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Orijinal araştırma (Original article)

Inhibitory effects of barley and wheat seed protein on digestive α-amylase and general protease activity of *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae)

Arpa ve buğday tohum proteininin *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae)'nın α-amilaz sindirimi ve genel proteaz aktivitesine inhibitör etkileri

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Summary

In the current study, the effects of barley and wheat seed proteins on Colorado potato beetle, *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) certain digestive enzymes and biological parameters were investigated. Ammonium sulfate precipitation proteinaceous fractions 0-30% and 30-50% of both seeds produced the greatest inhibition on L_4 α-amylase but no significant inhibition on the protease activity. The inhibitory effects of fraction of 0-30% on the L_1, L_2, L_3, L_4 and adult's α-amylase activity of barley were 64, 53, 59, 49, and 56%, respectively, and of wheat were 70, 63, 63, 57, and 67%, respectively. While the inhibitory effects of the effective fraction of 30-50% on the L_1, L_2, L_3, L_4 and adult's α-amylase activity of barley were 45, 46, 52, 48, and 53%, respectively, and of wheat were 66, 59, 70, 56, and 68%, respectively. Type of inhibition in both cases was determined as partial mixed at the enzyme-inhibition kinetic tests. Zymograms confirmed the inhibition of insect α-amylase activity. Bioassays were conducted using four cultivars of potato leaves treated with barley extract. Weight of L_4 in Picasso and the percentage of L_4 evolution in all cultivars were reduced and the developmental durations were significantly increased at Marx and Picasso, in comparison with control. However, there was no significant effect on gut amylase activity of survived individuals.

Keywords: α-amylase, cereal, *Leptinotarsa decemlineata*, protease, seed protein

Özet

Bu çalışmada, arpa ve buğday tohum proteinlerinin, Patates Böceği, *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae)'nın sindirim enzimleri ve biyolojik parametrelerine etkileri incelenmiştir. Her iki tohumdan elde edilen proteinlerin amonyum sülfatlı % 0-30 ve % 30-50'lik çözeltileri L4 lerin α-amilaz inhibasyonu üzerinde en yüksek etkiyi yaparken proteaz aktivitesi üzerindeki etkisi önemsiz bulunmuştur. Yüzde 0-30'luk protein çözeltilerinin L1, L2, L3, L4 ve erginlerin α-amilaz aktivitesine inhibasyon etkisi arpa proteinleri için sırasıyla % 64, 53, 59, 49 ve 56, buğday proteinleri için % 70, 63, 63, 57, ve 67 olarak bulunmuştur. Bununla birlikte % 30-50'lik protein çözeltilerinin L1, L2, L3, L4 ve erginlerin α-amilaz aktivitesine inhibasyon etkisi arpa ptoteinleri için sırasıyla % 45, 46, 52, 48, ve 53, buğday proteinleri için % 66, 59, 70, 56, ve 68 olarak tespit edilmiştir. Her iki durumda da enzim-inhibasyon kinetik testinde inhibasyonun kısmı karışık inhibasyon olduğu belirlenmişitir. Zymogram çalışmaları böceğin α-amilaz aktivasyonunun inhibe olduğunu onaylamıştır. Biyolojik denemeler arpa ekstraktlarının uygulandığı dört farklı patates çeşidinin yaprakları kullanılarak yapılmıştır. Picasso çeşidinde L4'lerin ağırlığı, tüm çeşitlerde gelişme yüzdesinin kontrole göre düştüğü, Marx ve Picasso çeşitlerinde gelişme süresinin kontrol ile karşılaştırıldığında belirgin şekilde uzadığı tespit edilmiştir. Bununla birlikte yaşayan bireylerin miğde amilaz aktiviteleri üzerinde belirgin bir etki bulunmamıştır.

Anahtar sözcükler: α-amilaz, tahıl, *Leptinotarsa decemlineata*, proteaz, tohum proteini

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Introduction

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae), is the most destructive pest of solanaceous crops (Kondrak et al*.,* 2005; Lawrence et al., 2007). The maintenance of digestive tract integrity of insects has to be ensured at all costs because it is the main food entry way, otherwise, their life cycles may be interrupted. Hence, the gut is a potential target for controlling economically important crop pests (Becker-Ritt & Carlini, 2012). Proteolytic enzymes, also called proteases, catalyze the hydrolytic cleavage of specific peptide bonds in their target proteins (Carlini & Grossi-de-Sa, 2002; Habib & Fazili, 2007). Midguts of many insects contain large amounts of proteases which play a vital role in providing free amino acids essential for insect's normal growth and development (Wolfson & Murdock, 1990). Proteolysis as a key process in all living organisms must be extremely controlled; otherwise, it could be very hazardous to their natural environment (Carlini & Grossi-de-Sa, 2002). Alpha-amylases (α-1,4-glucan-4-glucanohydrolases, EC 3.2.1.1) constitute a family of endo-amylases that catalyze the hydrolysis of α-D-(1→4)-glucan linkages in starch components and other carbohydrates. This process is an important step towards transforming sugar polymers into smaller units that can be assimilated by the organism (Carlini & Grossi-de-Sa, 2002; Franco et al., 2002; Becker-Ritt & Carlini, 2012). When the action of the amylases is inhibited, nutrition of the organism is impaired causing shortness in energy (Carlini & Grossi-de-Sa, 2002).

Pest control in modern agriculture is increasingly moving away from reliance on exogenously applied pesticides, towards a more 'environmentally friendly' methods (Gatehouse et al., 2002). Plant resistance against insects is due to a set of defense mechanisms acquired by plants during evolution (Franco et al., 2002). On the other hand, one of the most important pest control mechanisms involves interaction of the enzymes with proteins that inhibit their activities. These compounds form less active or fully inactive complexes with their cognate enzymes, and are called enzyme inhibitors (Habib & Fazili, 2007). They act on key insect gut digestive hydrolases, the α-amylases and proteases. Several kinds of α-amylase and protease inhibitors, present in seeds and vegetative tissues especially in cereals, including wheat (*Triticum aestivum* L.), barley (*Hordeum vulgareum* L.), sorghum (*Sorghum bicolor* L.), rye (*Secale cereal* L.) and rice (*Oryza sativa* L.) act to regulate numbers of phytophagous insects (Franco et al*.,* 2002; Chen, 2008). The transgenic expression of insecticidal proteins such as α-amylase and protease inhibitors is also being evaluated as a potential protective strategy against insects (Macedo & Freire, 2011) and in most cases resulted in detrimental effects on insect growth and development (Chen, 2008).

