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Original Contribution

α-AMYLASE ACTIVITY IN THE SALIVARY GLANDS AND THE MIDGUT OF *APODIPHUS AMYGDALI* GERMAR (HEMIPTERA: PENTATOMIDAE)

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ABSTRACT

In the current study, activities of α-amylase were determined and characterized in the salivary glands and the midgut of an orchard pest, *Apodiphus amygdali*. It was found that activity of the α-amylase in midgut was statistically higher than that of salivary glands. Optimal pH of amylolytic activities were obtained to be 8 for salivary glands and 5 for midgut. The enzymes extracted from salivary glands and midgut had the highest activities at temperatures of 45 and 40 °C, respectively. Mono- and di-valent cations significantly changed amylolytic activities in the midgut and salivary glands of *A. amygdali*. In case of salivary glands, Na^+, K^+ and Mg^{2+} showed no effects but Ca^{2+} and Cu^{2+} increased the enzymatic activity. Na^+ and Mg^{2+} decreased midgut α-amylase of *A. amygdali* but Ca^{2+} showed adverse results. Lieweaver-Burk analysis of the enzyme in salivary glands and midgut revealed V_{max} of 11.23 and 5.88 (U/mg protein) as well as K_m of 6.85 and 2.58 (%), respectively. Since α-amylase has critical role in carbohydrate digestion of insects, those have been targeted by several researches to develop inhibitors in resistant varieties. But characterization of the enzymes is the initial and major step to reach such an objective.

Key words: *Apodiphus amygdali*, α-amylase, characterization

INTRODUCTION

Digestion in insects is a multiple steps that ingested macromolecules are broken down as monomers to be absorbed via epithelial cells of midgut. Several macromolecules such as carbohydrates, proteases and lipids are ingested that require relevant enzymes, carbohydrases, proteases and lipases, to be digested. Carbohydrases are divided into several enzymes like amylases, β-Glucanases, Xylanases and Pectinases, Chitinases and Lysozymes, α-Glucosidases, β-Glucosidases and β-Galactosidases, Trehalases, Acetylhexosaminidases, β-Fructosidases and α-Galactosidases (1). In case, α-amylase seems to be more crucial since they are the first enzymes that encounter carbohydrates in the midgut because the enzymes break macromolecule from internal bonds. α-Amylases (EC 3.2.1.1) are a type of hydrolyzes that catalyze breaking-down of starch and glycogen from inner long α-1,4-glucan chains (2). General properties of the enzymes are molecular weight of 48–60 kDa, pI values of 3.5–4.0, and K_m values with soluble starch around 0.1% (1). Meanwhile, the enzyme is calcium-dependent and it is activated by chloride with displacement of the pH optimum (1).

Apodiphus amygdali Germar (Hemiptera: Pentatomidae) is an hemipteran pest of fruit trees by wide distribution in Europe and Middle East (3). Host trees contains plum, apricot, apple, olive, pear and pistachio in addition to non-fruit trees like poplar, pine, plane-tree, elm and willow bark (3). *A. amygdali* utilize salivary secretions to liquefy plant tissues, pump the liquid food to its midgut where the main digestive process are made by recruiting relevant enzymes. Feeding activities of the pest causes host weakness and attracts other insects to feed on host plants. Feeding on fruits causes complete degradation and yield loss (4).

Spraying of chemical insecticides leads to severe concerns like resistant of pest to used chemicals, environmental pollution, effects on

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non-target organisms, chemical residues in agricultural products and etc. This point goes to be more critical when direct spraying are made on trees where their fruits are consumes by human. Hence, adoption of other control procedures seems to be important. One of the promising pest control could be development of resistant varieties by using inhibitors of digestive enzymes. α-Amylase is one of the targeted digestive enzymes to develop inhibitors. There are Six different α-amylase inhibitors in insect control including lectin-like, knottin-like, cereal-type, Kunitz-like, c-purothionin-like, and thaumatin-like that may be useful in pest control (5). These inhibitors show structural diversity, different modes of inhibition, and different specificity profiles against a diverse range of α-amylases (5). To reach such an objective, characterization of target enzymes is mandatory to design or screen of an inhibitor. So, the objective of the current study were to determine amylolytic activity in the salivary glands and the midgut of *A. amygdali*, optimal pH and temperature for enzymatic activity, effects of cations and kinetic study.

