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RESEARCH REPORT

Biochemical characterization of α-amylases from gut and hemolymph of *Rhynchophorus ferrugineus* Olivieri (Col.: Curculionidae) and their inhibition by extracts from the legumes *Vigna radiata* L. and *Phaseolus vulgaris* L.

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Abstract

α-amylase inhibitors represent an important tool in engineering crop plants against insect pests. For achieving this goal, it is necessary to find the nature of α-amylases and their properties for possible use in a pest management procedure. Because *Rhynchophorus ferrugineus* Olivieri is a devastating pest of palm trees in the southeast of Iran, we attempted to characterize α-amylases from larval gut and hemolymph, and to study their interaction with inhibitors extracted from the common bean and the green mung bean. The optimal pHs for gut and hemolymph α-amylases were 4 - 5 and 5 - 6, respectively. Also, high gut amylolytic activity was found at temperatures of 40 – 50 °C. The highest and lowest specific α-amylase activities were detected in the guts of last instar and adult males, and in the hemolymph of last instar, respectively. As calculated from Lineweaver-Burk plots, the K_m values for gut and hemolymph α-amylases of the last instar were 0.54 and 2.15 %, respectively, when glycogen was used as the substrate. Also, when starch was used as the substrate, the K_m values for gut and hemolymph α-amylases were 1.37 and 0.15 %, respectively. Zymogram pattern in the native gel revealed that *R. ferrugineus* gut and hemolymph α-amylases had two isoforms. α-amylase inhibitors partially purified from *Vigna radiata* L. and *Phaseolus vulgaris* L. by ionic exchange DEAE cellulose column, inhibited the *R. ferrugineus* gut α-amylase activity by 19 ± 0.64 % and 11.56 ± 0.69 %, respectively.

Key Words: *Rhynchophorus ferrugineus*; gut and hemolymph α-amylases; common bean; green mung bean; inhibitors

Introduction

α-amylases (α-1,4-glucan-4-glucanohydrolases; E.C. 3.2.1.1) constitute a family of hydrolyses that cleave α-D-(1,4)-glucan linkages in starch components, glycogen and various other related carbohydrates. α-amylases are digestive enzymes that play an essential role in starch digestion and are consequently involved in energy production in insects (Pelegrini *et al*., 2006). Many studies have focused on the characterization of the digestive α-amylases of economically important insect pests including *Rhyzopertha dominica* (Baker, 1991), *Tenebrio molitor* (Buonocore *et al*., 1976), *Zabrotes subfasciatus* (Pelegrini *et al*., 2006), *Naranga aenescens* L. (Asadi *et al*., 2010), *Xanthogaleruca luteola* Mull. (Sharifi *et al*., 2011) and *Eurygaster integriceps* (Kazzazi *et al*., 2005).

Insect control strategies interfering with α-amylases, and thus food digestion, are known to reduce insect survival and growth, and, for this reason, many studies have focused on characterizing the effects of digestive α-amylase inhibitors on insect α-amylases. Disruption of carbohydrate digestion by transformation of plant genomes with α-amylase inhibitors represents an alternative approach to the control of insect pests. α-amylase inhibitors naturally occur in many plants and are particularly abundant in cereals and legumes, as part of natural defense mechanisms against herbivores insects (Carlini and Grossi-de-Sá, 2002; Franco *et al.,* 2002). Many insecticidal proteins and molecules of plant origin such as lectins, and α -amylase and protease inhibitors can retard insect survival, growth and development when ingested (Boulter, 1993; Ussuf *et al.,* 2001). When the α-amylase inhibitor gene from the common bean was expressed in transgenic peas, seeds became resistant to the infestation of bruchid

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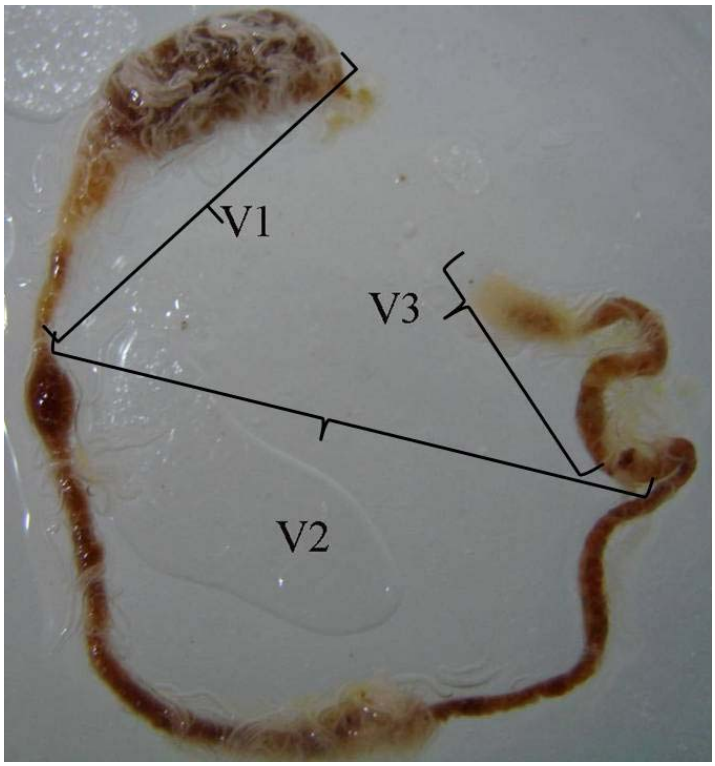

