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# Influence of Red Kidney Bean Seed Proteins on Development, Digestive α-amylase Activity and Gut Protein Pattern of *Leptinotarsa decemlineata* (Say)

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## ABSTRACT

The effects of red kidney bean, *Phaseolus vulgaris* cv. Goli (L.), seed proteinaceous extract on larval development, digestive α-amylase and gut protein pattern of Colorado potato beetle, *Leptinotarsa decemlineata* (Say) were investigated. The results showed that four proteinaceous ammonium sulfate precipitated fractions (30, 50, 70, and 100%) of bean seed extract caused significant inhibition, *in vitro*, on α-amylase activity of all larval stages and adults up to 69%. Affinity chromatography using larval gut sample as ligand and silicon dioxide as matrix, were used to identify enzyme inhibitor proteins, but purified protein was not recognized on SDS-PAGE. Alpha-amylase activity of larvae fed on the treated leaves of four different potato cultivars (Agria, Burren, Picasso, and Marx) by bean seed extract was inhibited significantly up to the 60% on Burren. In the bioassay, weight of fourth instar larvae was reduced 19 mg and the larval growth was decreased by 45.35% on Picasso, as compared to the control. In two-dimensional electrophoresis and SDS-PAGE, the gut protein patterns were changed in effects of feeding on leaves treated with bean seed extract. These data revealed that red kidney bean seed extract suppressed digestive α-amylase activity *in vitro* and *in vivo*, caused side effects on the actual parameters monitored and had an impact on the gut protein pattern of *L. decemlineata*.

*Keywords:* Colorado potato beetle, red kidney bean protein, gut enzyme inhibition, 2-D electrophoresis.

## INTRODUCTION

Alpha-amylases (α-1,4-glucan-4-glucanohydrolases, EC 3.2.1.1) constitute a family of endo-amylases that catalyze the polysaccharide hydrolysis with α-1,4 type bonds, such as starch and glycogen (Franco *et al.,* 2002; Becker-Ritt and Carlini, 2012). When the action of the amylases is inhibited, nutrition of the organism is impaired causing shortness in energy (Carlini and Grossi-de-Sa, 2002).

Enzyme inhibitors inhibit the digestive enzyme activities and impede digestion through their action on key insect gut digestive hydrolases (Franco *et al.*, 2002), hence they reduce the quantity of digested macromolecules, and also cause hyperproduction of the enzymes, as a result of which, the insects become weak with underdeveloped

growth and at last die (Lawrence and Koundal, 2002). These inhibitors present an interesting potential for the development of insect-resistant transgenic plants that express heterologous α-amylase inhibitors as they show insecticidal effect on several agricultural pests (Becker-Ritt and Carlini, 2012). This techniques offer an alternative strategy of pest control that may eliminate the reliance on chemical pesticides and have proven to be a promising approach. All need to be explored and prudently tapped for their implementation in integrated pest management programs (Fan and Wu, 2005). So far, some extensive studies have been carried out to identify proteins with insecticidal properties against major economic crop pests (Karimi *et al.*, 2010).

Alpha-amylase inhibitors occur in leguminous and some other plants which valued as potential protective strategy against insects (Carlini and Grossi-de-Sa, 2002; Franco *et al.*, 2002). Seeds of the common bean (*Phaseolus vulgaris* L.) contain a plant defense protein that inhibits the α-amylases of insects (Pueyo *et al.*, 1993). Powers and Culbertson (1983) purified single band protein from red kidney bean by affinity chromatography which had inhibitory effect on the yellow meal worm (*Tenebrio molitor* L.) α-amylase activity. Ishimoto and Kitanura (1989) found side effects of red kidney bean protein on the larval growth and α-amylase activities of two weevil species, the chines bruchid (*Callosobruchus chinensis* L.) and the cowpea weevil (*C. maculatus* F.).

Amylases are considered as one of the most active carbohydrases of a major pest of potato, Colorado potato beetle (CPB), *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae). Properties of some amylases of CPB were investigated, but the study on their specific plants inhibitors remains an imperative task (Tsvetkov *et al.*, 2015). The purpose of this investigation was to get in depth the effect of proteinaceous extract of the red kidney bean seed on the gut α-amylase activity of *L. decemlineata*. Moreover, larval development was assessed by feeding larvae on four different potato cultivar leaves treated by red kidney bean seed extract in bioassays. Subsequently, changes in these larval gut protein patterns were visualized in 2-D electrophoresis and SDS-PAGE.