MATERIALS AND METHODS

Fifth nymphal instars of *A. amygdali* was collected from elm trees in Shiraz (Fars province) and transferred to laboratory. Then, the nymphs were reared on elm leaves at 28±1 °C, 70% of humidity and 16 L:8D photoperiods. Adults were randomely selected and used for biochemical experiments.

Sample preparation

Dissection of adults were carried out by the method of Cohen (6). Salivary glands and midgut of *A. amygdali* were removed by dissection using a stereomicroscope in ice-cold saline buffer (NaCl, 10 mM). Removed tissues were rinsed in 1mL of ice-cold distilled water in the portions of five midgut and ten salivary glands. Tissues were grounded by an homogeniszer and centrifuged in 13,000 rpm for 20 min at 4 °C. Supernatant was carefully removed and transferred to new tubes and stored at -20 °C for subsequent experiments.

α-Amylase assay

The method described by Bernfeld (7) was used to determine α-amylase activity in the salivary glands and the midgut of *A. amygdali*. Briefly, 10 µl of the enzyme were incubated for 30 min at 35 °C with 50 µl of universal buffer (20 mM, Glycin, Succinate and 2-morpholinoethan sulfuric acid, pH 7) and 40 µl of soluble starch (1%). The reaction was stopped by adding of 100 µl dinitrosalicylc acid and heating in boiling water for 10 min prior to read absorbance at 545 nm. One unit of α-amylase activity was defined as the amount of enzyme required to produce 1 mg maltose in 30 min at 35 °C. A blank without substrate but with α-amylase extract and a control containing no α-amylase extract but with substrate were measured at the same time as the reaction mixtures.

Determination of optimal temperature and pH of the enzyme

The effects of temperature and pH on α-amylase activities in *A. amygdali* were determined by incubation of the reaction mixture in various temperature and pH sets. The effect of temperature was determined by incubating the reaction mixture at 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 70 °C for 30 min. Optimal pH was determined using universal buffer with pH set at 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12. Other steps were carried out as described earlier.

Effect of different cations on α-amylase activity

Different concentrations of mono- and di-valent cations (0, 1, 3 and 5 mM) were assayed to find their possible effects on the enzymatic activity. Used cations were Na+, K+, Ca2+, Cu2+ and Mg2+. Reaction mixture contained 50 µl of universal buffer, 20 µl of substrate, 20 µl of ion concentration and 10 µl of the enzyme. The experiment continued as described earlier.

Determination of kinetic values

Different concentrations of starch (0.2, 0.4, 0.6, 0.8 and 1%) were prepared and enzymatic activity was determined from samples of salivary glands and midgut. Obtained data were inserted in Sigma-Plot software to calculate V_{max} and K_m values.

Protein assay

Protein concentrations were assayed according to the method described by Lowry et al. (8).

Statistical analysis

All data were compared by one-way analysis of variance followed by Tukey's test when significant differences were found at $p \geq 0.05$ and marked in figures and tables with letters.

RESULTS AND DISCUSSION

Adults of *A. amygdali* was dissected under a stereomicroscope revealing an alimentary canal consists a four-sectioned midgut (V_1-V_4) **(Figure 1).** V1, V3 and V4 are the slender sections but V2 is a bulk-like section **(Figure 1).** Results of the biochemical experiments revealed significant presence of α-amylase in the salivary glands and the midgut of *A.*

amygdali adults so that the enzyme had higher activity in the midgut than the salivary glands **(Figure 2).** The obtained results were expectable since *A. amygdali* fed on plant tissues in which starch is the main storage components. Meanwhile, amylase of hemipteran salivary glands has major role in liquefying of plant tissues and the final and complete digestion of starch is made in midgut (9, 10). Several studies have reported presence of amylases in salivary glands and midgut of hemipterans. Mehrabadi et al. (11) determined amylolytic activity in the salivary glands and the midgut of *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae). Zibaee et al. (9) and Sorkhabi-Abdolmaleki et al. (12) reported amylolytic activity in saliva and midgut of *Andrallus spinidens* Fabricius (Hemiptera: Pentatomidae).