Fig. 1 Different parts (V1, V2 and V3) of the gut in the larvae of *R. ferrugineus.*

weevils (Shade *et al.,* 1994; Shroeder *et al.,* 1995). The α-amylase inhibitor markedly inhibited the α-amylase activity in the larval midgut of the weevils (Ryan, 1990; Ishimoto and Chrispeels, 1996).

The red palm weevil, *Rhynchophorus ferrugineus* is widely considered to be the most damaging insect pest of palms in the world. This pest is usually attracted from unhealthy palm trees, but they will often attack healthy palms too. *R. ferrugineus* larvae feed within the apical growing point of the palms, causing extensive damage to palm tissues and to the structure of the palm trunk. Good sanitation practices are needed to prevent the red palm weevil spreading from infested palms (Murphy and Briscoe, 1999). Because of the concealed nature of the larvae, effective methods for the management of the red palm weevils have been difficult to develop. Current methods recommended for the management of *R. ferrugineus* have focused on integrated pest management (IPM) strategies involving surveillance, pheromone lures, cultural control methods and chemical treatments (Murphy and Briscoe, 1999). Insecticides are probably the most common control tools used against the red palm weevil in Iran, and can be applied in a variety of ways for *R. ferrugineus* suppression including applications as dusts, and/or liquid sprays. Trunk injections or soil applications of systemic insecticides that move inside the palm poisoning weevil larvae and adults may also be effective (Murphy and Briscoe, 1999). However, because of the many problems associated with the use of synthetic insecticides in

Table 1 The mean α-amylase specific activity (±SE) in guts of adults and last instars and hemolymph of last instars of *R. ferrugineus.*

Tissue	Activity of α-amylase (µmol/min/mg protein)
V1 segment of larval gut	3.16 ± 0.022^{a}
V2 segment of larval gut	2.21 ± 0.033^{b}
V3 segment of larval gut	1.32 ± 0.0055^{c}
Adult female whole gut	2.06 ± 0.011^{b}
Adult male whole gut	3.05 ± 0.03^{a}
Larval gut	2.98 ± 0.03^{a}
Last instars hemolymph	0.27 ± 0.003^{d}

Different letters indicate that the activity of enzymes in different tissue are significantly different from each other by Tukey's test ($p < 0.05$).

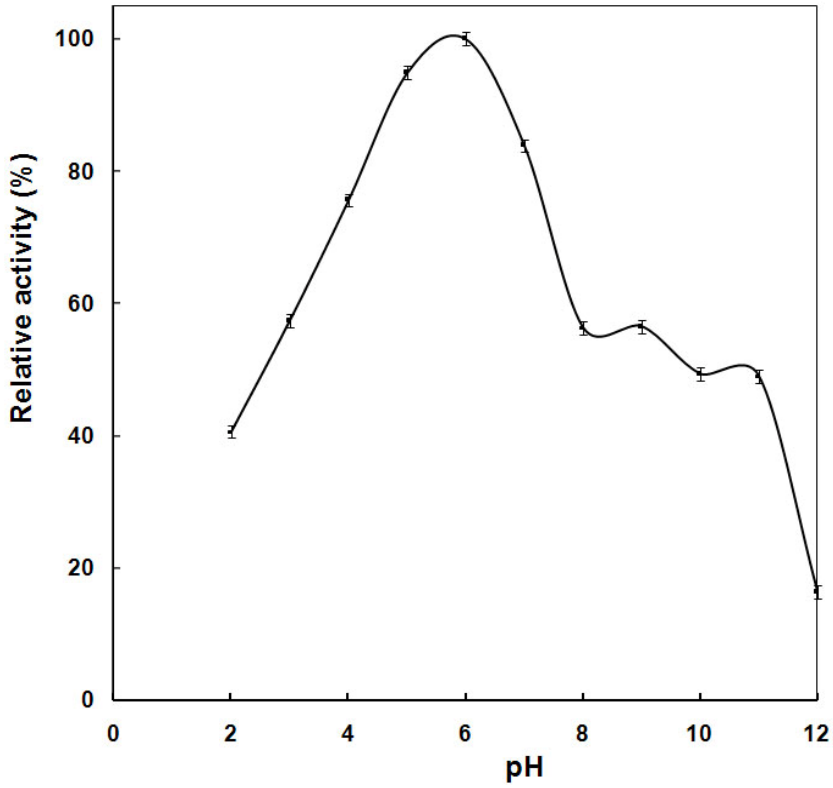

Fig. 2 The effect of pH on hemolymph α-amylase activity in larvae of *R. ferrugineus.*

integrated pest management approaches, the use of chemicals against this pest presents some difficulties. Therefore, the use of genetic engineering to produce pest resistant transgenic plants offers an alternative strategy for the control of this pest. Due to the significant damage caused by the red palm weevil, we attempted to characterize its gut and hemolymph α-amylases and to study its inhibition by inhibitors extracted from the common bean and the green mung bean.

