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RESEARCH REPORT

Purification and biochemical properties of a salivary α-amylase in *Andrallus spinidens* Fabricius (Hemiptera: Pentatomidae)

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Abstract

α-amylase is one of the enzymes that has crucial role in extra-oral digestion (EOD) of hemipteran insects. An α-amylase was purified and biochemically characterized from the salivary glands of *Andrallus spinidens* showing its considerable role in EOD process. It was found an enzyme by specific activity of 4.22 U/mg protein, recovery of 14.67 % and purification fold of 13.83-fold as well as molecular weight of 26 kDa. By using two buffer solutions, optimal pH of the purified α-amylase was found to be 9 for both universal and Tris-HCl buffers. Our findings revealed that the purified α-amylase had the highest activity at the temperatures of 35 and 40 °C, and were stable for 96 h at these temperatures. Kinetic parameters of the purified enzyme show that both starch and glycogen, are the suitable substrates for the enzymatic assay, but a lower K_m demonstrated glycogen as a more appropriate substrate. Among the cations used to show their possible involvement in active site of the enzyme, Ca^{2+}, Mg^{2+} and one concentration of Cu^{2+} increased the α-amylase activity but Na^{+} decreased the enzyme activity. Triton X-100 increased the enzyme activity but SDS, EDTA, EGTA and TTHA decreased it, indicating involvement of metal ions in the active site of the purified α-amylase.

Key Words: α-amylase; salivary gland; *Andrallus spinidens*

Introduction

Extra-oral digestion (EOD) is a tool used by the majority of terrestrial predaceous arthropods to feed on relatively large preys. These predators obtain prey extraction and nutrient by refluxing or non-refluxing application during injection of hydrolytic enzymes (Cohen, 1995). Abbreviation of prey handling time and increase in nutrient density of food are two major advantages for EOD allowing small predators to consume preys larger than their body size. The basis of EOD is a highly coordinated combination of biochemical, morphological, and behavioral adaptations that vary with different taxa (Cohen, 1995).

Andrallus spinidens is a potential bio-control agent of caterpillars that has been widely distributed around the world (Nageswara Rao, 1965). The insect has especially reported as a potential predator of rice pests in India, Malaysia and Iran (Nageswara Rao, 1965; Manley, 1982; Mohaghegh and Najafi, 2003). Both nymphs and adults feed on several caterpillars like *Chilo suppressalis* Walker (Lepidoptera: Crambidae), *Naranga aenescens* Moore (Lepidoptera: Noctuidae) and *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) (Mohaghegh and Najafi, 2003). Najafi-Navaee *et al.* (1998) reported that *A. spinidens* is a specific caterpillar predator of rice fields in northern Iran that has five generations per year having a critical role in regulation of rice pests' population.

Proteases, lipases and α-amylases are the three major enzymes involved in EOD of hemipterans. Our findings in a previous study demonstrated that salivary glands of *A. spinidens* have two anterior, two lateral and two posterior lobes (Zibaee *et al*., 2012a). General proteolytic activity in the salivary glands demonstrated optimal pH of 8 and optimal temperature of 40 °C when azocasein was used as substrate. By using specific substrates, it was found that trypsin-like, chymotrypsin-like, aminopeptidase and carboxypeptidase are the active proteases in the salivary glands of *A. spinidens* by maximal activity of trypsin-like protease in addition to their optimal pH of 8-9 (Zibaee *et al*., 2012a). Also, we characterized a

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TAG-lipase from salivary glands of *A. spinidens* that had optimal pH and temperature of 9 and 40 °C, respectively (Zibaee *et al*., 2012b). α-amylases (α-1,4-glucan-4-glucanohydrolases; EC 3.2.1.1) are the hydrolytic enzymes highly widespread in nature. They catalyze the hydrolysis of α-D-(1,4)-glucan linkages in glycogen, starch and other related carbohydrates (Strobl *et al*., 1998; Franco *et al*., 2000).

Regarding EOD importance in hemipterans especially *A. spinidens* as biocontrol agent, we have initiated a comprehensive study along with two other researches (Zibaee *et al*., 2012a, b). So, the aims of the current study were the complete purification and characterization of salivary α-amylase to find its role in EOD process of preys.