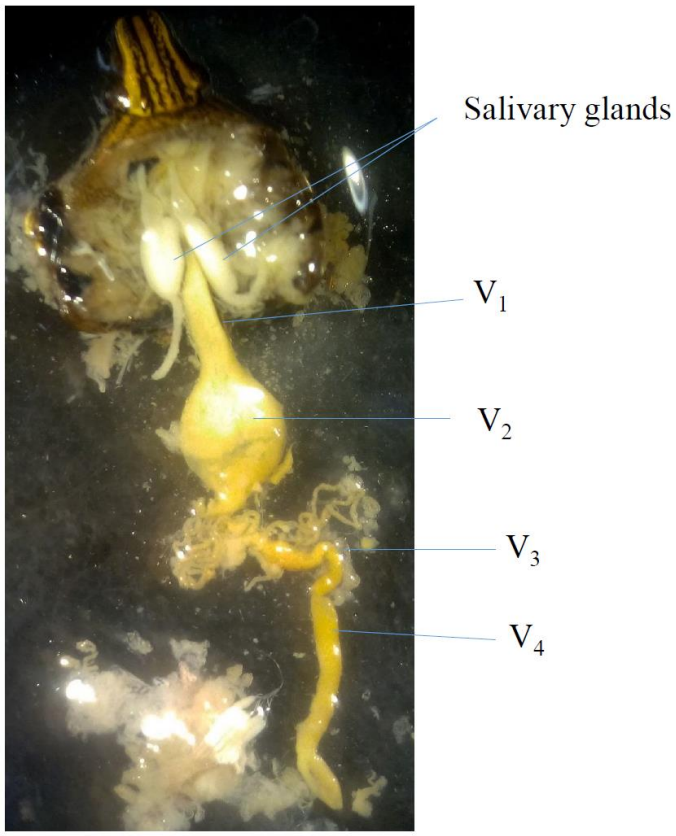

Figure 1. Morphology of the alimentary canal in the adults of *Apodiphus amygdali*.