Several extensive studies have been carried out to identify proteins with insecticidal properties against major economic pests (Karimi et al., 2010). The inhibitory effect of three proteinaceous inhibitors isolated from little millet (*Panicum sumatrense* Roth.) and finger millet (*Eleusine coracana* L.) (Poaceae) was tested on gut α-amylases of four stored grain and four phytophagous insect-pests (Sivakumar et al., 2006). Other studies were carried out using α-amylase inhibitors from different plant sources like beans (*Phaseolus* spp.) (Fabaceae) with inhibitory effect on α-amylase activities of *Tecia solanivora* Povolny (Lepidoptera: Gelechiidae) (Valencia-Jime´nez et al., 2008), Mungbean (*Vigna radiate* L.) (Fabaceae) on *Callosobruchus maculatus* F. (Coleoptera: Bruchidae) α-amylase activity (Wisessing et al., 2008), seeds of flamboyant (*Delonix regia* Bojer) (Fabaceae) tree on *C. maculatus*, *Anthonomus grandis* Boheman (Coleoptera: Curculionidae) and *Acanthoscelides obtectus* Say (Coleoptera: Bruchidae) α-amylase activity (Alves et al., 2009) and 24 species of Cerrado plants, against *Zabrotes subfasciatus* Bohemann (Coleoptera: Bruchidae) and *A. obtectus* α-amylase activity (Silva et al., 2009).

The purpose of this study was to investigate the effect of proteinaceous extract of the barley and wheat seeds on the α-amylase and protease activity and some biological parameters of the Colorado potato beetle.

Materials and Methods

Materials

Succinic acid disodium salt, bovine serum albumin (BSA), azocasein, ammonium persulfate for electrophoresis (APS) and dialysis bag (1 kDa cutoff, 28 mm) were purchased from Sigma (St Louis, MO, USA). Tris, phosphate buffer solution (pH: 7), 2-hydroxy-3,5-dinitrosalicylic acid (DNS), potassium sodium tartrate tetrahydrate, starch soluble, trichloroacetic acid (TCA), sodium hydroxide, ammonium sulfate, acrylamide, N,N′-methylene diacrylamide, dodecyl sulfate sodium salt (SDS), 2-morpholinoethanesulfonic acid (MES), sodium chloride, calcium chloride, phosphoric acid, glycerol, potassium iodide, iodine, coomassie brillant blue G 250, bromophenol blue, and N,N,N′,N′-tetramethyl ethylenediamine (Temed) were supplied by Merck (Darmstadt, Germany). Ethanol was from Arman Sina (Tehran, I.R.I); glysin from Scharlau (Barcelona, Spain) and Triton X-100 from Applichem (GmbH in Darmstadt, Germany). Spectrophotometric measurements were made using ELISA reader, BioTek® (Winooski, VT), ELx800.

Insect rearing

The colony of Colorado potato beetle was collected from potato farms of Ajabshir city, East Azarbayjan province, Iran. It was maintained on potato foliage cv. “Agria” at University of Tabriz, at 27±1 °C, 60±5% relative humidity, under 16:8 h (L:D) photoperiod and white fluorescent light. Insects were reared from egg hatch to adult in clear plastic dishes with fresh potato leaves.

Preparation of enzyme source

Enzyme source from guts of larvae and adults were prepared based on Bandani et al. (2009). Guts of adults, third and fourth instar larvae were excided by dissection in the ice-cold phosphate buffer solution (pH: 7). For first and second instar larvae, whole larvae were grounded according to Michaud et al. (1995). The samples were homogenized in cold distilled water (ddH_2O) and the mixtures were centrifuged at 13000 rpm for 30 min at 4 °C and stored at -20 °C before analysis. Protein concentration of the enzyme source was determined by protein assay and the protein content of the enzyme source was adjusted to 2 mg/ml for further use.

Preparation of seed protein extract

Seeds of barley (*H. vulgare* L. cv. Bahman) and wheat (*T. aestivum* L. cv. MV17) (Poaceae) were supplied by Seed and Plant Improvement Institute, Karaj, Iran. Proteinaceous seed extracts were extracted using the methods by Baker (1987), Melo et al. (1999) and Mehrabadi et al. (2010). Seed was grinded thoroughly, and 30 g was homogenized with 100 ml solution of 0.1 M NaCl for 2 h, followed by centrifugation at 10000 rpm for 30 min. Seed protein in the supernatant was extracted using a saturation of 0-30, 30-50, 50-70, 70-100% ammonium sulfate. A general fraction (0-70%) was prepared for preliminary inhibitory pH tests. After 45 min, the mixture of ammonium sulfate and precipitated proteins was centrifuged. At every fraction of the extraction, the supernatant was used for the next extraction, and the pellet was dissolved in a minimal volume of the Tris-HCl buffer (0.02 M and pH: 7). The pellet dialyzed against ddH_2O for 20 h with the dialysis water changed twice. Finally, the pellet was heated at 75 °C for 15 min to inactivate endogenous enzymes within extract and after centrifugation at 10000 rpm, before it was used as inhibitors in enzyme assays. Protein concentrations were achieved 0.6 and 1 mg/ml in barley and wheat protein extracts, respectively.

Amylase assay

The α-amylase activity was measured by the dinitrosalicylic acid (DNS) procedure (Bernfeld, 1955), using 1% soluble starch as substrate as described by Bandani et al. (2010). The enzyme source (10 µl) was incubated for 30 min at 37 °C with 65 µl universal buffer (0.02 M) containing succinic acid disodium salt, glycine and MES (pH: 5) and 25 µl soluble starch. The reaction was stopped by addition of 100 µl DNS and heated in a boiling water for 10 min. After cooling for 5 min, absorbance was read at 540 nm. Appropriate blanks (reaction without enzyme extract as control) were run for all investigations. Tests were performed in triplicate, and each of them was repeated three times.