Materials and Methods

Insect

The insect was collected from the palm trees in the Sistan and Baluchestan province of Iran. The final instars larvae and male and female adults were randomly selected for measuring the enzyme activity.

Enzyme sample preparation

Final instars larvae were immobilized on ice and dissected under a stereomicroscope in ice-cold saline buffer (10 µM NaCl, pH = 7). Whole parts of the gut (experimentally divided into V1, 2 and 3 segments) were removed from the body and thoroughly rinsed in ice-cold distilled water. Each digestive system was homogenized in a known volume of cold double-distilled water using a hand-held glass homogenizer before measuring the optimum pH. Also, the sample for measuring specific activity and effect of temperature on enzyme activity were prepared in buffer (optimum pH specific for each enzyme). The homogenates were centrifuged at 13,000×g for 15 min at 4 °C and supernatants were used for enzyme activity assays. Also the hemolymph was collected from larvae. Small incisions were made in the soft cuticle anterior to the second thoracic segment of larvae and the hemolymph was collected with a 75 µl glass capillary tube.

Chemicals

3,5-dinitrosalicylic acid (DNS), triton X-100 and EDTA (ethylenediaminetetra acetic acid) were purchased from Sigma (St. Louis, MO, USA). DEAE (Diethylaminoethyl) cellulose was obtained from Bio-Rad Laboratories Ltd. (UK). All other chemicals (reagent grade) were from Merck (Merck, Darmstadt, Germany).

Determination of α-amylase activity and protein concentration

α-amylase activity was determined at room temperature in 20 mM acetate-citrate (in optimum pH for each tissue). Supernatant (10 µl) was added to a tube containing 40 µl of the buffer and 50 µl of 1 % (w/v) starch. The concentration of reducing sugars obtained from the catalyzed reaction for 30 min was measured by the DNS method according to

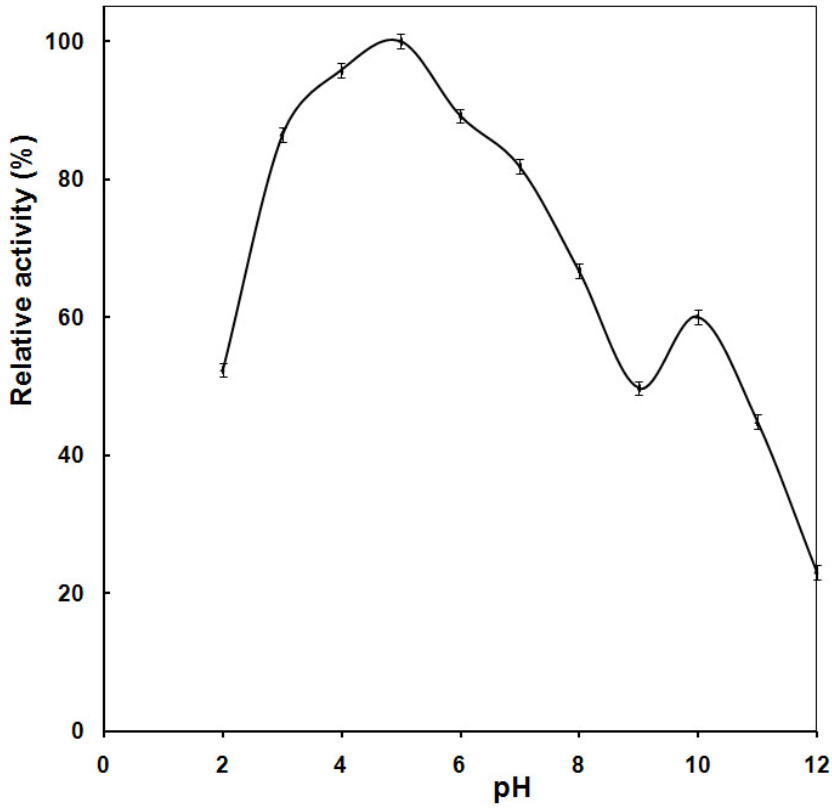

Fig. 3 The effect of pH on gut α-amylase activity in larvae of *R. ferrugineus*.