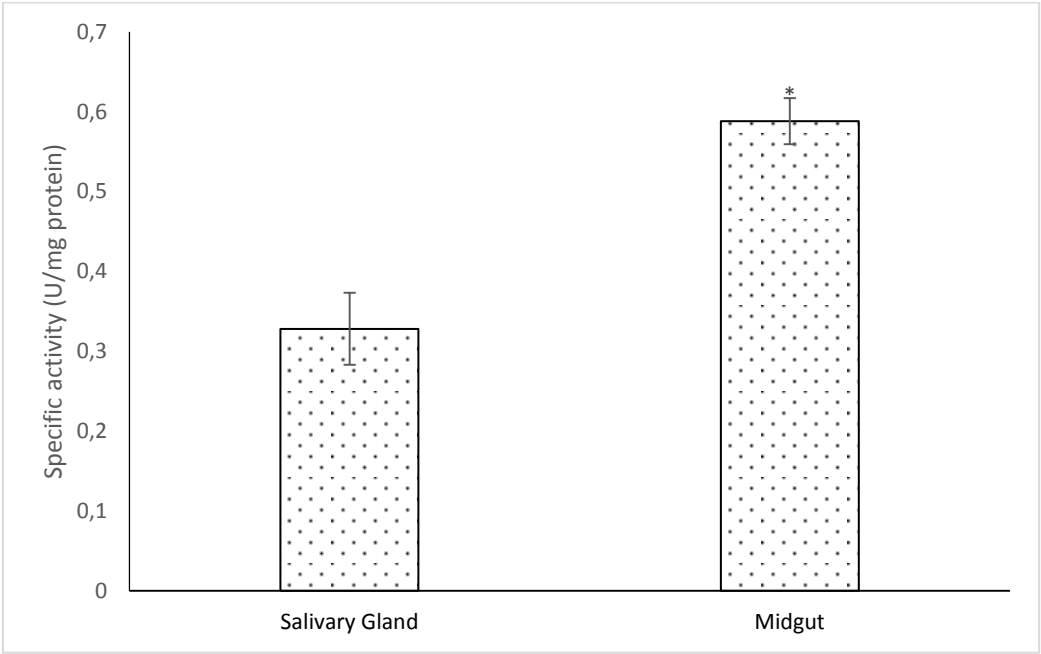

Figure 2. Comparison of amylolytic activity in the salivary glands and the midgut of *A. amygdali*. Statistical differences have been shown by asterisk (t-test, $p \leq 0.05$).

Enzymes showed various reaction toward substrates and inhibitors when they are exposed in various pH and temperature. Our results indicated that α-amylase in the salivary and the midgut of *A. amygdali* showed the highest activities in pHs 8 and 5, respectively (**Figure 3**; F: F: 74.32, Pr>F: 0.0001; F: 13.68, Pr>F: 0.0002). Meanwhile, activity of the enzyme from both sources increased from temperature 15 °C to optimal value then sharply decreased so that Optimal temperature of the salivary and midgut α-amylases were found 45 and 40 °C, respectively (**Figure 4**; F: 97.32, Pr>F: 0.0001; F: 64.56, F: 0.0001). Bandani et al. (13) found optimal pH of 6.5 and optimal temperature of 25-40 °C for purified α-amylase in the midgut of E.integriceps. Bezdi et al. (14) demonstrated optimal pH of 4.5 so salivary α-amylase activity in *E. integriceps*. Zeng and Cohen (15) reported optimal pH of 6 for α-amylase of *Lygus herperus* and *L. lineolaris*. Zibaee et al. (9) and Sorkhabi-Abdolmaleki et al. (12) found optimal pHs of 8 and 7 for salivary and midgut α-amylase of *A. spinidens*. In case of temperature, obtained values are similar to finding on other hemipterans like *E. integriceps*, *Lygus* spp and *A. spinidens* (9-15).