Materials and Methods

Andrallus spinidens rearing

Colony of *Andrallus spinidens* was established by adults collected from harvested rice fields in Amol (Mazandaran, North of Iran), late September 2011. Insects was reared on late instars of *C. suppressalis* L. (Lepidoptera: Crambidae) as prey and provided with wet cotton plugs fitted into small plastic dishes (2.5 cm diameter) as moisture sources at 25±1 °C and 80 % of relative humidity as laboratory conditions.

Sample preparation

Adults were randomly selected and the salivary glands were removed by dissection under a stereo microscope in ice-cold saline buffer 10 mM. Bodies were cut separately by a scalper and salivary glands appeared. Salivary glands were separated from the insect body, rinsed in ice-cold distilled water, placed in a pre-cooled homogenizer and grounded before centrifugation. An equal portion of tissue and distilled water were used to have a desirable concentration of the enzyme (W/V). Homogenate were separately transferred to 1.5 ml centrifuge tubes and centrifuged at 13,000 rpm for 15 min at 4 °C. The supernatant were pooled and stored at -20 °C for subsequent analyses (Zibaee *et al.*, 2012a).

α-amylase assay

α-amylase activity was assayed by dinitrosalicylic acid (DNS) procedure (Bernfeld, 1955), using 1 % soluble starch (Merck, Darmstadt, Germany) as substrate. Ten microliters of the enzyme were incubated for 30 min at 35 °C with 35 μl of phosphate buffer (0.02 M, pH 7.1) and 20 μl of soluble starch. The reaction was stopped by addition of 80 μl of DNS and heated in boiling water for 10 min prior to read absorbance at 540 nm. One unit of *α*-amylase activity was defined as the amount of enzyme required to produce 1 mg of maltose in 30 min at 35 °C. Used negative control contained all reaction mixture but pre-boiled enzyme (for 15 min) to prove the enzyme presence in the samples.

Purification process

Purification process of the salivary α-amylase in *A. spinidens* was carried out based on Englard and Seifter (1990) and Dennison (1999) procedures. The crude extract (40 ml) from salivary homogenates of *A. spinidens* adults was treated with ammonium sulfate at 4 °C to give fractions precipitated at 40 % and 80 % saturations. The precipitates were collected by centrifugation at 6,000 rpm for 15 min, diluted in 2 ml of Tris-HCl (20 mM, pH 8.8) and dialyzed overnight at 4 °C against the same buffer. The enzyme solution was applied to a Sepharyl G-100 column, equilibrated with the same buffer. The column was run at a flow rate of 0.5 ml/min and 1.5 ml. Amylase activity was measured as described in the previous section. Fractions containing enzymatic activity were pooled and applied to a diethylaminoethyl (DEAE)-cellulose column, equilibrated with Tris-HCl (pH 8.8). The enzyme elution was done at a flow rate of 0.5 ml/min with a linear NaCl gradient (0 - 0.6 mol). Fractions (1.5 ml/tube) were collected and their protein concentration and α-amylase activity were determined as described. In the final step, fractions containing the highest enzymatic activity were pooled and used as enzyme source.

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the procedure described by Laemmli, (1970). Acrylamide concentration was 10 % for the separating gel and 4 % for the stacking gel. After running the gel at 100 mV as constant voltage, proteins on the polyacrylamide gel were stained with 0.2 % Coomassie brilliant blue R-250 (Hames 1998). β-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35.5 kDa), restriction endonuclease Bsp 981 (25 kDa), β-lactoglobulin (18.4 kDa) and lysozyme (14.4 kDa) were used as molecular mass standards.

Effect of pH on enzyme activity

The optimal pH for amylase activity was determined using universal and Tris-HCl buffers. The tested pH values were 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13. The enzymatic assay was done as described in the earlier section.

Effect of temperature on enzyme activity

The effect of temperature on amylolytic activity was determined by incubating the reaction mixture for 30 min at the following temperatures: 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 70 and 80 °C. Also the thermo-stability of the enzyme at the optimal temperatures was determined over 192 h (8 days). Samples were maintained at 35 and 45 °C for 8 days followed by determination of residual activity as described in the earlier section.

Kinetic studies

Kinetic parameters of the purified α-amylase were calculated by using different concentrations of starch and glycogen as 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1 %. The assay procedure was carried out describing earlier. Obtained data were inserted in Sigma Plot software (Version, 6) and line regression was drawn by used concentrations of substrate and observed enzymatic velocity.

