at that range, and expressed as a percentage.

Extraction of Inhibitors from Seeds

The inhibitors were extracted from some Fabaceae seeds such as *Lathyrus sativus*, *Vicia faba*, *Prosopis farcta*, *Panecum miliaceum* and *Alhagi maurorum.* Extraction was performed by modified method of Ferrasson *et al*. (1997). For inhibitors extraction from seeds of plant, seeds were milled and the obtained powder was dissolved in sodium-acetate buffer (5 mM; pH 4.9) and stored at 4°C for 3 hours. Suspension was centrifuged at 9,000×g at 4°C for 30 minutes. To precipitate proteins, the resulting supernatant was saturated with ammonium sulfate (80% saturation) and stirred at 4°C for 2 hours. This solution was centrifuged at 9,000×g at 4°C for 15 minutes. The pellet was dissolved in a known volume of buffer, and dialyzed against TRIS-HCl buffer (5 mM pH 8.8) at 4°C for 24 hours on stirrer. Then, the suspension was centrifuged at 12,000×g at 4°C for 5 minutes, and the resulting supernatant was stored at -20°C for subsequence assays.

Effect of Plant Inhibitors on Total Proteolytic and Trypsin Activities

Inhibitory effect of inhibitors extracted from *L. sativus*, *V. faba*, *P. farcta*, *P. miliaceum* and *A.maurorum* were determined on total proteolytic and trypsin activities. Ten µL enzymes were added to 90 µL inhibitor and incubated for 30 minutes. After the incubation, 30 µL hemoglobin were added and incubated at 30°C for 2 hours; after that, the activity was determined as described previously.

For measurement of inhibitory effect on trypsin, 10 µL enzyme were added to 70 µL inhibitor and incubated for 30 minutes. Then, 20 µL universal buffer (with optimum pH of trypsin activity) and 5 µL BApNA (20 mM) were added. Then, absorbance was determined as described previously. The relative activities were expressed as the ratio of the activity obtained in the presence of inhibitor to the activity obtained in the control and expressed as a percentage.

Zymogram Analysis

Effect of Plant Inhibitors on Proteolytic Activity

Zymogram analysis of proteolytic enzymes was carried out using SDS polyacrylamide gel electrophoresis (4 and 10% polyacrylamide for the stacking and resolving gels, respectively) (Hosseininaveh *et al*., 2007). The samples were mixed with sample buffer and applied onto polyacrylamide gel. After electrophoresis, the gel was transferred to 50 mM glycine at pH 9 and 2.5% Triton (x-100) and was shaken. Then, the gel was washed with distilled water and soaked in casein 1% (pH 9.0) at 30ºC for 20 minutes. After that, it was stained in 0.1% Coomassie brilliant blue R-250 in a solution of 50% methanol, 10% acetic acid, and 40% water (50:10:40). Subsequently, the gel was distained in 20% ethanol and 10% acetic acid solution until the white bands appeared. For inhibition assay, the enzyme was mixed with inhibitor in the ratio of 1:3, and incubated at 30°C for 30 minutes, and then the electrophoresis was performed as described previously.

Effect of Plant Inhibitors on Trypsin Activity

Electrophoretic pattern of trypsin was performed using the methods of Vinokurov

et al. (2005). After native-PAGE electrophoresis, the gel was submerged in 50 mM Tris-HCl buffer at pH 9. Then, the nitro-cellulose membrane was immersed in BApNA for 40 minutes. Subsequently, the gel was quietly encased with a nitrocellulose membrane. Gel and a nitrocellulose membrane were incubated at 37°C until faint yellow bands became visible on the membrane. The membrane was then removed and, to make the bands appear, it was placed 5 minutes in each of 0.1% sodium nitrite, 0.5% ammonium sulfamate, and 0.05% N-(1-naphthyl) ethylenediamine solutions. Later, the membrane was scanned, when the pink bands of trypsin activity developed.

For trypsin inhibition, the enzyme was mixed with inhibitor (0.294 mg: 0.095 mg protein) and incubated for 30 min, after that, the electrophoresis was performed as described previously.