Bernfeld (1955). Absorbance was measured at 545 nm with a Microplate Reder Model Stat Fax® 3200 (Awareness Technology Inc.). One unit of α-amylase is defined as the amount of enzyme that liberates 1.0 µmol of reducing sugar/min with maltose as a standard. Protein concentration was determined by the Bradford's method (1976) with bovine serum albumin (BSA) as standard.

Effects of pH and temperature on α-amylase activity

The pH profiles of the α-amylase activity were determined at room temperature in a mixed buffer containing phosphate, glycine and acetate (25 mM of each) adjusted to various pHs (pH from 2 to 12) by adding HCl or NaOH for acidic and basic pH values, respectively. Before determining activity, the reaction mixtures were incubated at different pHs at room temperature for 5 min. The activity of α-amylase was determined by incubating the reaction mixtures in optimal pH value (20 mM acetate-citrate) at different temperatures ranging from 20 to 60 °C with 10 °C intervals.

Polyacrylamide gel electrophoresis and zymogram analysis

Non-denaturing polyacrylamide gel electrophoresis (PAGE) (8 %) was carried out as described by Davis (1964) and electrophoresis was performed applying a 100 V electric field at 4 °C. Afterward, the gel was incubated in 2.5 % (v/v) Triton X-100 for 30 min at room temperature with gentle agitation. Then, the gel was rinsed with distilled water and washed in 25 mM Tris-HCl (pH 7.4). The washed gel was incubated in fresh acetate buffer (pH 5) containing 1 % (w/v) soluble starch at 30 ºC for 60 min. After being washed with distilled water, the gel was subjected to staining with Lugol solution (I_2 1.3 % and KI 3 %) at room temperature until the appearance of clear zones in protein bands with α-amylase activity against a dark blue background.

Kinetic parameters of α-amylases

Catalytic activities of the enzymes were investigated at different concentrations of starch and glycogen over the range 0.05 - 1.5 % and 0.1 - 3 % (w/v) in 20 mM acetate (pH 5), respectively. The Michaelis-Menten constant (K_m) was estimated from the Lineweaver-Burk plots.

Purification of V. radiata *and* P. vulgaris *α-amylase inhibitors from seeds*

Seeds were ground into flour and extracted with 0.15 M NaCl with continuous stirring for 1 h at 4 °C. The materials were then centrifuged at 6,000×g at 4 °C for 30 min. Supernatants were submitted to 80 °C and centrifuged at 6,000×g for 15 min. The supernatant was then submitted to fractionation with ammonium sulfate (with 80 % saturation). After dialysis against a 20 mM Tris-HCl, pH 7, buffer, the

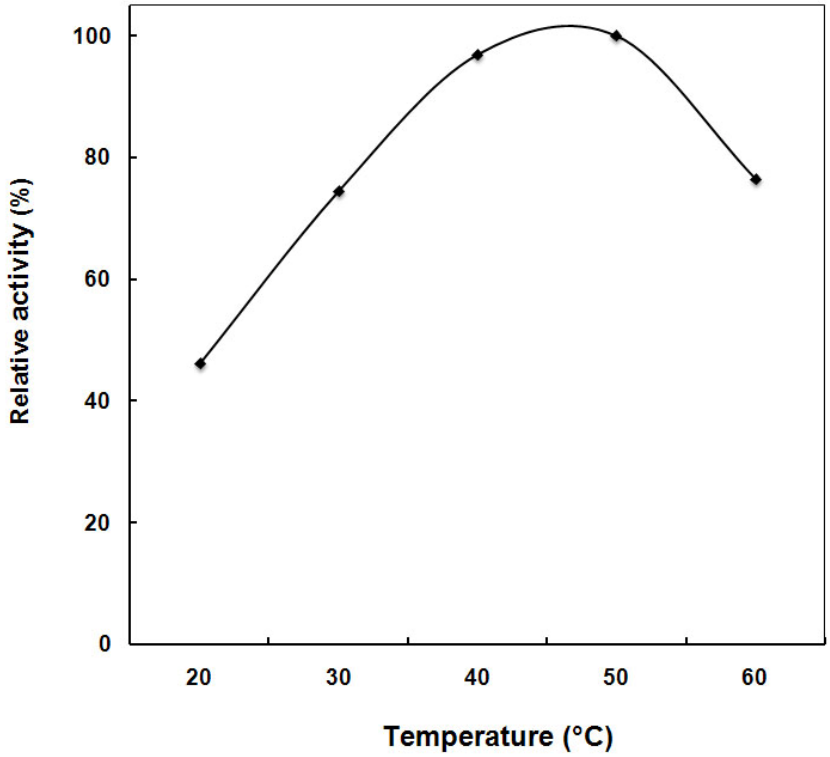

Fig. 4 The effect of temperature on gut α- amylase activity of last instar larvae *Rhynchophorus ferrugineus.* The relative activities were based on the ratio of the activity obtained at a temperature to the maximum activity obtained at that range and expressed as a percentage. Different letters indicate that the activity of enzymes in different temperature is significantly different from each other by Tukey's test ($p < 0.05$).