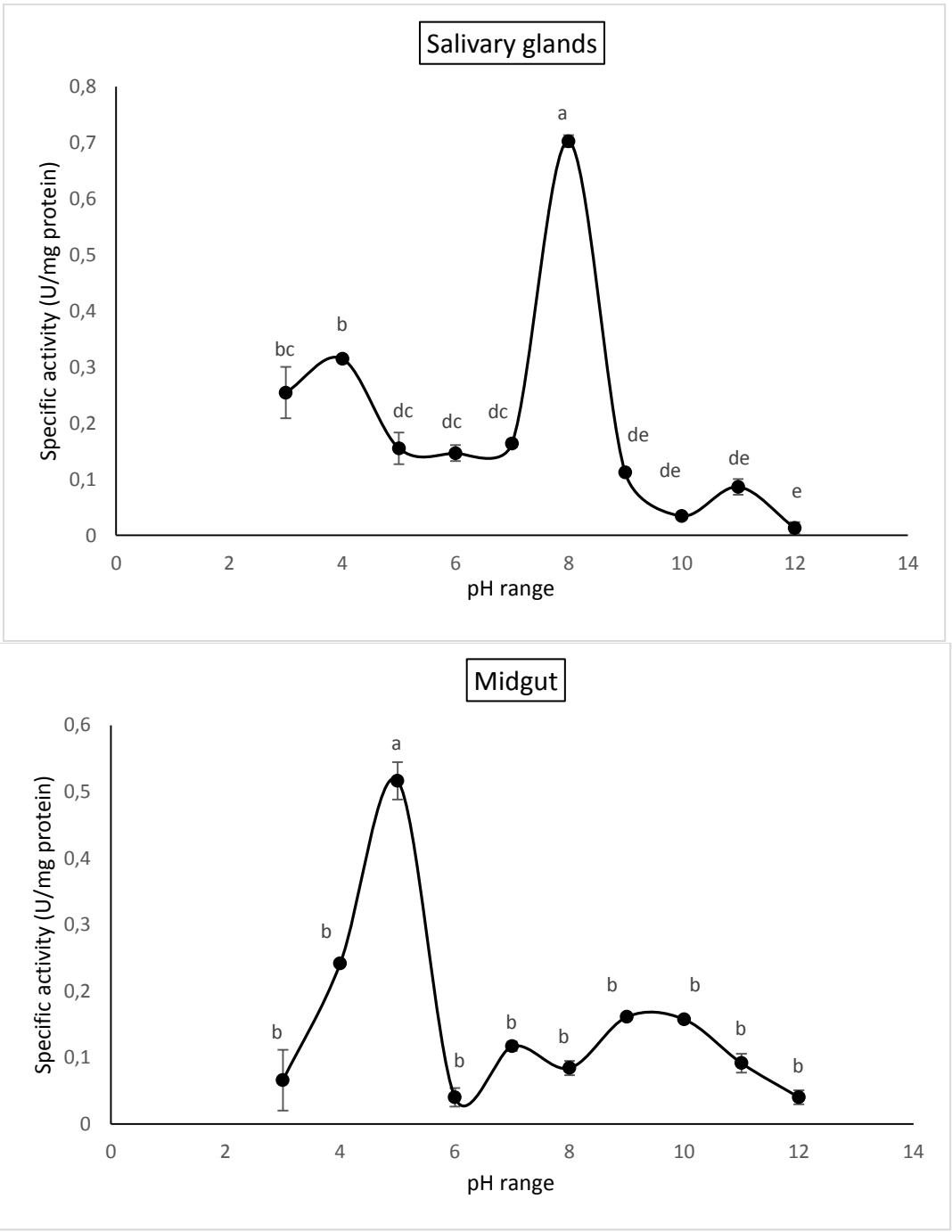

Figure 3. Optimal pH determination of α-amylase in the salivary glands and the midgut of *A. amygdali*. Statistical difefrences have been shown by various letter (Tukey test, $p \leq 0.05$).