Purification and Characterization of Inhibitor from *V. faba*

The crude extract containing the inhibitor was precipitated with ammonium sulfate at 80% saturation, and the precipitate was then segregated and dialyzed against buffer Tris-HCl. After dialysis, the concentrated solution of inhibitor was passed through Sephacryl® 100-HR gel filtration column (Sigma, USA).

Effect of purified inhibitor from *V. faba* was assayed for inhibition of trypsin activity as described above. The molecular mass of the purified trypsin inhibitor was determined by SDS-PAGE with standard protein (Unstained protein molecular weight marker: Fermentas, USA).

Inhibitory activity of the purified inhibitor on trypsin at different pH values was measured. In this assay, 15 µL inhibitor and 10 µL enzyme were mixed with 75 µL universal buffer (pH 3.0 to 11.0) at 37°C for 40 minutes, and then 5 µL BApNA (20 mM) as substrate were added. Absorbance was recorded at 405 nm continuously, as detailed previously.

The stability of protease inhibitor activity was determined at different pH values (5.0-12.0). At first, 20 µL inhibitor and 20 µL universal buffer with different pHs were incubated at 4°C for 120 minutes. When the incubation was finished, 10 µL enzyme (0.29 mg protein), 50 µL buffer with optimum pH of trypsin activity and 5 µL BApNA solution (20 mM) were added. Absorbance was recorded at 405 nm continuously for 2 minutes.

Stability of inhibitor activity was determined at different temperatures (20-70°C). In this assay, 20 µL of inhibitor were mixed with 20 µL universal buffer and stored at different temperatures for 30 minutes. Afterwards, 10 µL enzyme, 50 µL universal buffer optimum pH and 5 µL BApNA were added to this solution, and the increase in absorbance was recorded. The aforementioned assays were carried out in triplicate

Effect of Synthetic Inhibitors on Proteolytic Activity

Proteolytic activities in gut extracts of *O. elegans* were assayed in the presence of specific protease inhibitors, including PMSF (5 mM); TLCK (1 mM); TPCK (1 mM); EDTA (2 mM), Iodoacetate (5 mM) and Iodoacetamide. Ten µL of different inhibitors and 15 µL of enzyme were incubated at 35°C for 15 minutes. Then, 17 µL of azocasein (1%) were added and the reaction was kept at 35C° for 90 minutes. The rest of the steps were similar to those mentioned previously in the protease assays section. All inhibition assays were carried out in three replications.

Statistical Analysis

Data were compared by one-way analysis of variance (ANOVA) followed by Tukey's test using SAS program.

RESULTS

Effect of pH on Total Proteolytic and Trypsin Activities

Total proteolytic activity at different pH revealed the high activity at alkaline pH, with maximal at pH 9.0. In the range of pH from 8.0 to 11.0, proteolytic activity was higher than 5.0 to 7.0 (Figure 1-a). Trypsin activity was determined using BApNA as substrate at different pHs. Trypsin showed the high activity at alkaline pH, with maximal at pH 11. In the range of pH from 8.0 to 11.0, trypsin activity was higher than 7.0 to 8.0 (Figure 1-b).

Effect of Crude Extract of Inhibitors on Total Proteolytic and Trypsin Activities

Effect of inhibitors extracted from seeds of plants on proteolytic activity was measured and the inhibitor extracted from *V. faba* showed higher inhibitory effect on proteolytic activity compared with the inhibitors extracted from the others. Inhibitors extracted from *P. farcta, A. maurorum* and *P. miliaceum* showed negligible inhibitory effect on proteolytic activity. The inhibitor extracted from *V. faba* and *L. sativus* reduced the total proteolytic activity of *O. elegans* by 22.3 and ١٢.15%, respectively (Figure 2-a). Also, inhibitors extracted from seeds of *L. sativus* and *V. faba* were able to inhibit the trypsin activity in *O. elegans* by 82.3 and 91.5%, respectively (Figure 2-b).