fractions were applied to an ionic exchange DEAE cellulose column equilibrated with 20 mM Tris-HCl buffer (pH 7.0), with a flow rate of 0.5 ml/min. The column was eluted with a linear NaCl gradient of 0 - 0.5 M at the flow rate of 0.5 ml/min. The absorbance of the effluent was monitored at 280 nm in a BioPhotometer plus (Eppendorf, Germany)

Amylase inhibition assay

Ten µl of the enzyme sample (protein content = 45 µg) was pre-incubated with 10 µl of inhibitor and 30 µl of acetate buffer (pH 5) for 30 min at 37 °C; then the same procedure was applied to the amylase and its activity was determined by measuring absorbance at 540 nm. Experiments were performed in four replicates.

Statistical analysis

The data were compared by one-way analysis of variance (ANOVA) followed by Tukey's test when significant differences were found at $p = 0.05$ using SAS program (SAS Institute, 1997).

Results

α-amylase activity

The activity of α-amylases was characterized in crude extracts of *R. ferrugineus*. The results showed that there were significant differences between the α-amylase activities in tissues and that the highest and the lowest activities were detected in the guts of last instars and adult males, and in the hemolymph, respectively. The results showed that there were significant differences between the amylases activities in different parts of the digestive system and the highest activity was detected in the part V1 (Fig. 1, Table 1).

The effect of pH and temperature on enzyme activity

The influence of pH on the gut and hemolymph α-amylase activity is shown in the Figs 2 and 3. Hemolymph α-amylase was most active at pH 6, while α-amylase extracted from digestive systems showed highest activity at pH 5. The pH activity profile of hemolymph α-amylase was distributed along a very broad pH range (3 - 7) and the enzyme activity retained more than 55 % of its maximal activity in the pH range of 3 to 8. As is shown in Fig. 4, the optimal temperature for gut α-amylase was 40 - 50 °C. The substrate was hydrolyzed at a broad range of temperatures (20 - 60 °C).

Zymogram analysis of α-amylase at different pH

The crude *R. ferrugineus* larval extracts were analyzed by native PAGE. After α-amylase activity staining, two isoforms of α-amylase were clearly detected in the digestive system of the last instars. However, as depicted in Fig. 5, the light intensity of bands representing α-amylase activity was lower

with a relative mobility (rm) of 0.72 instead of 0.92. Zymogram of gut α-amylase from larval *R. ferrugineus* at different pHs is shown in Fig. 6. α-amylase activity was observed at pHs 3, 4, 5, 6, 8 and 9. Maximum in-gel α-amylase activity was determined at pH 5, which is consistent with the results from our tube assays (Fig. 6).

Kinetic parameters of α-amylases

Results showed that kinetic behavior of *R. ferrugineus* α-amylase toward starch and glycogen was significantly different. As calculated from Lineweaver-Burk plots, when starch and glycogen were used as substrates, the K_m values for hemolymph α-amylase were 0.1665 and 2.159 %, respectively. Also, when starch and glycogen were used as substrates, the K_m values for gut α-amylase from last instars were 1.375 and 0.541 %, respectively (Table 2).

Effects of P. vulgaris *and* V. radiata *α-amylase inhibitors on* R. ferrugineus α-*amylase activity*

Ammonium sulfate fraction of inhibitors was further fractionated on an ionic exchange DEAE cellulose column. The profile of *P. vulgaris* inhibitors showed three major peaks and three minor peaks (Fig. 7). Also, the chromatogram of *V. radiata* inhibitors showed three major peaks and 6 minor peaks. Assay of peaks revealed that one peak of *P. vulgaris* inhibited the *R. ferrugineus* gut α-amylase by 11.56 ± 0.69 %, while the others did not. In the chromatogram obtained from *V. radiate* the peak number 17 had the highest inhibitory effect on α-amylase activity.

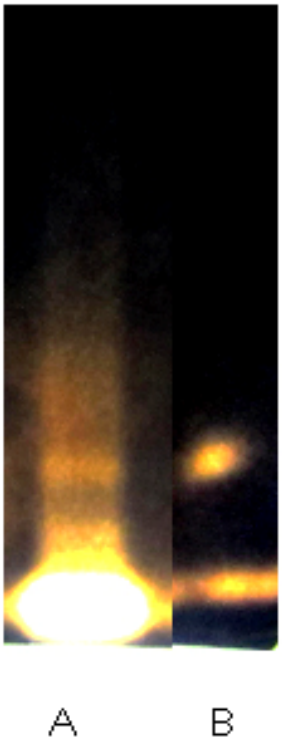

Fig. 5 Zymogram analysis of α-amylase activity from larval *R. ferrugineus*. A, gut; B, Hemolymph.