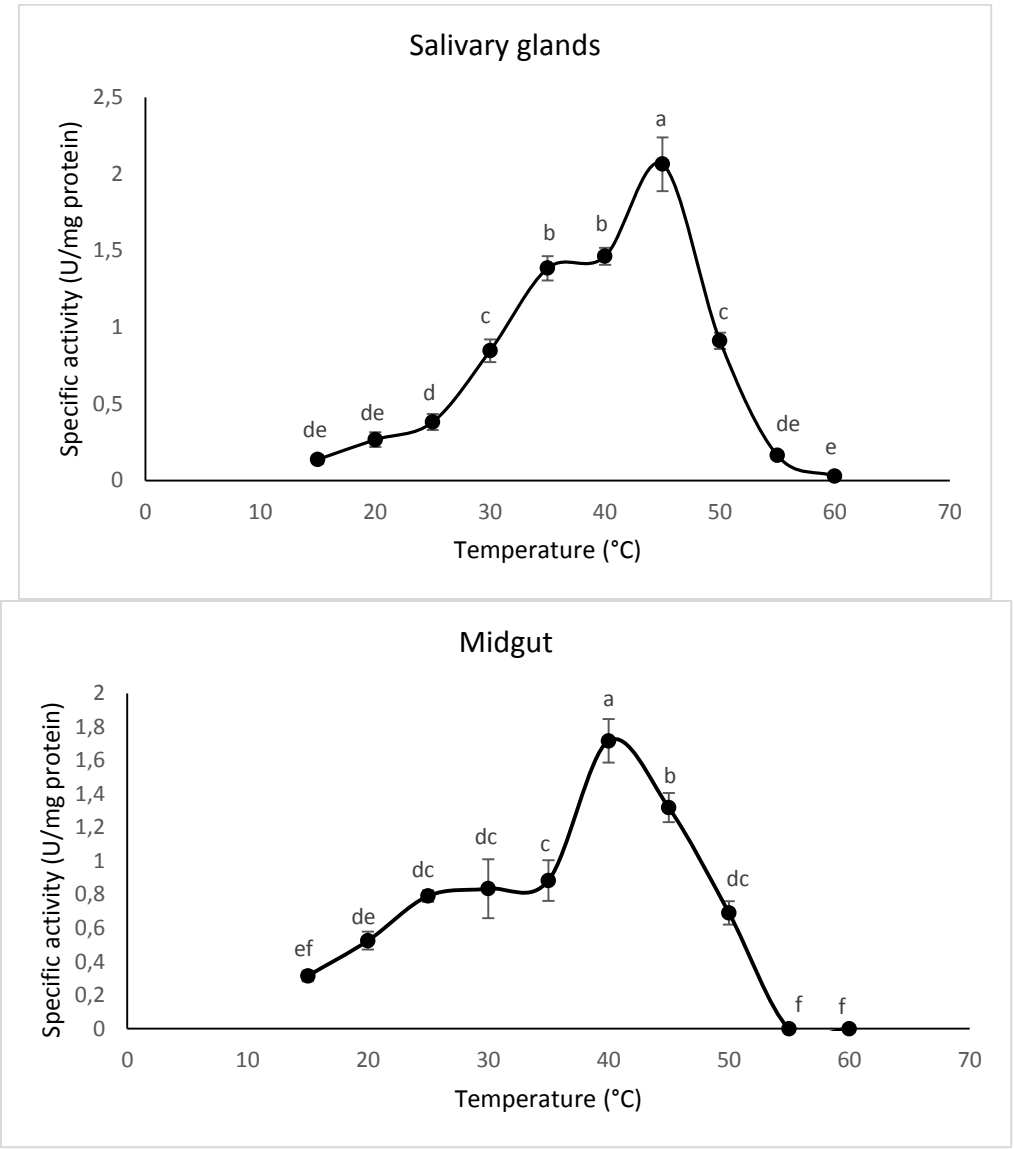

Figure 4. Optimal temperature (°C) determination of α-amylase in the salivary glands and the midgut of *A. amygdali*. Statistical difefrences have been shown by various letter (Tukey test, $p \leq 0.05$).

In agro-ecosystems, many fertilizers are used to improve quality and quantity of agricultural products. These compounds could affect various physiological processes of insects i.e. enzymatic activities. Moreover, many enzymes recruit ions in their active site or ions could serve as cofactors and increase or sometimes decrease the enzymatic activity. In the current study, Mon- and di-valent cations significantly changes amylolytic activities in the midgut and salivary glands of *A. amygdali*. In case of salivary glands, Na^+, K^+ and Mg^{2+} showed no effects but Ca^{2+} and Cu^{2+} increased the enzymatic activity **(Table 1).** Na^+ and Mg^{2+} decreased midgut α-amylase of *A. amygdali* but Ca^{2+} showed adverse results **(Table 1).** Taken collectively, it can be concluded that Ca^2 had critical role in amylolytic activity of *A. spinidens* salivary glands and midgut. Similar results have been observed in case of *E. integriceps* and *A. spinidens* (9, 12, 13).

Linweaver-Burk analysis to show kinetic parameters of salivary and midgut α-amylase in *A. spinidens* revealed V_{max} of 11.23 and 5.88 (U/mg protein) as well as K_m of 6.85 and 2.58 (%), respectively **(Figure 5).** K_m has an inverse relationship with the substrate concentration to saturate active sites of the enzyme. On the other hand, lower K_m reveal stronger binding of enzyme to substrate for degradation. Moreover, higher value of V_{max} demonstrates ability of the enzyme to reach the highest velocity for substrate degredation. In our study, α-amylase of salivary glands had the highet V_{max} but α-amylase of the midgut had the lower K_m. It could be concluded that higher velocity of salivary α-amylase enable insect to faster liquefaction of plant tissues but lower K_m of midgut α-amylase enable the insect to efficient digestion of ingested carbohydrates.