Electrophoretic Analysis

Zymogram pattern of proteolytic activity revealed one isoform of protease in the gut

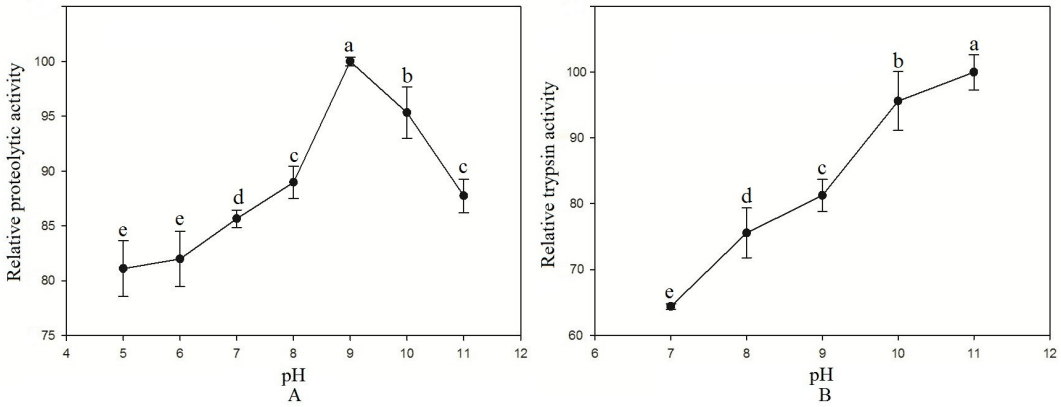


Figure 1. (A) Effects of pH on proteolytic, and (B) Trypsin activities of *O. elegans*.

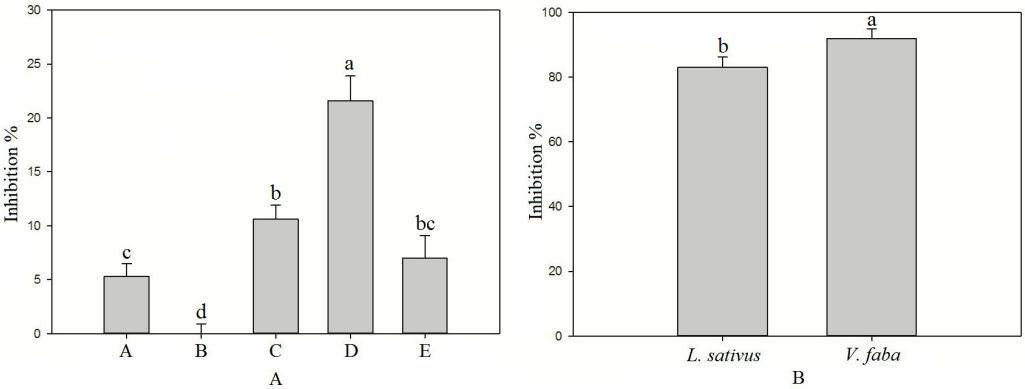
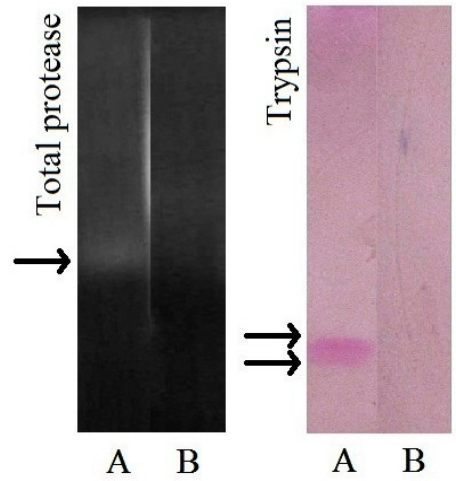
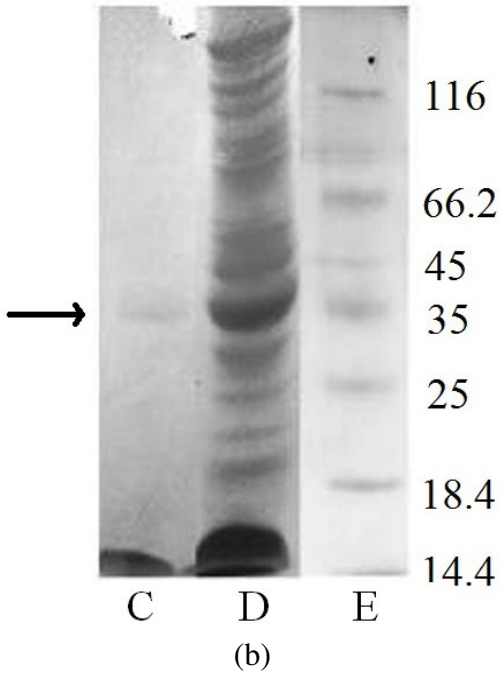