## MATERIALS AND METHODS

### Materials and equipment

Succinic acid disodium salt, bovine serum albumin (BSA), ammonium persulfate for electrophoresis (APS), silicon dioxide (nanopowder, 10 nm, 99.5%), acetic acid, ethanol, β-mercaptoethanol, sodium thiosulfate, formaldehyde, glutaraldehyde, sodium phosphate dibasic dihydrate and monobasic puriss, sodium carbonate, 5-7 ampholyte, 3-10 ampholyte, dithiothreitol (DTT), nonidet P 40, urea, 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, soluble starch, sodium hydroxide (NaOH), ammonium sulfate, acrylamide, N,N′-methylene diacrylamide, dodecyl sulfate sodium salt (SDS), 2-morpholinoethanesulfonic acid (MES), sodium chloride (NaCl), calcium chloride ($CaCl_2$), glycerol, potassium iodide (KI), iodine ($I_2$), coomassie brillant blue G-250 and R-250, bromophenol blue, phosphoric acid ($H_3PO_4$), sodium acetate trihydrate, silver

nitrate ($AgNO_3$), and N,N,N′,N′-tetramethyl ethylenediamine (TEMED) were supplied by Merck® (Darmstadt, Germany). Glycine was from Scharlau® (Barcelona, Spain) and Triton X-100 from Applichem® (GmbH in Darmstadt, Germany). Spectrophotometric measurements were made using ELISA reader, BioTek® ELx800 (Winooski, Vermont, USA).

**Insect rearing and preparation of enzyme samples**

The colony of CPB was maintained on potato foliage cultivar “Agria” at 27±1°C, 60±5% relative humidity, under 16:8 h (L:D) photoperiod and white fluorescent light, at University of Tabriz, Tabriz, Iran. Insects were reared from egg hatch to adult in clear plastic dishes containing fresh potato leaves. Enzyme samples from adults, fourth instar larvae ($L_4$) and third instar larvae ($L_3$) were prepared by dissection of guts under a light microscope in the ice-cold phosphate buffer solution (pH: 7). For first and second instar larvae ($L_1$ and $L_2$), whole larvae were grounded according to Michaud *et al.* (1995). The samples were homogenized in cold distilled water and the mixtures were centrifuged at 13,000 rpm for 30 min at 4°C. The supernatant was stored as an enzyme source at -20°C before analysis. The protein content of the enzyme source was adjusted to 2 mg/ml.

**Preparation of seed protein extract**

Seeds of red kidney bean, *P. vulgaris* cv. Goli (L.) were supplied by Seed and Plant Improvement Institute, Karaj, Iran. Thirty grams of grinded seed powder was homogenized with 100 ml solution of 0.1 M NaCl and stirred for 2 h, followed by centrifugation at 10,000 rpm for 30 min. Seed protein in the supernatant was extracted using a saturation of 0-30, 30-50, 50-70, 70-100% (w/v) (as gradient; 30, 50, 70 and 100%) ammonium sulfate. In every fraction extraction, the supernatant was brought to next saturation by further addition of ammonium sulfate. A general fraction 0-70% saturation of ammonium sulfate was prepared, too. After 45 min, the crude extract was centrifuged at same condition. The pellet was dissolved in the minimal volume of the Tris-HCl buffer (0.02 M and pH: 7) until the solid material dissolved completely. Then the extracts were dialyzed using dialysis bag against the distilled water for 20 h with changing the dialysis water at least twice. Finally, this dialyzed solution was heated at 70°C for 20 min to inactivate endogenous enzymes within extract and after centrifugation at same condition it was used as inhibitor.

**Amylase assay**

The α-amylase activity was measured by the dinitrosalicylic acid (DNS) procedure (Bernfeld, 1955), using 1% soluble starch as substrate. 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.

**Enzyme inhibition assay**

The inhibition assays followed procedure according to Mehrabadi *et al.* (2010). The enzyme extract was pre-incubated with proteinaceous seed extract before addition of substrate for 30 min at 37°C. Then the same procedure for the amylase assay was conducted. In this study, pH dependence of $L_4$ gut enzyme inhibition by fraction 0-70% of seed extracts and inhibitory activity of four proteinaceous fractions on all larval stages and adult's 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)]

**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 and Bandani (2010). Adult's enzyme extract was incubated with inhibitor (fraction 0-70%) 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 distilled water 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 distilled water, 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.

**Bioassay**

Newly hatched CPB larvae were reared on excised leaves of four potato cultivars (Agria, Burren, Picasso, and Marx), prepared from Ardabil Potato Research Institute, Ardabil, Iran. Fifty newly emerged larvae were placed in aerated plastic arenas. The leaves were painted with red kidney bean seed proteinaceous extract (fraction 0-70%) containing 0.6 mg protein per ml and replaced daily throughout the experiment. The developmental period from first up to forth instar larvae ($L_1$-$L_4$) and larval growth (from $L_1$ up to $L_4$) were recorded. Fourth instar larvae were weighed on the fourth day. The gut enzyme extracts of surviving individuals were prepared as above mentioned and kept on -70°C for further analysis (two-dimensional electrophoresis and α-amylase assay). Whole parameters were compared to that of insects fed with potato leaves painted with distilled water as control.