Discussion

This study has clearly demonstrated α-amylase activity in the digestive system and hemolymph of the red palm weevil's last instars, and male and female adults. The specific activity of gut α-amylase derived from adult males was found to be higher than that of females and hemolymph of last instars (Table 1). Sharifi *et al.* (2011) showed that the specific activity of gut α-amylase from last instars of *X. luteola* was 1.46-fold higher than that of adults. Reports of differences in digestive enzyme activities between male and female insects are contradictory. In numerous insects, females have additional enzymes along the digestive tract with respects to males, apparently to meet the metabolic demands of egg production. However, in some insect species, males show higher enzyme activity than females. Mandal *et al.* (1981) showed that the protease activity in *Schizodactylus monstrosus* was higher in adult males compared to females and larval stages. Investigation of midgut trypsin and chymotrypsin specific activities in adult castes of four *Polister* species showed that chymotrypsin activity in males of *P. mericus*, *P. fuscatus* and *P. exclamans* were 1.97-, 2.78- and 1.13- fold higher than in females, respectively. Also, trypsin activity in males of *P. mericus* and *P. fuscatus* were 2- and 2.65- fold higher than in females, respectively (Kayes, 1978). α-amylase is present in all regions of the alimentary canal of the last larval instar of *R. ferrugineus* (Table 1). Our results show a significant difference in the activity of α-amylase in V1, V2 and V3 gut regions of last larval instar (Fig. 1, Table 1). The rank order, from the highest to the lowest α-amylase activity was V1>V2>V3. The alimentary canal of the pistachio green stink bug, *Brachynema germari* Kolenati (Hemiptera: Pentatomidae) was divided into four distinct regions (v1, v2, v3 and v4) by Ramzi and Hosseininaveh (2010) and activities of α-amylase and α- and β-glucosidases were measured in these parts. Their results showed that the highest α-amylase and α- and β-glucosidase activities were observed in v3, whereas the lowest activities were measured in v4. Also, Sharifi *et al.* (2011) divided the alimentary canal of elm leaf beetle into three distinct divisions and their results showed that the α-amylase activity in the midgut of last instars was 3.125- and 4.16-fold higher than that in foregut and hindgut, respectively.

Our results show that the highest activities of gut and hemolymph α-amylase are in 4-5 and 5-6 pH ranges, respectively (Figs 2, 3). α-amylase in *R. ferrugineus* was active in acidic pH, which is consistent with the optimal neutral to slightly acidic conditions reported for other coleopteran species (Baker, 1983; Terra *et al.*, 1996). Optimal pH values for α-amylases in larvae of several coleopterans range from 4 to 5.8 (Baker, 1983). α-amylase in *H. postica* larvae have an acidic optimal pH (pHs 3 - 6) (Vatanparast and Hoseininaveh, 2010). Also, α-amylase extracted from *Hypothenemus hampei* has an optimal activity at pH 5 (Valencia-Jimenez *et al.*, 2000). The optimal pH values were found at pH 5.2

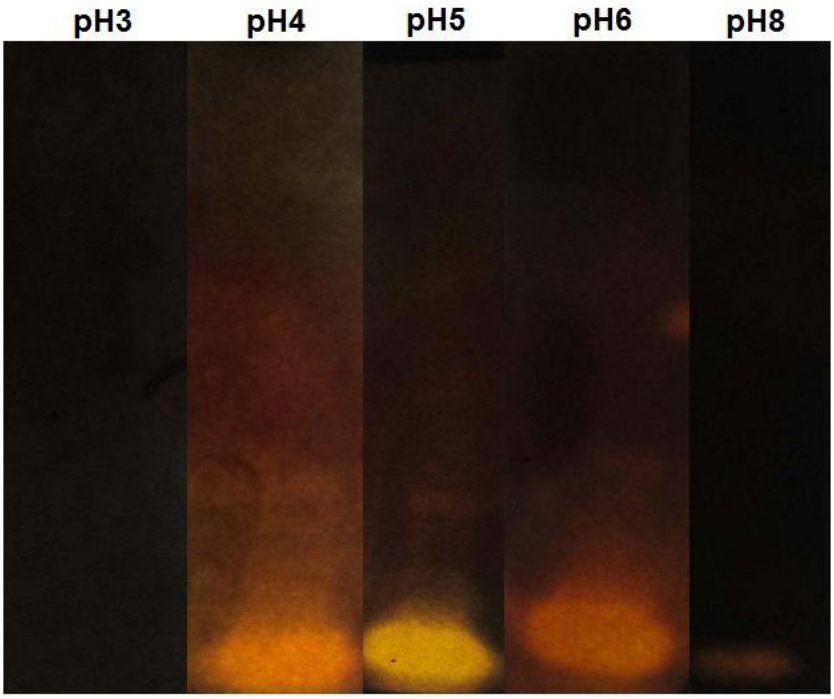

Fig. 6 Zymogram analysis of gut α-amylase activity from larvae of *R. ferrugineus* at different pHs. As shown, the α-amylase activity is affected by pH variations.