Table 1. *Effects of mono- and di-valent cations on α-amylase activities in the salivary glands and the midgut of A. amygdali.*

Cation	Concentration	Salivary glands	Midgut
Na^+	Control	100±10.59a	100±3.69a
	1	84±10.33a	99±2.57a
	3	81±3.65a	91±6.45ab
	5	84±1.94a	76±2.43b
K^+	Control	100±16.53a	100±6.27a
	1	100±8.83a	101±3.70a
	3	116±14.31a	99±2.68a
	5	127±2.88a	113±5.49a
Ca^{2+}	Control	100±5.51c	100±4.14b
	1	113±1.78bc	86±1.67b
	3	133±8.43b	93±6.92b
	5	174±7.03a	123±1.34a
Mg^{2+}	Control	100±5.41a	100±1.35ab
	1	80±4.82a	90±1.35b
	3	89±9.86a	117±4.86a
	5	88±3.87a	98±6.19b
Cu^{2+}	Control	100±3.14b	100±1.70a
	1	91±2.98b	105±13.52a
	3	112±1.64a	108±3.82a
	5	111±1.34a	104±0.89a

*. Statistical differences have been shown by various letters (Tukey test, $p \leq 0.05$).

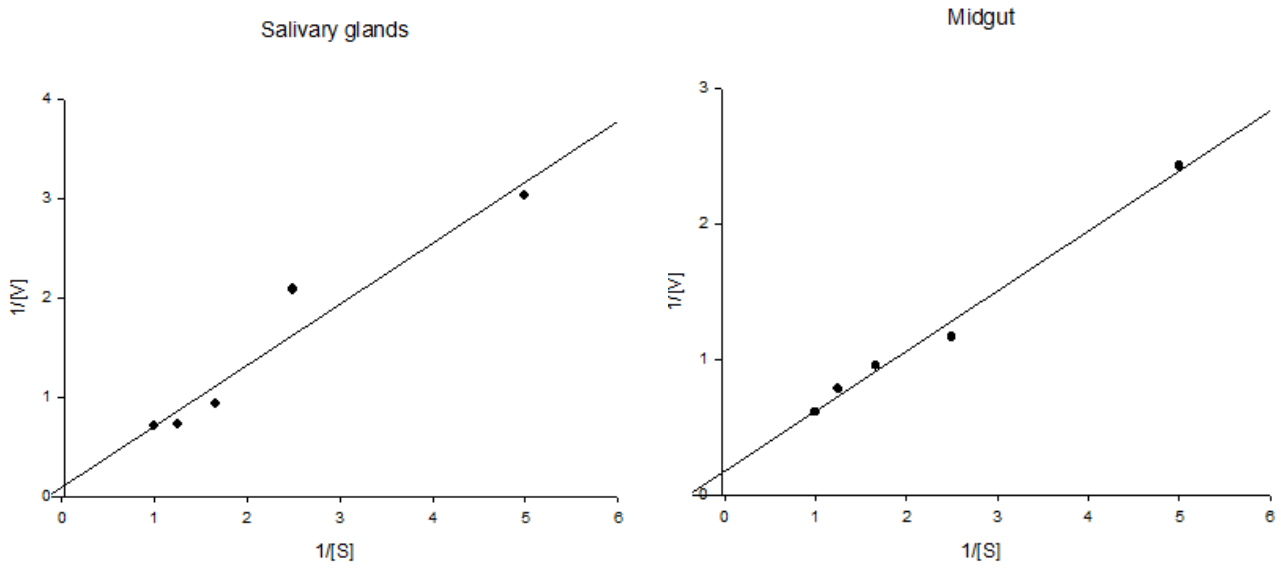

Figure 5. Linweaver-Burk plots sjowing kinetic parameters of α-amylase in the salivary glands and in the midgut of *A. amygdali*.

CONCLUSIONS

Results of the current study clearly depicted presence of α-amylase as one of the major enzymes in the midgut of *A. amygdali*. Determination of amylolytic activities in insects is one of the main steps to develop a safe and efficient pest control. Finding of enzyme properties will be helpful to obtain an inhibitor to decrease enzymatic activity leading to mal-nutrition of target pest. In case, screening of various medicinal plants will be helpful to extract inhibitors like lectins to inhibit amylolytic activity in *A. amygdali* and suppress population outbreaks of the pest.

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