**Affinity chromatography**

Affinity chromatography was done by using glutaraldehyde-activated silica according to Daglioglu and Zihnioglu (2012) with some modifications. Insect third instar larval gut protein mix was chosen as ligand. Immobilization of larval gut enzyme was performed on silicon dioxide activated with glutaraldehyde (25%) (silica-GA-enzyme): silicon dioxide (7 g) was incubated in 70 ml 0.1 M acetate buffer (pH: 4) containing

1% glutaraldehyde at 25°C and 200 rpm for 1 hour. Excess of glutaraldehyde was removed using centrifugation at 4,500 rpm for 30 min and subsequently washed with distilled water. Activated silica was incubated in 3 ml insect gut enzyme extract (2 mg protein/ml) and 7 ml 0.02 M sodium phosphate buffer (pH: 7.5) at 4°C and 200 rpm overnight. Finally, the immobilized enzyme preparation was washed with same buffer at least twice. Subsequently, 10 ml of 1 M glycine was added to the enzyme immobilized matrix and after 10 min excess of glycine was washed by centrifugation. Finally, 10 ml bean protein extract (fraction 0-70%) (1.5 mg protein/ml) was added in the immobilized enzyme preparation and shake for 1 hour at room temperature. Then the excess of unabsorbed inhibitor was removed by centrifugation for 30 min at 4,500 rpm and again was washed by 10 ml same buffer. Subsequently, 10 ml of same buffer containing 0.1 M NaCl was used to remove nonspecifically bound proteins. Finally, the matrix was eluted with 10 ml elution buffer, same buffer containing 0.1 M NaCl, pH: 11 at least three times. These three fractions were pooled, dialyzed and concentrated using ultrafiltration.

**Two-dimensional gel electrophoresis**

The protein pattern of $L_4$ gut enzyme samples obtained from bioassay was compared by using two-dimensional (2-D) electrophoresis. For this purpose, gut samples of larvae reared on the bean seed extract treated leaves of Picasso cultivar and related control was selected. In the first dimension (IEF), the proteins were separated based on isoelectric point (pI) using gel tubes. In the second dimension, gel strips were loaded on SDS-PAGE and proteins were separated based on molecular weight. Two-D electrophoresis was performed using Biorad® protean II xi 2-D cell (U.S.). Isoelectric focusing (IEF) tube gel solution contained 11 g urea (9.2 M final concentration), 3 ml acrylamide/bis stock (30%), 0.2 ml 5-7 ampholyte, 0.8 ml 3-10 ampholyte, and 1 ml detergent solution (100 µl triton X-100, 100 µl nonidet P 40, and 800 µl distilled water) which were reached to 20 ml by distilled water, also 40 µl 10% (w/v) APS and 20 µl TEMED. Upper and lower running electrolytes were contained 20 mM NaOH and 10 mM $H_3PO_4$, respectively. Iso-urea solution contained 0.1 g DTT, 100 µl triton X-100, 5.4 g urea, 500 µl 3-10 ampholyte, and 6 ml distilled water, was mixed with samples in the ratio of 1:10. The amount of protein in the samples was adjusted to 7 mg/ml. For denaturing, samples are heated at 95°C for 5 min. IEF was carried out at 200 V for 2 h, 500 V for 2 h, and 800 V for 16 h. After electrophoresis, the strip gels were transferred to the transfer solution contained 40% (v/v) glycerol, 3% (w/v) SDS, 0.01% (w/v) bromophenol blue, 1% (w/v) DTT (added prior the application), and 12.5% (v/v) 1 M Tris-HCl buffer (pH: 6.8). After 15 min, strip gels were loaded to the second dimension. SDS-PAGE was performed using 12% separating gel and carried out at 24 mA per gel for 6 h. After electrophoresis, the gels were silver stained. The protein spots in the gels were analyzed by “Bio-Profil Bio-2D” software.

**Silver staining**

Silver staining was carried out according to Jungblut and Seifert (1990) with slight modification; the gels were rinsed in the fixation solution (50% ethanol, 10% acetic

acid, and 40% distilled water) at least 1 h, then incubated in incubation solution (30% ethanol, 0.5 M sodium acetate trihydrate, 0.5% glutaraldehyde, and 0.2% sodium thiosulfate) for 2 h. In this step, gels were washed in distilled water for three times, 20 min each, and then rinsed in silver nitrate solution (0.1% $AgNO_3$ and 0.01% formaldehyde) for 30 min. Short washing (30 sec) with distilled water was performed and staining was developed by shaking in developer (2.5% sodium carbonate pH: 11, 0.002% sodium thiosulfate, 0.01% formaldehyde) up to staining the protein spots. After reaching to required color intensity the gels were transferred to stop solution contained 5% acetic acid.