for *Callosobruchus chinensis* (Podoler and Applebaum, 1971) and pH 5.4 for *Tribolium castaneum* (Applebaum and Konijn 1965). However, in some coleopterans such as *Trogoderma granarium* (Dermestidae), optimum α-amylase activity mostly occurs at alkaline pH range (*e.g.*, 6 - 9) (Hosseininaveh *et al*., 2007). Sharifi *et al.* (2011) showed that in the case of larval *X. luteola,* the optimum pH for gut α-amylase activity was 5. Asadi *et al*. (2010) showed that the optimum pHs for α-amylases midgut, salivary and hemolymph α-amylases of *Naranga aenescens* were 8, 9 and 9, respectively. The maximum amylase activity at pHs 5 - 6 observed in the hemolymph of *R. ferrugineus,* which is consistent with physiological pH of the hemolymph as reported for most insects. Also, the optimum pH 6.8 was reported for hemolymph amylase in *Bombyx mori* (Abraham *et al.*, 1992). Activity of digestive α-amylase in the larval gut of *R. ferrugineus* was highest at 40 - 50°C. Optimum temperature for *H. posticae* α-amylase has been reported as 35°C (Vatanparast and Hosseininaveh, 2010).

The activity of α-amylase was also characterized by zymogram analysis after native PAGE which allowed visualization of the enzyme activity *in situ*. The results indicated two isoforms of α-amylase in crude gut and hemolymph of the last instar with the same electrophoretic pattern (Fig. 5). However, as depicted in Fig. 5, intensity of both bands showing α-amylase activity in hemolymph were less than that in the gut, which is correlated well with α-amylase activities in crude extracts.

Our results from a zymogram analysis of α-amylase activity at different pHs f show two bands in the extracts from the larval digestive system of *R. ferrugineus*. No α-amylase activity was observed at pH 2. Maximum in-gel α-amylase activity was determined at pH 5 based on the intensity of bands (Fig. 6). In other coleopteran insects, the gut α-amylases of *X. luteola* (Sharifi *et al.*, 2011) and *Osphranteria coerulescens* Redt. (Saberi Riseh and Ghadamyari, unpublished results) has present just one isoform. The number of α-amylases identified in different insect species varied from 1 to 8 isoforms *e.g., Helicoverpa armigera*, *Spodoptera litura*, *Callosobruchus chinensis* and *Carcyra cephalonica* exhibited more than five isoforms whereas *Sitophilus oryzae* and *Tribolium castaneum* possess only one isoform (Sivakumar *et al*., 2006). Wisessing *et al*. (2008) showed that *Callosobruchus*

Table 2 K_m values of α-amylases from gut and hemolymph of larval *R. ferrugineus* on starch and glycogen.

	Substrate	
Tissue	Glycogen	Starch
Gut	0.541±0.08	1.375±0.02
Hemolymph	2.159±0.01	0.1665±0.005