Figure 2. (A) Effect of inhibitors extracted from: (A) *P. farcta*, (B) *P. miliaceum*, (C) *L. sativus,* (D) *V. faba,* and (E) *Alhagi maurorum,* on total proteolytic activity of *O. elegans,* and (B) Effect of plant inhibitors on trypsin activity of *O. elegans* larvae gut extracts.

of *O. elegans* (Figure 3-a). Additionally, the protease band disappeared when it was treated with inhibitor extracted from *V. faba* (Figure 3-a). Trypsin zymogram was performed using BApNA as substrate, and trypsin in *O. elegans* showed two isoforms



(a)



(b)

Figure 3. (a) Zymogram pattern of proteolytic and trypsin activities in *O. elegans* (A) and inhibitory effect of inhibitor extracted from *V. faba* on proteolytic and trypsin activities (B). (b) Electrophoresis of inhibitor extracted from *V. faba*; purified inhibitor (C), crude plant extract (D), and molecular mass marker (E).

(Figure 3-a). Moreover, electrophoretic analysis of inhibitory effect of *V. faba* inhibitor on trypsin activity showed that this inhibitor was able to completely eliminate the two isoforms after treatment (Figure 3a).

Purification and Characterization of Inhibitor from *V. faba*

Fractions were emerged from Sephacryl® 100-HR column and their absorbance is presented in Figure 4-a. Fractions number 10 and 13 inhibited 57.1 and 72.7% of trypsin activity, respectively. Therefore, fraction number 13 had the highest inhibitory activity (Figure 4-b).

Electrophoretic analysis of the crude and purified inhibitor on SDS-PAGE revealed 17 (Figure 3-b (lane D)) and 1 bands (Figure 3-b (lane C)), respectively. The molecular

mass of inhibitor was determined by SDS-PAGE and protein standard (marker). As shown in Figure 3b, the molecular weight of inhibitor purified from *V. faba* seems to be 35 kDa.

Optimum pH for trypsin inhibition by inhibitor at different pHs was measured, and results showed that optimum pH for inhibitory activity occurred at pH 11.0 (Figure 5-a).

Stability of *V. faba* inhibitor at different pHs (2.0-12.0) was measured. Results showed the maximal stability of *V. faba* at pH 11.0 (Figure 5-b).

Additionally, the stability of the inhibitor in a broad range of temperatures (20-70°C) was measured. Results determined thermal stability of *V. faba* at 30 and 40°C. By continued increasing of temperature, the inhibitory activity of this inhibitor declined (Figure 5-c).