### SDS-PAGE

General influence of red kidney bean seed extract on $L_4$ gut proteins was detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). For this purpose, larval gut extracts prepared in the bioassays on all four cultivars were used. SDS-PAGE was performed according to Laemmli (1970) using 12% stacking and 4% resolving gels. Samples were denatured by heating at 95°C for 5 min and β-mercaptoethanol is used to break disulphide bonds. The amount of protein in all samples was adjusted to 2 mg/ml. After running for 2 h at 16 mA and 4 h at 24 mA per gel, the gels were transferred to coomassie brilliant blue R-250 staining (0.05% w:v in methanol, water, and acetic acid 50:40:10) overnight and destaining (methanol, water, and acetic acid 40:50:10) up to forming of clear bands. The apparent molecular mass was measured in the presence of unstained protein ladder (5-250 kDa, Thermo scientific®, Cat number: 26630) using “Infinity Capture” software. Also, determination of apparent molecular mass of eluted α-amylase inhibitor proteins from affinity chromatography was carried out using protein ladder (14.2-66 kDa, Sigma-aldrich®: Cat number: SDS7).

### Estimation of protein concentration

The protein content was estimated according to the method of Bradford (1976), using bovine serum albumin (BSA).

### Statistical analysis

Analyses of Variance (ANOVA) were employed on the data using the MSTAT-C statistical package. Means of the three replicates in different assays were tested by Tukey’s or Duncan’s test for significant differences.

## RESULTS

### Inhibitory activity of red kidney bean seed protein extract on CPB α-amylase

The inhibition of *L. decemlineata* fourth instar larval gut α-amylase by general fraction, 0-70% ammonium sulfate precipitated bean seed protein extract was not dependent on the pH value of the assay medium. The inhibition values at pH 4, 5, 6, 7, and 8 were measured as 57.28, 58.08, 56.46, 51.94, and 48.58%, respectively

(data not shown), which showed no significant difference ($p < 0.05$) between them based on Tukey's test.

In order to fractionate red kidney bean seed proteins, we used ammonium sulfate precipitation method with saturations; 30, 50, 70, and 100%. First the inhibitory effect of these fractions was evaluated on the $L_4$ gut α-amylase activity and there was not a significant difference ($p < 0.05$) between their inhibitory effects based on Tukey's test. The inhibition of these fractions was recorded as 42.39, 42.12, 40.85, and 41.35%, respectively (data not shown). Because of good potential of all fractions for inhibition of this enzyme, the inhibitory effects of them were examined on all larval and adult's enzyme activities. As shown in Fig. 1, there was differential inhibition of digestive α-amylase activities by fractions on all developmental stages of CPB ($L_1$, $L_2$, $L_3$, $L_4$, and adults). Generally, all fractions had significant inhibitory effects on them. However, the adult's α-amylase activity was not inhibited significantly by the fraction of 100%, as compared to the other fractions.

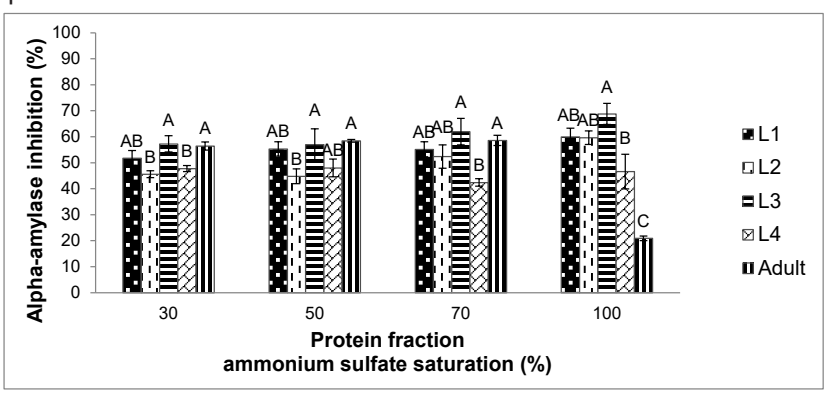


Fig. 1. The inhibitory activities of four proteinaceous fractions of red kidney bean on the digestive α-amylase activity of the first instar larvae (L1), second instar larvae (L2), third instar larvae (L3), fourth instar larvae (L4), and adults of *Leptinotarsa decemlineata*. Means followed by the different letters in each fraction indicate significant differences ($p < 0.05$) between data based on Tukey's test.