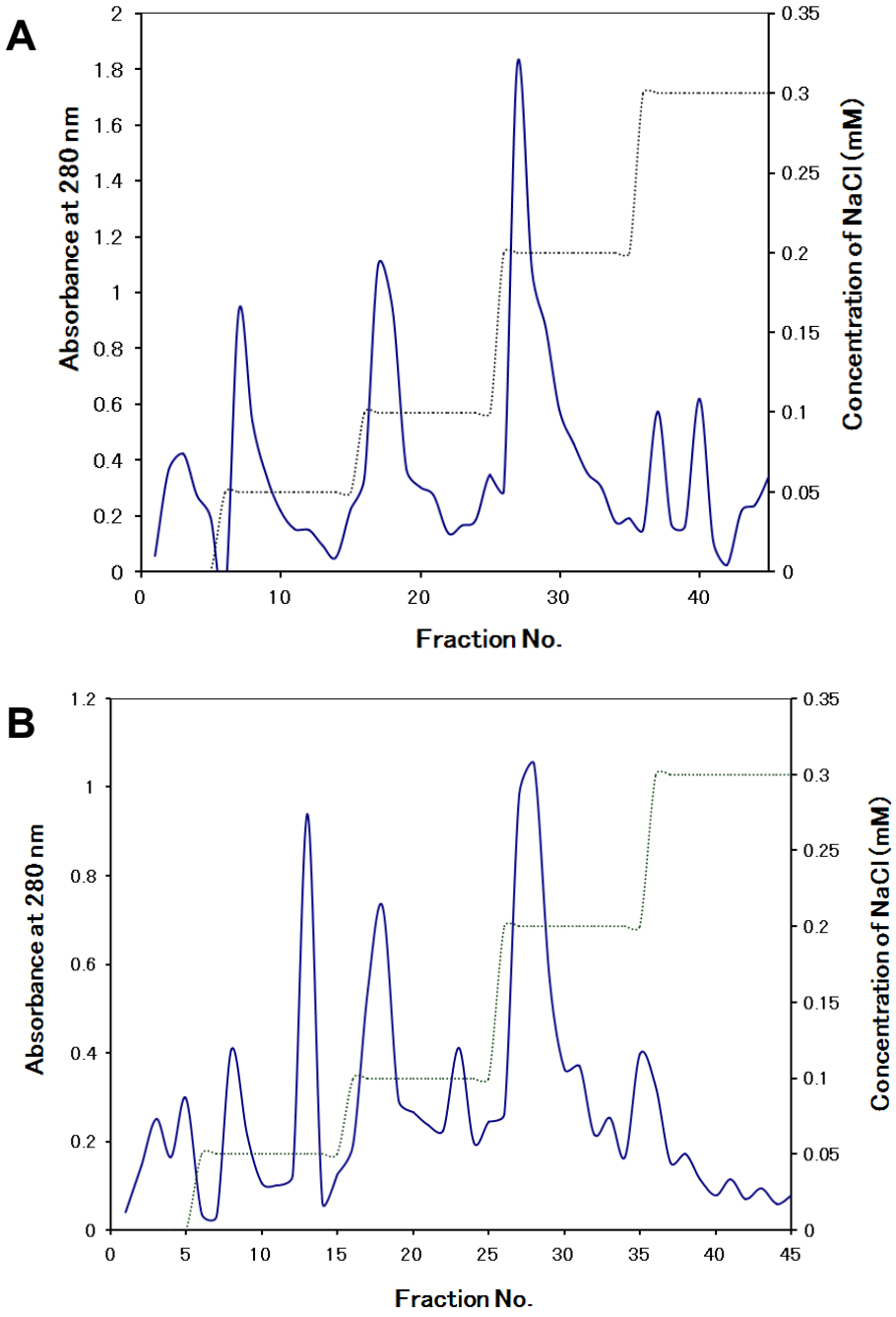

Fig. 7 Retained fraction obtained from ionic exchange DEAE cellulose chromatography, equilibrated with 20 mM Tris-HCl buffer, pH 7.0, with a flow rate of 0.5 ml/min. Dashed line represents 0 - 0.5 M NaCl linear gradient. a) and b) chromatograms for inhibitors extracted from *P. vulgaris* and *V. radiata*, respectively.

maculatus α-amylase had one isoform with a molecular weight of 50 kDa. Two α-amylase isoforms in the gut extracts of two species of *Sitophilus*, including *S. zeamais* and *S. granarius* (Baker,1983), *Prostephanus truncatus* (Mendiola-Olaya *et al.,* 2000) and *Hypothenemus hampei* (Valencia-Jimenez *et al.,* 2000) were reported.

V. radiata and *P. vulgaris* seeds seem to contain a number of *R. ferrugineus* α-amylase inhibitors that can be separated by ion-exchange chromatography. These proteins show α-amylase inhibitory activity against *R. ferrugineus* gut α-amylases. Our results confirmed that α-amylase inhibitors, purified from *V. radiata* and *P. vulgaris* by ionic exchange DEAE cellulose column, exert inhibitory activity on *R. ferrugineus* gut α-amylase. The peak number 37 (NaCl concentration 0.3 mM; protein concentration = 0.93 mg/ml) from *P. vulgaris* and peak number 17 (NaCl concentration 0.1 mM; protein concentration = 1.17 mg/ml) from *V. radiata*

inhibited α-amylase activity by 11.56 ± 0.69 % and 19.8 ± 0.64 %, respectively. Plant α-amylase inhibitors show a great potential as tools to engineer the resistance of crop plants against pests. The *in vivo* effect of the α-amylase inhibitor on mortality of *C. maculatus* showed that the α-amylase inhibitor purified from *V. radiata* acts on *C. maculatus* during the developmental stage, by reducing carbohydrate digestion necessary for growth and development, rather than during the egg laying/hatching stage (Wisessing *et al.,* 2010). Also, Engkagul *et al.* (2004) reported that crude protein extracts from the Kamphaengsaen 1 variety of mung bean (KPS1) inhibited the *C. maculatus* α-amylase, which in turn prevents growth and development of insect larvae infesting seeds. These α-amylase inhibitors, are attractive candidates for seed weevil biocontrol, and have been purified and characterized from different varieties of common bean including the white kidney bean (Yamaguchi, 1991), red kidney bean and black kidney bean (Lajolo and Finardi-Filho, 1985).

In conclusion, protein inhibitors active against different types of hydrolytic enzymes are widely distributed in plants. These inhibitors play a protective role against insect attack. The transgenic expression of insecticidal proteins such as α-amylase inhibitors is being evaluated as a potential protective strategy against insects (Schuler *et al*., 1998). Our results show that the inhibitor extracted from *V. radiata* seeds has a more potent inhibitory activity against *R. ferrugineus* gut α-amylase compared to the inhibitor extracted from *P. vulgaris* seeds.

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