Effect of mono- and divalent cations on α-amylase activity

The effects of various cations on purified α-amylase activity were investigated using $CaCl_2$, $MgCl_2$,

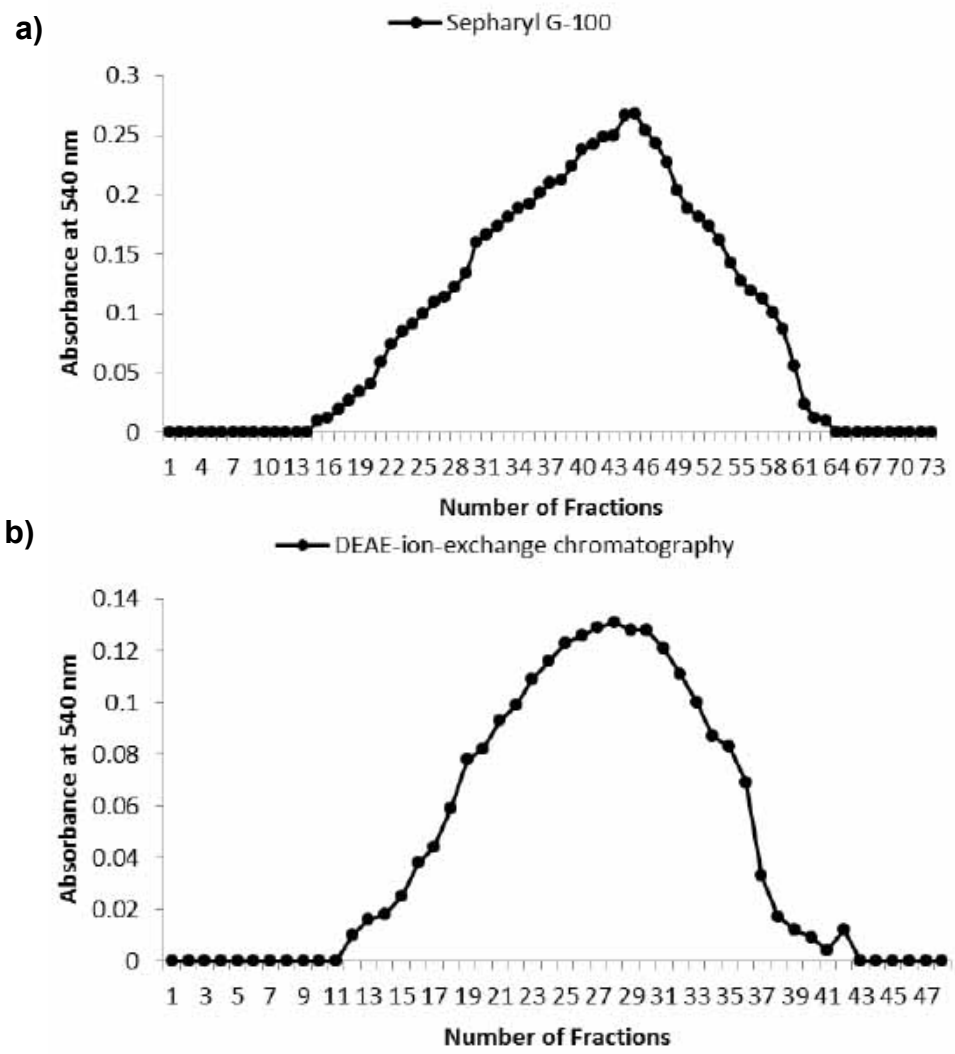

Fig. 1 Column chromatography of the salivary purified α-amylase from adults of *A. spinidens*. a) Sepharyl G-100 gel-filtration of the enzyme after ammonium sulfate (40 and 80 %) treatment. Fractions 41 - 47 contained the highest enzymatic activity on starch (1 %) and collected for next steps. b) DEAE ion-exchange chromatography of the gel-filtrated α-amylase from *A. spinidens*. Fractions 25 - 32 contained the highest enzymatic activity on starch (1 %) and used for other steps of the experiments.

NaCl, KCl, CuSO4 and ZnSO4. In case, 10 µl of a solution containing each concentration of ions (0, 0.5, 1, 3, 5 and 10 mM) and 10 µl of enzyme were pre-incubated for 10 min at pH 9 of universal buffer and 35 °C as optimal temperature. Thirty microliters of starch were added to the mixture and experiment was continued as described above.