Effect of Synthetic Inhibitors on Proteolytic Activity

The effect of various commercial inhibitors on protease activity is shown in

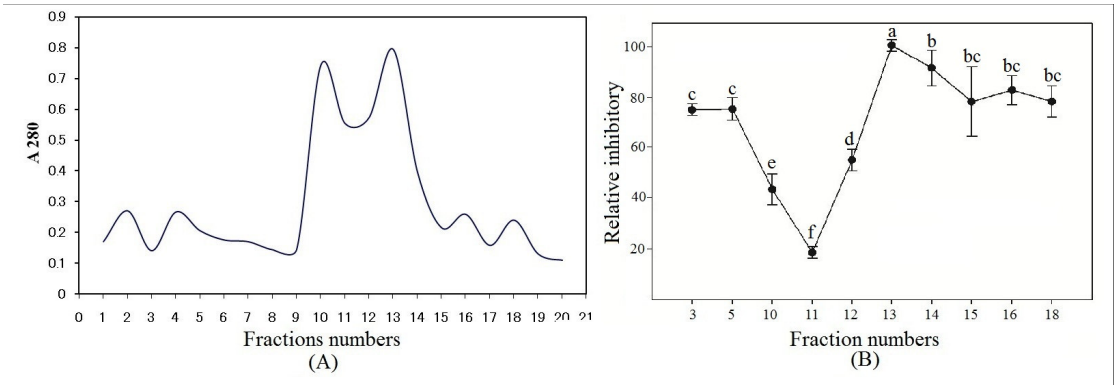


Figure 4. (A) Sephadex G-100 gel filtration chromatography of inhibitor from *V. faba* and (B) Effect of purified inhibitor from *V. faba* on trypsin activity of *O. elegans* larvae gut extracts.

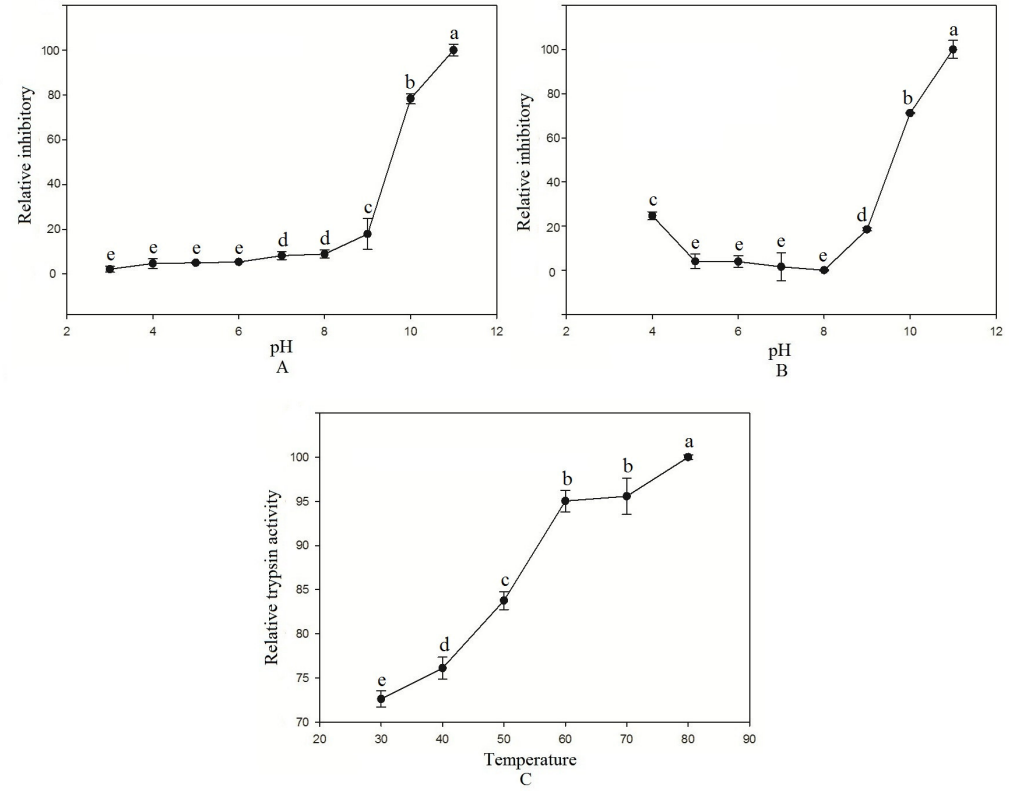


Figure 5. (A) Effect of pH on inhibitory activity of the purified inhibitor on trypsin of *O. elegans*, (B) pH stability, and (C) Thermal stability of *V. faba* purified trypsin inhibitor.