### In zymogram inhibition assay

For approving the results of colorimetric inhibition assays, the inhibition of incubated adult's α-amylase with fraction 0-70% was visualized in the semi-denaturing native PAGE (Fig. 2). The gel inhibition assays proved that gut α-amylase of the adults was affected by the inhibitor and the intensity of the bands (two isoforms) decreased in comparison to the control.

### Characterization of inhibitor proteins by affinity chromatography

Following ammonium sulfate precipitation, red kidney bean seed protein extract (fraction 0-70%) and $L_3$ gut extract was applied in affinity chromatography using silicon dioxide. Analysis of the eluted proteins by SDS-PAGE is shown in Fig. 3. The apparent molecular weight of crude and eluted proteins was estimated (between 14 and 66 kDa). As a result, SDS-PAGE indicated nine protein bands in eluted fraction. With

regards to these result, the purification fold and yield were not evaluated. However, the crude extract with 1.5 mg protein/ml protein caused 65% inhibition on gut α-amylase activity of $L_3$, despite the concentrated eluted fraction with 1 mg protein/ml had 73% inhibitory effect on this enzyme activity (data not shown). It is possible that one of the observed protein bands could be the CPB amylase inhibitor.

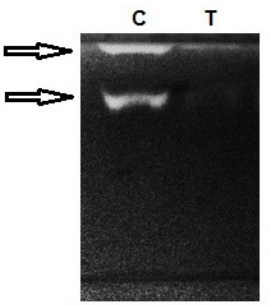


Fig. 2. In gel inhibition assay of proteinaceous extracts of red kidney bean seed on the adult's digestive α-amylase of Leptinotarsa decemlineata. C; control related to α-amylase activity without seed extract and T: treatment related to inhibited enzyme activity with seed extract.

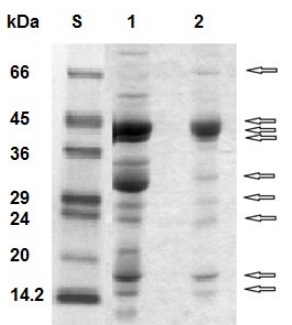


Fig. 3. SDS-PAGE analysis for the purification of red kidney bean proteins by affinity chromatography. S; Standard, Lane 1; crude extract (fraction 0-70%) and lane 2; eluted fraction of affinity chromatography. Coomassie brilliant blue was used to localize the protein bands.

## Development and α-amylase activity of larvae fed on bean protein extract coated leaves

In order to understand the effect of red kidney bean seed extract on larval development and α-amylase activity *in vivo*, insect feeding trials were conducted and results were shown in Table 1. Weight of $L_4$ fed on treated leaves of Picasso and Marx cultivars were significantly lower than the control ($P<0.05$), and the larval growth ($L_1$ to $L_4$ evaluation) in Picasso cultivar was reduced significantly ($P<0.05$), as compared to the control. There was no significant effect on developmental durations up to $L_4$ molting ($L_1$ to $L_4$), on all cultivars in comparison to the control. On the other hand α-amylase activity of surviving individuals feeding on treated leaves of all cultivars was inhibited significantly ($P < 0.05$), as compared to the control.

Table 1. The effects of red kidney bean proteinaceous extract on larval developmental parameters and digestive α-amylase of Leptinotarsa decemlineata by using four different cultivars of potato leaves.

| Parameters | Treatment | Potato cultivars | | | |
|---|---|---|---|---|---|
| | | Agria | Burren | Picasso | Marx |
| Larval weight (mg) | Control | 112.33±2.33 bc | 110.33±2.91 bc | 126±4.73 a | 122±5.29 ab |
| | Treated | 100.33±4.33 c | 108.33±6.01 bc | 106.67±9.28 bc | 103.33±8.33 c |
| Larval growth (%) | Control | 42.67±4.35 abc | 29.33±4.61 c | 57.33±4.79 a | 51.33±5.51 ab |
| | Treated | 32±1.42 bc | 25.33±1.80 c | 31.33±0.41 bc | 32.67±2.48 bc |
| Duration up to $L_4$ molting (day) | Control | 8.31±0.28 abc | 8.91±0.77 abc | 7.33±0.09 c | 8.02±0.41 bc |
| | Treated | 9.33±0.20 a | 8.37±0.21 abc | 7.97±0.32 bc | 7.92±0.17 bc |
| α-amylase activity (U/mg protein) | Control | 2.18±0.04 a | 2.25±0.06 a | 2.24±0.1 a | 2.26±0.13 a |
| | Treated | 1.03±0.21 b | 0.91±0.15 b | 1.14±0.28 b | 1.18±0.17 b |

Means followed by the same letters indicate no significant differences ($P < 0.05$) between data based on Duncan's test. U: ΔAbs 540 nm/min. The effect of ingested inhibitor proteins on larval gut protein pattern.