Protease assay

General protease assay was conducted according to Elpidina et al. (2001) and Gatehouse et al. (1999), with slight modification, using 2% azocasein as substrate. The mixture contains 40 µl universal buffer (pH: 5), 10 µl enzyme source and 50 µl substrate solution. After 90 min incubation of reaction mixture at 37 °C, 100 µl of 30% trichloroacetic acid (TCA) was added, and the incubation mixture was kept at 4 °C for 30 min, centrifuged at 13000 rpm for 20 min to precipitate non-hydrolysis substrate. One hundred microliters of 1 M NaOH was added to 100 µl supernatant and the absorbance at 405 nm was recorded. Appropriate blanks (reaction without enzyme extract as control) were run for all assays. Tests were performed in triplicate, and each of them was repeated three times.

Enzyme inhibition assay

The inhibition assays followed procedure according to Mehrabadi et al. (2010). The enzyme extract was pre-incubated with proteinaceous seed extracts before addition of substrate for 30 min at 37 °C. Then the same procedure for the amylase and protease assays was conducted. In this study, pH dependence of the last instar larval gut enzymes inhibition by fraction 0-70% of two seed extracts, inhibitory activity of four proteinaceous fractions on the last instar larval α-amylase and the activity of the most effective fractions of both extracts on the developmental stages α-amylase activities were investigated. The inhibition percentage (%I) was calculated as follows;

% I=100[1−(absorbance at 540 nm experiment/absorbance at 540 nm control)]

Kinetic of enzyme inhibition

The inhibition was measured with different concentrations of substrates (mg/ml) and proteinaceous seed extracts (mg protein/ml) according to the description of Mehrabadi et al. (2010). The type of inhibition was determined by Lineweaver-Burk plot. The inhibitory constant (K_i), Michaelis constant (K_m) and total maximum velocity (V_{max}) values were determined. There were three replications.

Amylase zymogram

The visualization of amylase activity was carried out by semi-denaturing native polyacrylamide gel electrophoresis (PAGE) using the procedure described by Laemmli (1970) and Mehrabadi & Bandani (2010). Enzyme extract was incubated with inhibitors for 30 min at 37°C, and then the remaining amylase activity was determined. Electrophoresis was performed in 5 and 10% polyacrylamide for stacking and resolving gels, respectively, with a 1% starch solution as substrate, at 4 °C and a voltage of 120 V. The gel was rinsed with ddH_2O and washed by shaking gently with 1% (v/v) Triton X-100 for 15 min. Then, the gel was incubated in MES buffer (pH: 5) containing 2 mM $CaCl_2$ and 10 mM NaCl for 30 min. Consequently, after rinsing the gel with ddH_2O, it was soaked with a solution of 1.3% I_2 and 3% KI to stop the reaction and to stain the unreacted starch background. Zones of amylase activity appeared as a light band against the dark background of the gel.

Feeding trials

Newly hatched Colorado potato beetle larvae were reared on excised leaves of four potato cultivars (Marx, Picasso, Burren and Agria). Fifty newly emerged larvae were placed in aerated plastic arenas. The leaves were painted with barley proteinaceous extract containing 0.6 mg protein per ml and replaced daily throughout the experiment. The developmental period of first up to forth instar larvae (L_1-L_4) and the percentage of L_4 were recorded. Last instar larvae were weighed on the fourth day. The gut enzyme extracts were prepared as mentioned and their amylase activity was determined. Whole parameters were compared to that of insects fed with potato leaves painted with ddH_2O as control.

Estimation of protein concentration

The protein concentration of all samples was estimated according to the method of Bradford (1976), where bovine serum albumin (BSA) was used as a standard protein.

Statistical analysis

Analyses of Variance (ANOVA) were employed on the data using the MSTAT-C statistical package. Means of the three replicates were tested by Tukey's and Duncan's test for significant differences. Kinetic analysis was conducted using SigmaPlot 12.5 (Exploratory Enzyme Kinetics).

Results

in vitro analysis

pH influence on enzyme inhibition: The inhibition of *L. decemlineata* α-amylase and protease activities was pH dependent (Fig 1a and b). There were significant differences between pHs, except the inhibition of α-amylase activity with wheat seed extract. The highest inhibitory effect by both seed extracts on enzyme activities was observed at pH 6. The trend of both enzyme inhibitions in all cases was similar in pH 4-8.

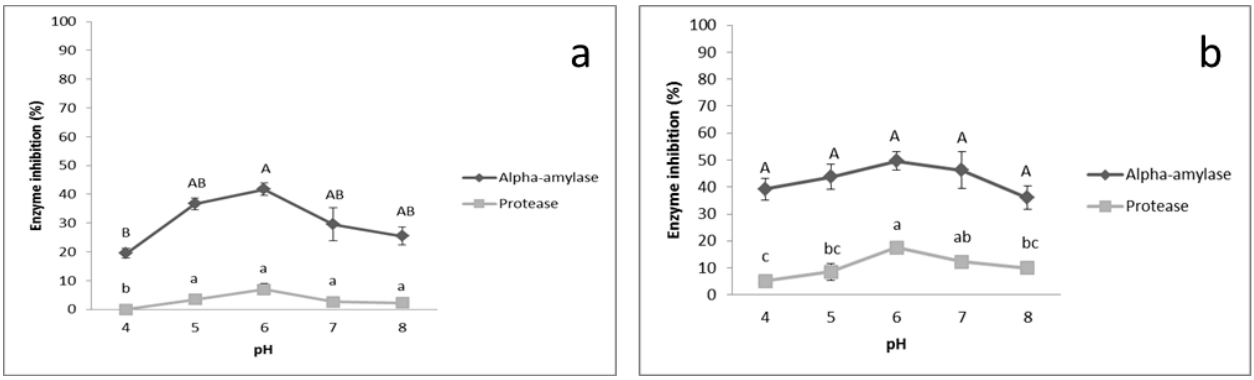

Figure 1. Influence of pH on the inhibition of the last instar larval digestive α-amylase and protease of *Leptinotarsa decemlineata* by barley (a) and wheat (b) proteinaceous seed extracts. Means followed by the different letters indicate significant differences ($p<0.05$) between data based on Tukey's test .

Enzyme inhibitory activity by proteinaceous fractions of seed extracts: Four ammonium sulfate precipitation proteinaceous fractions; 0-30, 30-50, 50-70, and 70-100% were used. These four fractions of barley seeds showed inhibitory effect with the percentage of 44, 41, 17, and 17% on the last instar larval α-amylase activity and 11, 29, 15, and 21% on the protease activity, respectively (Fig 2a). These inhibited values by wheat fractions were 50, 49, 21, and 21% on the α-amylase activity and 2, 19, 7, and 4% on the protease activity (Fig 2b). From the data, fractions from wheat seeds did not show significant inhibition on protease activity, while fractions from barley showed significant inhibition but the percentages were not substantial for further analysis. Since fractions 0-30 and 30-50% from both barley seeds and wheat produced the greatest inhibition on α-amylase activity, these fractions were used for further analysis.