Effect of specific inhibitors on α-amylase activity

The effects of enzyme inhibitors on amylolytic activity were studied using different concentrations (0, 0.5, 1, 3, 5 and 10 mM) of ethylene glycol-bis (β-aminoethylether) N, N, N′, N′-tetraacetic acid (EGTA), triethylenetetramine hexaacetic acid (TTHA), Ethylenediamine tetraacetic acid (EDTA), SDS and Triton X-100. The purified enzyme (10 µl) was pre-incubated for 10 min at pH 9 and 35 °C with 10 µl of inhibitors (each concentration). Fifty microliter of starch was added to the mixture and experiment was continued as earlier. Other steps were carried out as mentioned earlier.

Kinetic parameters by using IC50 concentration of EGTA and TTHA

To measure further involvement of EGTA and TTHA, IC_{50} concentration of each inhibitor were added to different concentration of starch (0.1, 0.2, 0.4, 0.6, 0.8 and 1%). Then, observed activities were inserted in Sigma Plot software to calculate V_{max} and K_m values.

Protein determination

Protein concentration was determined either by measuring absorbance by the method of Bradford (1976) using bovine serum albumin as standard.

Statistical analysis

All data were compared by one-way analysis of variance (ANOVA) followed by Tukey's studentisized test when significant differences were found at $p \leq 0.05$ (SAS, 1997). Differences between samples were considered statistically significant at a probability less than 5 % and marked in figures and tables with letters and asterisks.

Results and Discussion

Saliva is the main source of enzymes involving in EOD process of hemipterans. Miles (1968) divided insect saliva into three categories; i) saliva of hemipterans contains almost exclusively α-amylase like in *Lygus disponsi* Linnavuori (Hemiptera: Miridae), while other carbohydrases are just present in the alimentary canal, ii) phloem feeder insects like aphids in which carbohydrases are the only salivary enzymes, iii) seed feeder insects whose saliva contains proteases and esterases. After amonium sulphate precipitation, samples were subjected to sepharyl G-100 column. Among 73 collected fractions, fractions 41 - 47 showed the highest amylolytic activity (Fig. 1a). These fractions were pooled and loaded onto DEAE ion-exchange chromatography. Fractions 25 - 32 out of 47 gathered fractions showed the highest amylolytic activity (Fig. 1b) then, they were pooled and used for SDS-PAGE showing purity of the enzyme. SDS-PAGE analysis revealed purity of the enzyme by molecular weight of 26 kDa (Fig. 2). After confirming purity of the enzyme by SDS-PAGE, biochemical assays revealed an enzyme with 4.22 U/mg protein of specific activity, a recovery percentage of 14.67 and a 13.83 purification fold (Table 1). Terra and Ferriera (2005) reported that α-amylases in insects have a molecular weight of 48 - 52 kDa. In our study, it was found a lower molecular weight that coincides with the hypothesis that salivary α-amylase is involved in preliminary digestion of starch and glycogen and it definitely has a few isozymes in comparison with midgut α-amylase that is involved in complete digestion of carbohydrates by considering the point that proteases

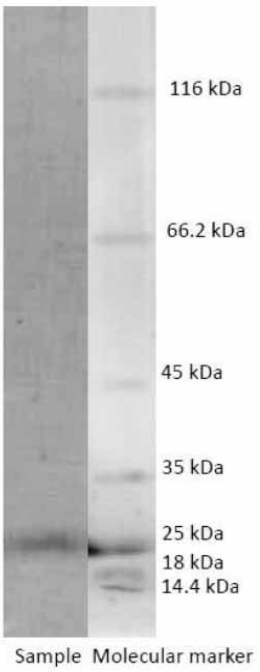

Fig. 2 Denaturing SDS-PAGE to find purity and molecular weight of the salivary purified α-amylase from the salivary gland of adult *A. spinidens.*

are the main salivary enzymes in predaceous bugs (Zibaee *et al.*, 2012a).

pH and temperature are the two major factors that affect enzymatic reactions in several ways like substrate and enzyme stability, their combination, tertiary structure of the enzyme etc. It was observed that 9 is the optimal pH value for activity of purified α-amylase in *A. spinidens* by using universal and Tris-HCl buffers (Fig. 3). Terra and Ferriera (2005) believe that enzyme optimal pH should be determined using different buffers to discount the

Table 1 Purification of α-amylase in the salivary glands of *A. spinidens* adults