In this investigation, the gut protein pattern of larvae reared on the bean extract treated leaves of Picasso cultivar and control, in which leaves treated with distilled water, was compared using 2-D electrophoresis. The experiment was repeated five times and protein spots on each gel were matched with their repeats (data not shown). The final gels were provided in Fig. 4.

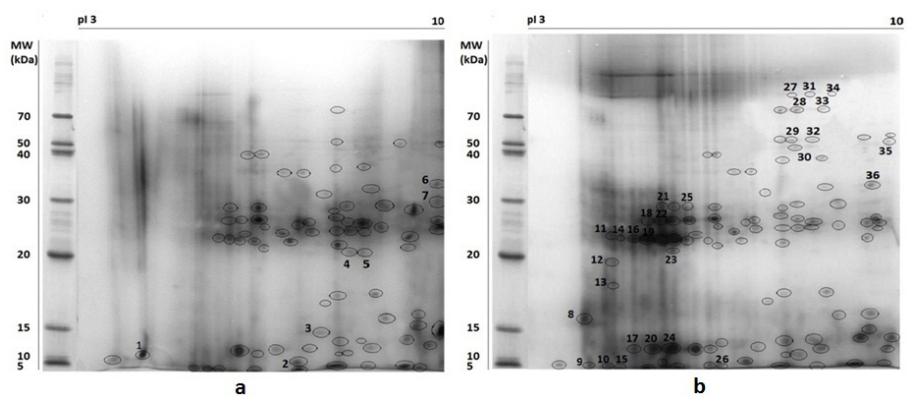


Fig. 4. *Leptinotarsa decemlineata* larval gut protein pattern in Two-dimensional electrophoresis a; control and b; red kidney bean seed protein extract treatment. The isoelectric point (pI) was obtained in the range of 3-10. The molecular weight (MW) was estimated by the standard. Numbered spots are the distinct spots on each gel and unnumbered spots are the similar spots in the control and treatment. Silver nitrate is used for staining proteins. From a total of 107 protein spots in the control and treatment, seven spots in the control (number 1-7) (6.54%) (Fig. 4.a) and 29 spots in the treatment (number 8-36) (27.10%) (Fig. 4.b) were unique spots and 71 spots (66.36%) were identical in two gels. It was concluded that the protein patterns were changed in effects of feeding on leaves treated with red kidney bean seed protein extracts, and approximately 29 proteins had their expression up regulated and 7 were down regulated.

In addition to 2-D electrophoresis, gut proteins of larvae fed on the leaves of potato cultivars, Agria, Burren, Picasso, and Marx, treated with red kidney bean seed extract and distilled water (as control) were also evaluated on SDS-PAGE, as shown in Fig. 5. A number of protein bands were different between control and treatment, and also

between the different cultivars. As a general, the most obvious differences among the bands related to all cultivars were seen clearly at bands with molecular weight approximately 19 and 31 kDa.

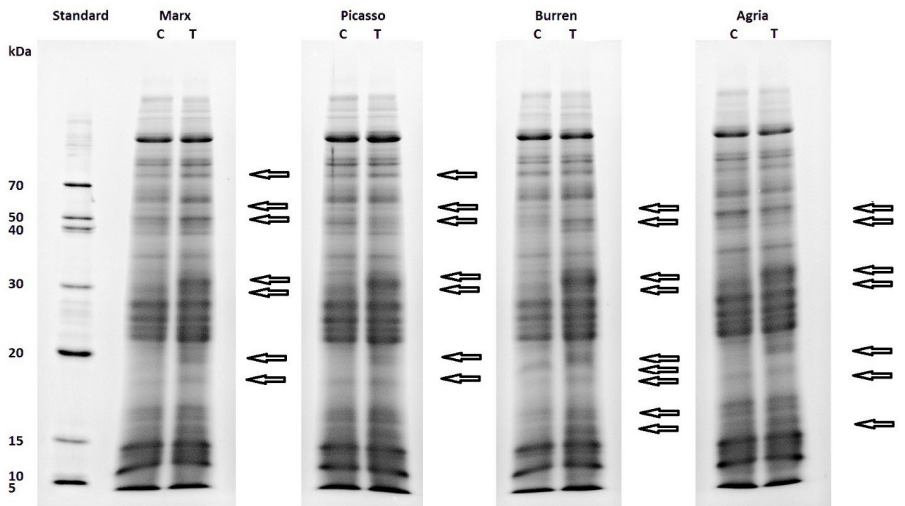


Fig. 5. SDS-PAGE for gut protein of *Leptinotarsa decemlineata* larvae fed on the leaves of different potato cultivars Agria, Burren, Picasso and Marx, C; treated with distilled water and T; treated with red kidney bean seed protein extract. Coomassie brilliant blue was used for staining proteins. Differential protein bands in different samples showed with arrows.