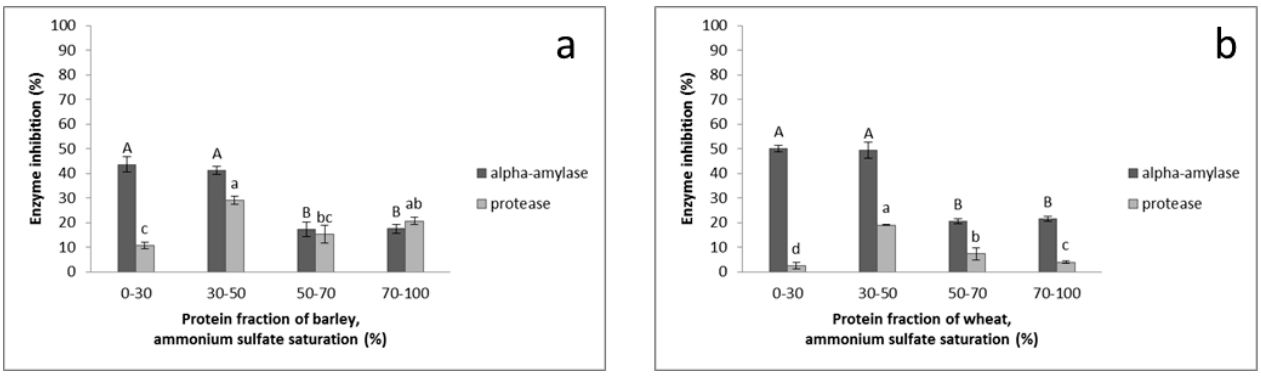

Figure 2. The effect of four ammonium sulfate precipitation proteinaceous fractions; 0-30, 30-50, 50-70, and 70-100% of barley (a) and wheat (b) on the inhibition of the last instar larval digestive α-amylase and protease of *Leptinotarsa decemlineata*. Means followed by the different letters indicate significant differences ($p<0.05$) between data based on Tukey's test .

Inhibition of α-amylase activity by effective proteinaceous fractions on CBP developmental stages: Differential inhibition of digestive α-amylase activity was seen in all developmental stages of CPB (L_1, L_2, L_3, L_4 and adults), by effective fractions of both seed extracts (Fig 3).

In barley, fraction 0-30% showed a significant inhibition on L_1 as compared to L_4 and fraction 30-50% did not show any significant differences ($p<0.05$) in inhibition of digestive α-amylase activity of all developmental stages (Fig 3a).

In wheat, the highest inhibition percentage was seen on L_1 at fraction 0-30%, and it is significant when compared to that of the L_4 (Fig 3b). A significantly high inhibitory activity of the second fraction of wheat was seen on L_3 and adults as compared to that L_2 and L_4 (Fig 3b).

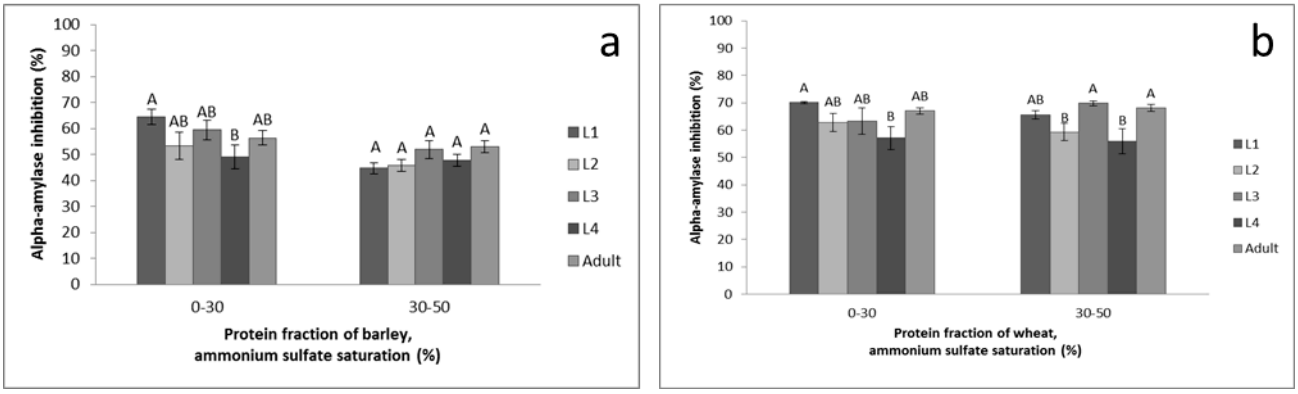

Figure 3. The inhibitory activities of effective proteinaceous fractions of barley (a) and wheat (b) on the digestive α-amylase of the first instar larvae (L1), second instar larvae (L2), third instar larvae (L3), fourth instar larvae (L4), and adults of *Leptinotarsa decemlineata*. Means followed by the different letters indicate significant differences ($p<0.05$) between data based on Tukey's test.

Kinetic of α-amylase inhibition: The detail of inhibition kinetic of the digestive α-amylase by the inhibitors was plotted using Lineweaver-Burk plots. As shown in these plots (Fig 4a and b), in the presence of inhibitors, α-amylase activity (V_{max}) was decreased with dose dependent manner; the highest velocity was recorded in the absence of inhibitors while minimum velocity was achieved in the presence of the highest dose of inhibitors. The lowest Ki value of wheat extract showed the highest potential of inhibitory activity. The interception of lines at a single point in the third quadrant ($\alpha<1$) indicates a mixed inhibition (competitive and uncompetitive inhibition) for barley (Fig 4a) and wheat extracts (Fig 4b). In both cases β value was in the interval $0<\beta<1$, that shows partial instead of full inhibition ($\beta\neq0$). These results were also supported by using "enzyme kinetics model comparison" of SigmaPlot software that compares the appropriate equations of inhibition mode by ranking R^2 and AICc (Akaike's Information Criterion), which draws median trajectory behavior plot known as direct linear plot (data not shown).