Figure 6. Results showed that iodoacetate caused a significant inhibitory effect on hydrolyzing azocasein, implying that cystein proteases play the most important roles in protein digestion in the gut of *O. elegans*. The mild inhibition by TLCK, TPCK, and PMSF showed that serine proteases comprised the main digestive enzymes in the digestive system of *O. elegans.*

DISCUSSION

Inhibition assays with synthetic inhibitors showed that metalloprotease, chymotrypsin, and cysteine proteases were present in the digestive system of *O. elegans*, with cysteine proteases being dominant. The

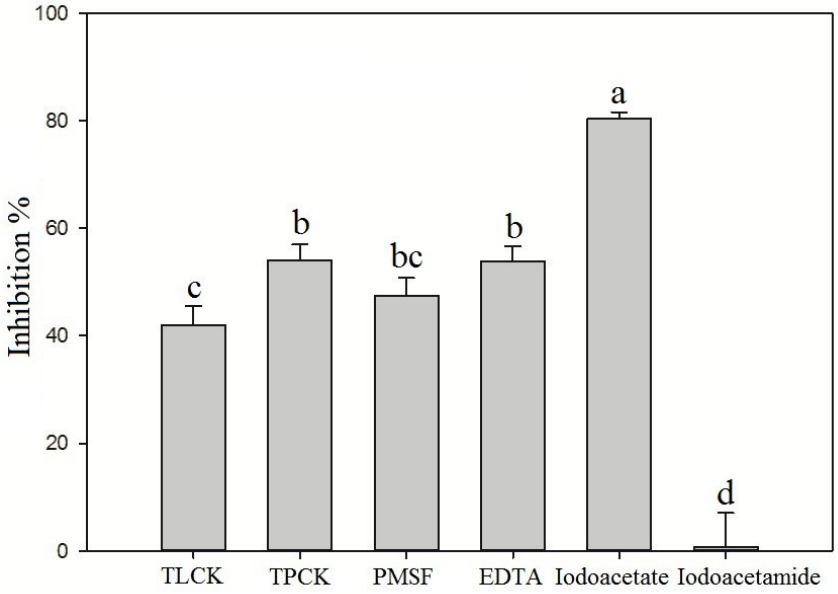


Figure 6. Effect of some commercial protease inhibitors on the total proteolytic activity of *O. elegans* larvae gut extracts.

major enzyme in coleopterans was cystein proteinases (Marshall *et al*., 2008). Nevertheless, serine and aspartic proteinases were distinguished in some coleopterans (Wolfson and Murdock, 1990). Also, Mochizuki (1998) stated that serine and cystein proteinases comprised the main digestive enzymes in Coleoptera. Although, coleopterans use aspartic and cystein proteases for long term, serine protease activity in some of them has been also observed (Sharifi *et al.,* 2012). Thus, different enzymes can act on protease which may overlap with each other (Bahagiawati *et al*., 2007). Cystein proteinases are reported in digestive system of *Tribolium castaneum* (Coleoptera: Tenebrionidae) as major proteases (Oppert *et al*., 2010). Both serine and cystein proteinases are present in digestive system of *T. molitor* for digestion of proteins and serine proteases in its posterior midgut obviously are more important for protein digestion than those in *T. castaneum* larvae (Vinokurov *et al*., 2009). Also, in *Homalinotus coriaceus* (Col.: Curculionidae) trypsin-like proteinases were the major enzyme in digestion of proteins (Macedo *et al*., 2010). Loncar *et al*. (2009) demonstrated that trypsin has over 90% activity in the foregut of *Morimun funereus* (Col.: Cerambycidae) larvae and activity of chymotrypsin and elastase in this part of digestive system is lower than trypsin. Our inhibition assay revealed that inhibition of total protease by TPCK was higher than TLCK. These results showed that the activity of chymotrypsin in digestive system of *O. elegans* was higher than trypsin. Also, trypsin and chymotrypsin were the main protease in digestion of proteins in *Dermestes maculatus* (Col.: Dermestidae), implying high activity of serine proteases in the digestive system of this insect (Caldeira *et al*., 2007). Our results showed that the main protease in digestive system of *O. elegans* was cystein protease. Hosseininaveh *et al*. (2007) also showed that proteases, especially serine proteases comprised the main digestive proteases in *Trogoderma granarium* (Col.: Dermestidae).

Data showed the maximal activity of total proteolytic and trypsin in digestive system of *O. elegans* occurred at alkaline pH. Results showed optimal proteolytic activity in *O. elegans* at pH 9. Zhang and Brune (2004) demonstrated that proteases in

Pachnoda ephippiata (Col.: Scarabaedae) had the maximal activity at pH 12. Also, Osuna-Amarillas *et al*. (2012) revealed that proteases in digestive system of *Rhyzoptera dominica* (Col.: Bostrichidae) had maximal activity at pH 10.