## DISCUSSION

Inhibitors of digestive enzymes can be used to protect plants against insects and for this purpose effects of red kidney bean seed proteins on α-amylase activity of all larval and adult stages, larval development, and larval gut protein pattern of Colorado potato beetle were assessed *in vitro* and *in vivo*. According to the results, this proteinaceous extract showed significant inhibitory activity on *L. decemlineata* $L_4$ digestive α-amylase activity at wide range pH (4-8). So there was not the exact optimum pH for inhibition, but it was matched with optimum pH of amylase activity (Ashouri *et al.*, 2015) and the gut pH of this insect which was found acidic (pH: 5-6) (Novillo *et al.*, 1997). Sharifi *et al.* (2011) showed that α-amylase inhibitors purified from *P. vulgaris* exhibited good inhibitory activity on the elm leaf beetle (*Xanthogaleruca luteola* Muller) gut α-amylase activity up to 72%. Khan (2011) extracted proteinaceous inhibitors from kidney bean and found 67.8% inhibitory activity against α-amylase from the red flour beetle (*Tribolium castaneum* Herbst). Dastranj *et al.* (2013) stated that common bean seed extract, inhibited the α-amylase activity of *T. molitor* with the percentage of 71. In accordance with these findings, in this study all fractions of kidney bean seed protein (30, 50, 70, and 100%) had significant inhibitory effect on all larval and adult's α-amylase activity of *L. decemlineata* up to 69% on $L_3$ by fraction 100%.

So, we have this hypothesis that with bean seed extracted proteins we can suppress the activity of α-amylase in all destructive stages of *L. decemlineata*. However, the adult's α-amylase activity was less sensitive to be inhibited by the fraction 100%. When the adult's enzyme was incubated with fraction 0-70% on zymogram, which was a qualitative experiment for enzyme inhibition reported by other researchers (Mehrabadi *et al.*, 2010-2011-2012; Dastranj *et al.*, 2013) as well, the intensity of two α-amylase bands were declined strongly. It confirms that incubated enzyme with inhibitor did not yet had activity in the electrophoretic gel containing starch as substrate.

Chromatographic techniques are used to identify and purify enzyme inhibitor proteins; Bonavides *et al.* (2007) isolated the cowpea weevil (*C. maculatus* F.) α-amylase inhibitors from the baru seeds (*Dipteryx alata* Vogel) (Fabaceae) by affinity chromatographic procedures and for attempt further purification, they applied this fraction onto a reversed-phase high performance liquid chromatography (HPLC) column. They purified four proteins by different molecular weights with ability of α-amylase activity inhibition and said that several α-amylase inhibitor classes with biotechnological potential can be isolated from a single plant species. Gupta *et al.* (2014) purified α-amylase inhibitor from bean (*P. vulgaris*) cultivars using ammonium sulfate precipitation, gel filtration chromatography (Sephadex G-100), and ion exchange chromatography (DEAE-Sephadex) which consisted of three subunits of molecular weight 15, 18, and 26 kDa and were effective against α-amylases extracted from larvae of *C. chinensis*, *T. castaneum*, and adults of the egyptian cotton leaf worm (*Spodoptera littoralis* Boisduval). Powers and Withaker (1978) purified *T. molitor* larval midgut amylase inhibitor from red kidney bean seed with apparent molecular weight of 49 kDa by Sephadex gel filtration. In current study, affinity chromatography was examined for this purpose and $L_3$ gut sample was used as ligand and silicon dioxide as matrix. From the mixed protein bands in crude extract of red kidney bean just about nine bands were seen in eluted extract. By the SDS-PAGE the apparent molecular weight were estimated 15, 17, 23, 27, 33, 41, 43, 45, and 66 ~kDa. Beside the inhibition of insect digestive enzymes by plant proteinaceous extract *in vitro*, the effect of these proteins on the larval development and α-amylase activity were investigated in feeding trials. Moreover, these trials were carried out by using four different cultivars of potato as larval food, because potato cultivars can vary in terms of nutrients for pests. When the extracts were ingested by larvae on the treated leaves, weight of $L_4$ were reduced 19 mg on Picasso and Marx cultivars as compared to the control. Also, the larval growth on Picasso was decreased by 45.35% as compared to the control. As a consequence, the highest effect of extracts on larval development parameters has been on Picasso and Marx cultivars. It is probably due to the suitability of these cultivars for larval feeding. In accordance with our findings, in other literatures bean proteins had side effects on insect's development; Ishimoto and Kitanura (1989) stated that larvae of *C. chinensis* and *C. maculatus* were unable to grow on azuki bean which mixed with kidney bean seed protein. Gupta *et al.* (2014) said that larvae of *T. castaneum* fed on flour mixed with purified inhibitor from common bean for five days showed 100% larval mortality.