Amylase zymogram: For confirmation of the colorimetric inhibition assay results, the inhibition of incubated enzyme with first proteinaceous fraction of seeds was visualized in the semi-denaturing native PAGE (Fig 5). The gel inhibition assay confirmed that gut α-amylase of the adults was affected by the inhibitors. Gel assay using barley extract as the enzyme inhibitor showed that α-amylase activity was affected to some extent (Fig 5a). While for the wheat extract as the enzyme inhibitor, the intensity of the bands (two isozymes) were decreased completely, in comparison to the control (Fig 5b).

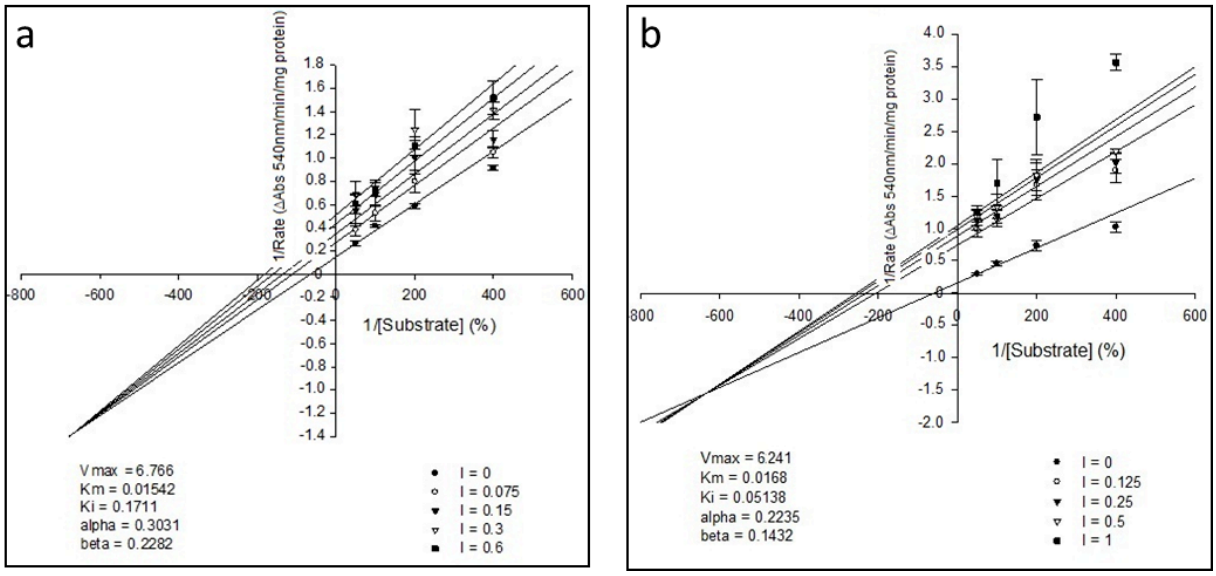

Figure 4. Lineweaver-Burk plot of *Leptinotarsa decemlineata* α-amylase in the presence of barley (a) and wheat (b) protein extracts, which give an estimation of K_i with variable concentrations of starch (0.25, 0.5, 1 and 2 %) and each crude enzyme inhibitors (I) (mg/ml).

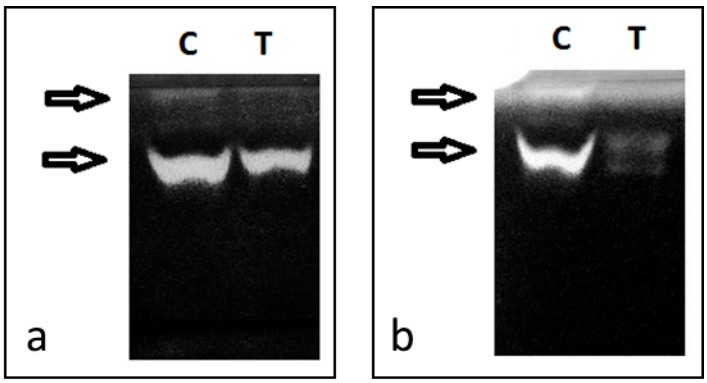

Figure 5. Gel inhibition assay of proteinaceous fraction 0-30% of barley (a) and wheat (b) on the adult's digestive α-amylase of *Leptinotarsa decemlineata*. Left columns showed controls α-amylase activity without seed extracts (C), and right columns to inhibited enzyme activity with seed extracts (T).

in vivo analysis

Diet bioassays: In order to understanding the effect of barley proteinaceous extract *in vivo*, insect feeding trials were conducted. Larvae of the Colorado potato beetle were reared from first instar on potato leaves of four cultivars (Marx, Picasso, Burren and Agria) as control and on leaves coated with extract. The developmental parameters of larvae were followed.

Weight of last instar larvae fed on treated leaves of Picasso cv. (103 mg) was lower than control (126 mg) significantly ($P<0.05$), and the L_1 to L_4 evaluation percentage in all cultivars were reduced significantly ($P<0.05$), as compared to the control (Table 1). Developmental durations up to L_4 molting (L_1 to L_4) were increased significantly when reared on Marx and Picasso ($P<0.05$), in comparison to the control. But there was no significant effect on gut α-amylase activity of survived individuals feeding on all cultivars (Table 1).

Table 1. The effects of barley proteinaceous extract on larval biological parameters and digestive α-amylase of *Leptinotarsa decemlineata* by using four different cultivars of potato leaves

Parameters	Treatment	Potato cultivars			
		Marx	Picasso	Burren	Agria
Larval weight (mg)	Control	122±5.3 ab	126±4.7 a	110.33±2.9 bc	112.33±2.3 bc
	Treated	116±4.5 abc	103.33±1.8 c	108.33±4.9 c	107.67±2.4 c
L_1 to L_4 evaluation (%)	Control	51.33±5.51 a	57.33±4.79 a	29.33±4.61 bc	42.67±4.35 ab
	Treated	16.67±1.043 cd	15.33±0.54 cd	10±1.11 d	23.33±1.61 cd
Duration up to L_4 molting (day)	Control	8.023±0.409 bc	7.333±0.086 c	8.906±0.774 abc	8.309±0.285 abc
	Treated	9.953±0.149 a	9.575±0.901 ab	9.267±0.371 ab	9.383±0.96 ab
L_4 gut α-amylase activity (U/mg protein)	Control	2.258±0.127 a	2.239±0.1 a	2.252±0.059 a	2.185±0.036 a
	Treated	2.32±0.051 a	2.248±0.121 a	2.173±0.089 a	2.223±0.057 a

Means followed by the different letters indicate significant differences ($p<0.05$) between data based on Duncan's test.