The optimal pH for the trypsin activity in *O. elegans* larvae were generally similar to those described previously for other coleopterans. The maximal trypsin activity in midgut of *T. granarium* was reported at pH 11 (Hosseininaveh *et al*., 2007). Also, Castro-Guillén *et al.* (2012) observed that the highest activity of trypsin in *Prostephanus truncates* (Col.: Bustrichidae) was at pH 10.5.

The inhibitors extracted from *V. faba* and *L. sativus* showed the highest inhibitory effects on trypsin activity in digestive system of *O. elegans*. Trypsin activity was inhibited 91.5 and 82.3% by inhibitors extracted from *V. faba* and *L. sativus*, respectively. Franco *et al*. (2004) revealed that protease inhibitors extracted from soybean seeds inhibited 64.7% of trypsin activity in larvae of *Anthonomus grandis* (Col.: Curculionidae). Also, protease inhibitors extracted from *Opuntia streptacantha* (Cactaceae) have significant effect on trypsin activity in *Prostephanus truncates* (Col.: Bostrichidae) and *Periplaneta americana* (Col.: Blattidae) and 85% and 75% of the trypsin activity in *P. truncates* and *P. americana* was inhibited by this inhibitor, respectively (Torres-Castillo *et al*., 2009). Macedo *et al*. (2002) revealed that inhibitors extracted from *Dimorphandra mollis* were (Fabaceae) able to inhibit 80.5% of trypsin-like activity in *Callosobruchus maculatus* (Col.: Chrysomelidae). Oliveira *et al*. (2002) extracted serine proteinases (Kunitz-type) inhibitors from seeds of *Prosopis juliflora* (Legominosae). Their results showed that trypsin activity in some bruchid species was inhibited by this inhibitor.

Zymogram pattern of proteolytic activity in *O. elegans* provided only one isoform and the inhibitor extracted from *V. faba* was able to inhibit this isoform completely. Franco *et al*. (2004) showed that one band from the isoforms of proteolytic activity was inhibited by the inhibitors extracted from seeds of soybean. George *et al*. (2008), in-gel assays (gelatin-PAGE) of *Busseola fusca* (Lepidoptera: Noctuidae), demonstrated the presence of six distinct protease forms, five of which were inhibited by SBTI. Also, trypsin in digestive system of *O. elegans* showed two isoforms in native-PAGE. Electrophoresis analysis of trypsin showed that inhibitor extracted from *V. faba* completely inhibited both isoforms. Zymogram pattern of *Helicoverpa armigera* (Lep.: Noctuidae) showed three isoforms of trypsin activity, and inhibitors extracted from soybean seeds inhibited one of them (Ghodkea *et al*., 2013).

Stability of inhibitor purified from *V. faba* was measured at different pH and the highest stability of inhibitor occurred at pH 11. Pre-incubation of the inhibitor extracted from seeds of *Crotalaria pallid* (Fabaceae) (CpaTI) at different pH (2.0–12.0) showed that trypsin inhibitor was stable in all pH values (Gomes *et al*., 2005). Also, the effect of a wide range of pH (2.0–8.0) on ability of *Terminalia arjuna* (Combretaceae) (TaTI) to inhibit trypsin showed the highest inhibitory at pH 8.0 (Rai *et al*., 2008). Stability of *V. faba* at different temperatures showed the highest stability to inhibit trypsin activity at 30-40°C and, when the temperature was increased (50-80°C), stability of the inhibitors declined. The inhibitor extracted from *T. arjuna* showed stability over a temperature range from 4 to 60°C, and a slight decrease in activity was observed from 75 to 100°C, to inhibit the trypsin activity of *Antheraea mylitta* (Lep.: Saturniidae). The ability of TaTI to retain its activity over a temperature range of 4–100°C indicated that TaTI was tolerant to extreme temperatures (Rai *et al*., 2008). Also, trypsin inhibitors extracted from Oat (Mikola and Mikkonen, 1999) and inhibitors isolated from cabbage (Broadway and Colvin, 1992) showed stability in a wide range of temperatures (0–100°C). Study of the temperature effect on *C. pallid* showed that the inhibitory activity was stable at 80°C, losing only 40% of activity at 100°C (Gomes *et al*., 2005).