On the other hand, in this study, α-amylase activity of surviving individuals fed on treated leaves of all cultivars was inhibited significantly ($P < 0.05$), as compared to the control; 53, 60, 49, and 48% on Agria, Burren, Picasso, and Marx, respectively. In the other words red kidney bean seed protein showed inhibitory activity on *L. decemlineata* gut α-amylase *in vivo*. Ishimoto and Kitanura (1989) found that larvae of *C. chinensis* and *C. maculatus* midgut α-amylase activity were suppressed by kidney bean seed protein. Their results suggest that the resistance of kidney beans to these weevils is closely related to the inhibition of the larval amylase activity by the α-amylase inhibitor. They also found that larvae of the mexican bean weevil (*Zabrotes subfasciatus* Boheman), a pest of kidney bean seed, developed on the treated beans and the midgut amylase activity was not appreciably affected. Moreover, data of the biological parameters in control trials showed that the most resistant cultivars to CPB damage was identified Burren and then Agria, Marx, and Picasso, respectively. Also, there was seen no significant differences between α-amylase activities of larvae fed on different cultivars. In contrast, Mardani-Talaee *et al.* (2015) found differential α-amylase activity of *L. decemlineata* on six different cultivars of potato; possibly it is because of the differences in the used cultivars.

The impact of ingested red kidney bean seed protein on the larval gut protein pattern in the control and treatment were compared by 2-D electrophoresis. According to the results, a large number of spots in both treatments and control were similar (66.38%). Only a small number of spots (6.54%) in the control were not seen on the treatment, and a lot more protein spots (27.1%) in the treatment were added. Most of these added spots were observed in the isoelectric points 5 and 6. Tsvetkov *et al.* (2015) claimed that inhibitors of amylases have relatively large diversity of molecular content, with isoelectric point in the pH range from 5 to 6. The increase or decrease in the size of the spots was observed in some cases. Also in other literatures, 2-D electrophoresis was used for analyzing gut protein pattern of insects; Parde *et al.* (2012) observed the gut protein contents of the pod borer (*Helicoverpa armigera* Hubner), using 2-D electrophoresis to visualize activation of inactive isoforms of proteinase when larvae feed on a cocktail of synthetic inhibitors incorporated in artificial diet. In their results, at least four pro-proteinases showed activation, and the apparent molecular weights of the activated proteinases were 47, 44, 32, and 26 kDa, which showed acidic pI. Merzendorfer *et al.* (2012) also used 2-D electrophoresis for comparison of proteins extracted from midgut of control and diflubenzuron treated *T. castaneum* larvae and in their investigation among 388 proteins analyzed in duplicate gels only 26 proteins (7%) were significantly changed. Saadati and Toorchi (2015) were separated gut proteins of the sunn pest (*Eurygaster integriceps* Puton) adults fed from wheat (*Triticium aestivum* L.) by 2-D electrophoresis and from 212 detected spots, they found one α-amylase inhibitor with molecular weight 13.8 kDa and pI 5.7 and claimed that identification of gut proteins can be helpful for finding of new target proteins in pest control. According to the 2-D electrophoresis results, we can conclude that gut protein pattern of *L. decemlineata* was changed with feeding from treated leaves with red kidney bean seed protein. Furthermore, in SDS-PAGE, it was seen differences in larvae gut protein bands, which fed from different cultivar potato leaves treated

by red kidney bean extract and distilled water as control. The molecular weight of the differentiated protein bands was variable but in the bands with molecular weight 19 and 31 kDa differences were more obvious in all treatments. According to 2-D electrophoresis results of Tsvetkov *et al.* (2015), amylases of *L. decemlineata* are presented by four proteins or groups of proteins with similar isoelectric point with a molecular weight in the range from 30 to 50 kDa. To ensure the relevance of these spots and bands to the α-amylase enzyme, the sequencing of amino acids is required.

## CONCLUSIONS

Due to dependence on α-amylases for survival, these enzymes can be good target candidates for bio-insecticides via α-amylase inhibitors. These data revealed that red kidney bean seed protein extracts can interfere with digestive α-amylase of the Colorado potato beetle and had impact on larval developmental parameters and gut protein pattern, present an interesting potential for being considered in the integrated management of this pest.

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