Discussion

Plants have evolved a wide array of defensive compounds, most of which are accumulate in the seed tissues constitutively or after induction, that confer resistance against phytophagous insects (Carlini & Grossi-de-Sa, 2002). Using naturally occurring plant enzyme inhibitors to target insect digestive enzymes has received serious consideration as a means of insect pest management (Zhu-Salzman et al., 2005). These inhibitors are the most extensively studied group of anti-insect chemicals (Chen, 2008).

It is considerable that *in vivo* conditions may crucially modulate α-amylase specificity. For example, the acidic optimum inhibitory pH may be responsible for their inhibition of amylases in coleoptera, whose intestinal contents are acidic (Franco et al., 2002). For this purpose, the inhibitory pH of proteinaceous extracts of barley and wheat on digestive α-amylase and protease of CPB was studied in current paper. In accordance with the optimum pH of enzymes activity, the maximum inhibitory effects of both extracts were observed at acidic pH (6 and 5). It was found that interaction between enzymes and seed extracts was also pH dependant. The accordance between gut lumen pH, amylase optimal pH, and pH optimum for amylase inhibition by plant amylase inhibitors has been described in other insect studies (Mehrabadi et al., 2010).

In preliminary tests, after achievement to optimum inhibitory pH with general fraction (0-70%) of ammonium sulfate precipitated protein of seeds, four other fractions (0-30%, 30-50%, 50-70% and 70-100%) were prepared and their inhibitory effects on both enzymes were compared. Results showed that digestive protease of the last instar larvae of CPB was less sensitive to these inhibitors, while starch hydrolysis was inhibited by fractions 0-30 and 30-50% of both seeds. When the sensitivity of digestive α-amylase activity of all developmental stages was compared, it was obvious that all stages were affected significantly. As a whole statement, the last instar larvae digestive α-amylase activity is less sensitive to be inhibited in most cases. Maybe this is due to the high activity of enzymes in the last instar larvae as compared to others. The hydrolytic activity of the amylase in the presence of different inhibitors and substrate concentrations was analyzed. To determine the mode of inhibition Lineweaver-Burk plots were drawn for the inhibited enzyme. In both seed extracts, the type of inhibition was found to be mixed (partial, not full inhibition), so the inhibitor can bind to enzyme or to the enzyme-substrate complex. According to the K_i values of both inhibitors, the wheat proteinaceous extract (K_i=0.05 mg/ml) had inhibitory activity higher than the barley (K_i=0.17 mg/ml). The gel assay showed two amylolytic bands in the insect gut, confirmed the inhibition of the adult's α-amylases by inhibitors and reduction in the bands intensity.

In the feeding assays, all studied biological parameters were affected slightly when larvae was fed with barley protein coated leaves of the four cultivars of potato. Several significant differences were shown in controls of different cultivars in larval weight and evaluation parameters values were in this order; Picasso>Marx>Agria> Burren. The best affected parameters were found in Picasso-inhibitor treatment but there was no significant reduction on gut α-amylase activity of survived individuals which suggest that they may be able to overcome α-amylase inhibitor. However, the reduction in enzyme activity of the survived larvae is difficult to be accessed *in vivo*.

In other literatures, Gutierrez et al. (1990) prepared crude α-amylase inhibitor from the endosperms of wheat and barley and found that 50µg of them causes 53% and 38% inhibition on Colorado potato beetle larval enzyme, respectively, which are in accordance with our findings. Feng et al. (1996) purified four α-amylase inhibitors from wheat flour that inhibited α-amylases of *Sitophilus oryzae* L. (Coleoptera: Curculionidae), *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae), and *Tenebrio molitor* L. (Coleoptera: Tenebrionidae). They observed that the growth rate of *T. castaneum* larvae was slowed when purified inhibitor was included in the diet at a level of 10%. Franco et al. (2000) isolated five α-amylase inhibitors from wheat kernels and showed its inhibitory effect on the enzyme of *A. obtectus*. Warchalewski et al. (2002) showed that wheat proteinaceous α-amylase inhibitors in diets of adults of *Sitophilus granarius* L. (Coleoptera: Curculionidae), did not affect their survival, but it lengthened the development time of *T. confusum* Duval (Coleoptera: Tenebrionidae) larvae by 15 days. In their study, the wheat proteinaceous extract in a diet consisting of 50% crude inhibitors also caused *Ephesitia kuehniella* Zeller (Lepidoptera: Pyralidae) larvae failed to develop. However, in our results, barley proteinaceous extract had a limited influence on the developmental duration of CPB. It extended the duration of CPB molting on two cultivars of potato, Marx and Picasso, from 8.0 days in the control trial to 9.9 days in the treatment, and from 7.3 days to 9.6 days, respectively.