In conclusion, we studied the properties of digestive proteases in *O. elegans* larvae to

provide a better understanding of the development of new method based on plant resistance to pests. We found that midgut extracts of *O. elegans* larvae have azocaseinolytic activity in neutral and alkaline conditions, suggesting that this species has a midgut based on proteases of different classes. Sensitivity to protease inhibitors confirmed the presence of trypsin, chymotrypsin, cysteinproteases, and metalloproteases. Cysteinprotease is the dominant protease in the digestive system of *O. elegans*. Plant proteinase inhibitors extracted from *V. faba* showed the highest potential for trypsin inhibition. The molecular weight of inhibitor purified from *V. faba* was 35 kDa, and this inhibitor showed the maximal stability at pH 11. Plant protease inhibitors can interfere with absorption of proteins and can retard the normal growth of insects (Lecardonnel *et al.* 1999). Therefore, these results provide useful knowledge needed for developing transgenic plants resistant to this pest via trypsin inhibitors that are available in seeds of *V. faba*.

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اثر بازدارنده‌های بذور گیاهی روی فعالیت پروتئازی لارو سوسک کرگدنی خرما *Oryctes elegans* Prell (Coleoptera: Scarabaeidae)

ن. صابری ریسه، م. قدمیاری، و. حسین نوه، ب. معتمدی نیا، و ن. آقاعلی

چکیده

سوسک کرگدنی خرما یکی از آفات مهم خرما در سرتاسر جهان است. در این تحقیق ویژگی‌های بیوشیمیایی پروتئازهای گوارشی لارو این آفت مورد بررسی قرار گرفت و فعالیت بهینه پروتئازی کل و تریپسین به ترتیب در pH ۹ و ۱۱ بود. رنگ‌آمیزی پروتئاز در SDS-PAGE، یک ایزوفرم را نشان داد. همچنین الگوی زایموگرامی تریپسین با استفاده از غشای نیتروسلولزی دو ایزوفرم را نشان داد. اثرات بازدارندگی PMSF، TLCK، TPCK، EDTA، یدولستات و یدواستامید روی فعالیت پروتئازی سوسک کرگدنی خرما بررسی شد. یدواستامید بیشترین درصد بازدارندگی را روی فعالیت پروتئولیتیک کل نشان داد. بنابراین سیستئین پروتئاز، پروتئاز عمده در لوله گوارش سوسک کرگدنی است. فعالیت پروتئولیتیک کل به وسیله بازدارنده‌های استخراج شده از *Vicia faba* و *Lathyrus sativus* به ترتیب ۲۲/۳ و ۱۲/۱۵ درصد کاهش یافت. هرچند که بازدارنده‌های استخراج شده از بذور *Prosopis farcta*، *Panecum miliaceum* و *Alhagi maurorum* بازدارندگی ناچیزی روی فعالیت پروتئازی کل نشان داد. درصد بازدارندگی تریپسین توسط *V. faba* و *L. sativus* به ترتیب برابر با ۹۱/۵ و ۸۲/۳٪ بود. آنالیز الکتروفورزی نشان داد که بازدارنده *V. faba* شدت فعالیت پروتئلیتیک کل و تریپسین را کاهش می‌دهد. بازدارنده از *V. faba* با استفاده از رسوب با سولفات آمونیوم و ژل فیلتراسیون خالص شد و وزن ملکولی آن ۳۵ کیلودالتون تخمین زده شد. این بازدارنده خالص شده قادر به مهار ۷۲/۷ درصدی فعالیت تریپسین بود. بعلاوه، بیشترین فعالیت بازدارندگی تریپسین به وسیله بازدارنده *V. faba* در pH ۱۱ مشاهده شد. همچنین پایداری بازدارنده *V. faba* در pHها و دماهای مختلف بررسی شد که این بازدارنده در pH ۱۱ و دمای ۳۰ درجه سلسیوس پایدار بود.