Piasecka-Kwiatkowska et al. (2007) studied inhibitory activities of three cereal grains; wheat, rye (*Secale cereale* L.) and triticale (X *Triticosecale wittmack*) against some stored-product pest α-amylases. They concluded that inhibitory effects were different and dependent on genus and cereal varieties. Priya et al. (2010) showed that wheat α-amylase inhibitors inhibits *Rhyzopertha dominica* F. (Coleoptera: Bostrichidae) α-amylase and stated that this inhibitor showed mixed type of inhibition mainly competitive with some non-competitive behavior that is similar to our results. Mehrabadi et al. (2010) examined the effect of triticale extract on midgut amylases of *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae) and showed that it had inhibitory effects on α-amylases, with dose dependent manner. Mehrabadi et al. (2011) studied the effect of seven plant species extracts including *Punica granatum* L. (Punicaceae), *Rheum officinale* B. (Polygonaceae), *Rhus coriaria* L. (Anacardiaceae), *Artemisia sieberi* B. (Compositae), *Peganum harmala* L. (Nitrariaceae) , *Datura stramonium* L. (Solanaceae) and *Thymus vulgaris* L. (Lamiaceae) on α-amylase activity of four stored insect pests including *C. maculatus*, *R. dominica*, *S. granarius*, and *Trogoderma granarium* E. (Coleoptera : Dermestidae) and showed that plant extracts can inhibit activity of insect α-amylases varying from nearly 4% to 95% inhibition. Khan (2011) extracted proteinaceous inhibitors from wheat, chick pea (*Cicer arietinum* L.) (Fabaceae), kidney bean (*Phaseolus vulgaris* L.) (Fabaceae), maize (*Zea mays* L.) (Poaceae), and millet (*Pennisetum typhoides* Burm) (Poaceae) and found that inhibitors isolated from millet (57.1%), wheat (75%), maize (82.1%) and kidney bean (67.8%) exhibited inhibitory activity against α-amylase from *T. castaneum*. Borzoei et al. (2013) showed 15.3% and 91.2% inhibition of α-amylase of *Plutella xylostella* L. (Lepidoptera: Plutellidae) in a low and high dose of the wheat seed extracts. In accordance with our findings, they found that interaction between the insect α-amylase and seed extracts is also pH dependant. They also stated that physiochemical environment of the insect gut affect interaction between the insect α-amylase and the inhibitors. Borzoui & Bandani (2013) found that the effect of wheat is greater than the triticale seed extract on both α-amylase and protease of *Ectomyelois ceratoniae* Zeller (Lepidoptera: Pyralidae). In their assays the lowest concentration of wheat and triticale seed extracts (0.106 mg protein/ml) inhibited 39% and 18% and the highest concentration (1.7 mg protein/ml) inhibited 82% and 75% of the amylase activity, respectively. Dastranj et al. (2013) stated that bean, and wheat cultivars; MV17, Aflak, Sivand, Saymon, and Zare inhibited the α-amylase activity of *T. molitor* with the percentage of 70.9, 58.3,56.2, 58.5, 57.2, and 48.5, respectively. Most of the results in current study were in accordance with mentioned literature.

Due to importance of α-amylases in insect survival, since larvae development would be suppressed by lower carbohydrate intake rates, consequently, reducing energy availability required for insect development, these enzymes can be good target candidates for bio-insecticides via their inhibitors from plant sources (Sivakumar et al., 2006; Silva et al., 2009). Searching for new enzyme inhibitors, has increasingly lead to more studies aimed at genetic modification techniques for the insertion of genes of resistance to insects in plant species, or for the development of specific bio-insecticides (Silva et al., 2009). If an increase in effectiveness of α-amylase inhibition achieves, further engineering of these proteins can be made to produce more effective inhibitor for application as bio-control agent instead of the currently employed insecticides (Wisessing et al., 2008).

These data revealed that wheat and barley seed extracts contain proteinaceous molecules that can interfere with digestive α-amylase and developmental parameters of the Colorado potato beetle, present an interesting potential for the development of insect-resistant transgenic plants that express heterologous α-amylase inhibitors. Many of these plants extract α-amylase inhibitors should be tested in field conditions and commercialized.

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References

Alves, D. T., I. M. Vasconcelos, J. T. A. Oliveira, L. R. Farias, S. C. Dias, M. D. Chiarello, S. Maria-Neto, & O. L. Franco, 2009. Identification of four novel members of Kunitz-like α-amylase inhibitors family from *Delonix regia* with activity toward Coleopteran insects. Pesticide Biochemistry and Physiology, 95: 166-172.

Baker, J. E., 1987. Purification of isoamylases from the rice weevil, *Sitophilus orizae* L. by HPLC and their interaction with partially purified amylase inhibitor from wheat. Insect Biochemistry, 17: 37-44.

Bandani, A. R., M. Kazzazi & M. Mehrabadi, 2009. Purification and characterization of midgut α-amylases of *Eurygaster integriceps*. Entomological Science, 12: 25-32.

Bandani, A. R., F. Maleki, S. Rahmani & M. Fazeli-Dinan, 2010. Characterization of α-amylase in the alimentary canal of *Naranga aenescens* Moore (Lepidoptera: Noctuidae), the Rice green caterpillar. Munis Entomology and Zoology, 5: 716-725.

Becker-Ritt, A. B. & C. R. Carlini, 2012. Fungitoxic and insecticidal plant polypeptides. Biopolymers (Peptide Science), 98: 367-384.

Bernfeld, P., 1955. Amylases, α and β. Method Enzyme, 1: 149-158.

Borzoui, E. & A. R. Bandani, 2013. Wheat and triticale proteinacious seed extracts inhibit gut α-amylase and protease of the carob moth, *Ectomyelois ceratoniae*. Molecular Entomology, 4: 13-21.

Borzoei, E., A. R. Bandani, M. Dastranj & M. Belbasi, 2013. Effect of proteinaceous extract of wheat seeds on α-amylase activity of diamondback moth, *Plutella xylustella* (L.) (Lepidoptera: Plutellidae). SOAJ Entomolgical Studies, 2: 1-10.

Bradford, M. M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72: 248-254.

Carlini, C. R. & M. F. Grossi-De-Sa, 2002. Plant toxic proteins with insecticidal properties: A review on their potentialities as bioinsecticides. Toxicon, 40: 1515-1539.

Chen, M. S., 2008. Inducible direct plant defense against insect herbivores: A review. Insect Science, 15: 101-114.

Dastranj, M., A. R. Bandani & M. Mehrabadi, 2013. Age-specific digestion of *Tenebrio molitor* (Coleoptera: Tenebrionidae) and inhibition of proteolytic and amylolytic activity by plant proteinaceous seed extracts. Journal of Asia-Pacific Entomology, 16: 309-315.

Elpidina, E. N., K. S. Vinokurov, V. A. Gromenko, Y. E. Rudenskaya & D. P. Zhuzhikov, 2001. Compartmentalization of proteinases and amylases in Nauphoeta cinerea midgut. Archive of Insect Biochemistry and Physiology, 48: 206-216.

Feng, G. H., M. Richardson, T. M. S. Chen, K. J. Kramer, T. D. Morgan & G. R. Reeck, 1996. α-Amylase inhibitors from wheat: Amino acid sequences and patterns of inhibition of insect and human α-amylases. Insect Biochemistry and Molecular Biology, 26: 419-426.

Franco, O. L., D. J. Rigden, F. R. Melo, C. B. Jr, C. P. Silva & M. F. Grossi-De-Sa, 2000. Activity of wheat α-amylase inhibitors towards bruchid α-amylases and structural explanation of observed specificities. European Journal of Biochemistry, 267: 2166-2173.

Franco, O. L., D. J. Rigden, F. R. Melo & M. F. Grossi-De-Sa, 2002. Plant alpha-amylase inhibitors and their interaction with insect alpha-amylases, structure, function and potential for crop protection. European Journal of Biochemistry, 269: 397-412.

Gatehouse, A. M. R., E. Norton, G. M. Davison, S. M. Babbe, C. A. Newell & J. A. Gatehouse, 1999. Digestive proteolytic activity in larvae of tomato moth, *Lacanobia oleracea*, effects of plant protease inhibitors *in vitro* and *in vivo*. Journal of Insect Physiology, 45: 545-558.

Gatehouse, L. N., J. T. Christeller, H. S. Gatehouse & X. Y. Zou, 2002. A strong inhibitor of chymotrypsin/elastase is highly antimetabolic to *Helicoverpa armigera* larvae. New Zealand Plant Protection, 55: 421-428.

Gutierrez, C., R. Sanchez-Monge, L. Gomez, M. Ruiz-Tapiador, P. Castafiera & G. Salcedo, 1990. α-Amylase activities of agricultural insect pests are specifically affected by different inhibitor preparations from wheat and barley endosperms. Plant Science, 72: 37-44.

Habib, H. & K. M. Fazili, 2007. Plant protease inhibitors: a defense strategy in plants. Biotechnology and Molecular Biology Reviews, 2: 68-85.

Karimi, J., E. Haubruge & F. Francis, 2010. Development of entomotoxic molecules as control agents: illustration of some protein potential uses and limits of lectins (Review). Biotechnology Agronomy Society and Environment, 14: 225-241.

Khan, N., 2011. In vitro effects of protienaceous alpha amylase inhibitors on red flour beetle, *Tribolium castaneum.* Science Research Reporter, 1: 101-104.

Kondrak, M., J. Kutas, B. Szenthe, A. Patthy, Z. Banfalvi, M. Nadasy, L. Graf & B. Asboth, 2005. Inhibition of Colorado potato beetle larvae by a locust proteinase inhibitor peptide expressed in potato. Biotechnology Letters, 27: 829-834.

Laemmli, U. K., 1970. Cleavage of structural proteins during the assembly of bacteriophage T4. Nature, 227: 680-685.

Lawrence, S. D., N. G. Novak & M. B. Blackburn, 2007. Inhibition of proteinase inhibitor transcripts by *Leptinotarsa decemlineata* regurgitant in *Solanum lycopersicum*. Journal of Chemical Ecology, 33: 1041-1048.

Macedo, M. L. R. & M. D. G. M. Freire, 2011. Insect digestive enzymes as a target for pest control. Invertebrate Survival Journal, 8: 190-198.

Mehrabadi, M. & A. R. Bandani, 2010. New approach toward α-amylase electrophoresis and isoamylase detection. Munis Entomology and Zoology, 5: 1085-1087.

Mehrabadi, M., A. R. Bandani & F. Saadati, 2010. Inhibition of Sunn pest, *Eurygaster integriceps*, α-amylases by α-amylase inhibitors (T- αAI) from Triticale. Journal of Insect Science, 10: 179-191.

Mehrabadi, M., A. R. Bandani, F. Saadati & M. Mahmudvand, 2011. Alpha-amylase activity of stored products insects and its inhibition by medicinal plant extracts. Journal of Agricultural Science and Technology, 13: 1173-1182.

Melo, F. R., M. P. Sales, L. S. Silva, O. L. Franco, J. R. C. Bloch & M. B. Ary, 1999. α-Amylase inhibitors from cowpea seeds. Protein and Peptide Letters, 6: 387-392.

Michaud, D., N. Bernier-Vadnais, S. Overney & S. Yelle, 1995. Constitutive expression of digestive cysteine proteinase forms during development of the Colorado potato beetle, *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae). Insect Biochemistry and Molecular Biology, 25: 1041-1048.

Piasecka-Kwiatkowska, D., D. Madaj & J. R. Warchalewski, 2007.The biological activity of wheat, rye and triticale varieties harvested in four consecutive years. Acta scientiarum Polonorum/Technologia alimentaria, 6: 55-65.

Priya, S., N. Kaur & A. K. Gupta, 2010. Purification, characterization and inhibition studies of a-amylase of *Rhyzopertha dominica*. Pesticide Biochemistry and Physiology, 98: 231-237.

Silva, E. M., A. Valencia, M. F. Grossi-de-Sa, T. L. Rocha, E. Freire, J. E. De Paula, & L. S. Espindola, 2009. Inhibitory action of cerrado plants against mammalian and insect α-amylases. Pesticide Biochemistry and Physiology, 95: 141-146.

Sivakumar, S., M. Mohan, O. L. Franco, & B. Thayumanavan, 2006. Inhibition of insect pest alpha-amylases by little and finger millet inhibitors. Pesticide Biochemistry and Physiology, 85: 155-160.

Valencia-Jime´nez, A., A. J. W. Arboleda, A. Lopez Avila, & M. F. Grossi-de-Sa, 2008. Digestive α-amylases from *Tecia solanivora* larvae (Lepidoptera: Gelechiidae): response to pH, temperature and plant amylase inhibitors. Bulletin of Entomological Research, 98: 575-579.

Warchalewski, J. R., J. Gralik, Z. Winiecki, J. Nawrot & D. Piasecka-Kwiatkowska, 2002. The effect of wheat a-amylase inhibitors incorporated into wheat-based artificial diets on development of *Sitophilus granarius* L., *Tribolium confusum* Duv., and *Ephestia kuehniella* Zell. Journal of Applied Entomology, 126: 161-168.

Wisessing, A., A. Engkagul, A. Wongpiyasatid, & K. Chuwongkomon, 2008. Proteomic and characterization of α-amylase inhibitor from Mungbean (*Vigna radiate*). Kasetsart Journal (Natural Science), 42: 245-250.

Wolfson, J. L. & L. L. Murdock, 1990. Diversity in digestive proteinase activity among insects. Journal of Chemical Ecology, 16: 1089-1102.

Zhu-Salzman, K., J. L. Bi & T. X. Liu, 2005. Molecular strategies of plant defense and insect counter-defense. Insect Science, 12: 3-15.