Purification steps	Total activity (µmol/min)	Total protein (mg)	Specific activity (µmol/min/mg protein)	Recovery (%)	Purification fold
Crude extract	2.59 ± 0.86	8.47	0.30 ± 0.07	100	1.00
$(NH4)_2So_4$ (0 - 40 %)	2.08 ± 0.49	5.12	0.41 ± 0.03	80.03	1.33
$(NH4)_2So_4$ (40 - 80 %)	1.53 ± 0.32	3.14	0.74 ± 0.10	59.07	2.43
Sepharyl G-100	0.80 ± 0.05	0.53	1.51 ± 0.17	31.00	4.95
CM-Sepharose	0.38 ± 0.03	0.09	4.22 ± 0.34	14.67	13.83

All experiments were carried out at 30 °C.
Salivary gland homogenates were used in this experiment.
Starch (1 %) was used as substrate to find amylolytic activity.

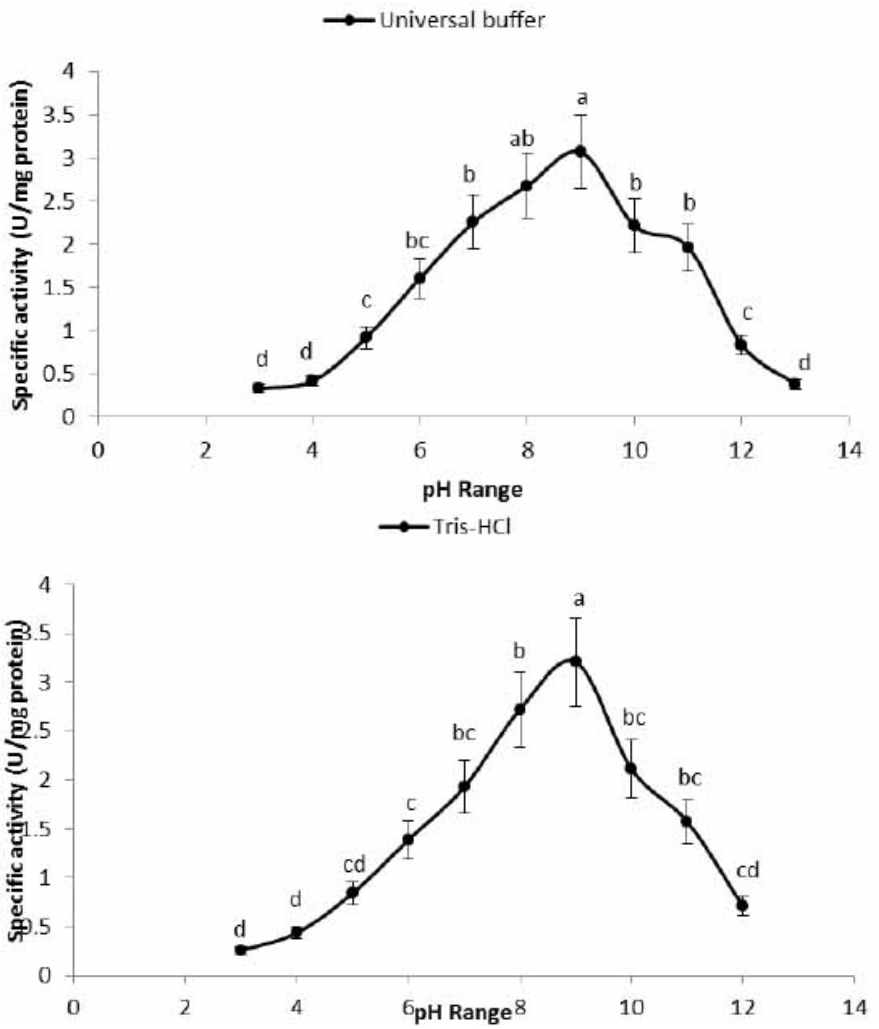

Fig. 3 Optimal pH determination of the purified α-amylase from salivary glands of *A. spinidens* adults. Two bufferic solutions were used and statistical differences have been shown by the different letters (Tukey's test, $p \leq 0.05$).

effects of chemical constituents present in the buffers and their ionic strength on enzyme activity. Bezdi *et al*. (2008) reported pH 4.5 as the optimal pH of salivary α-amylase in *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae). Zeng and Cohen (2000) reported that optimal pH 6 for α-amylase from *L. herperus* and *L. lineolaris*. Mehrabadi and Bandani (2009) found that salivary α-amylase of *E. maura* had the highest activity at pH 6 - 7. *A. spinidens* feed on caterpillar larvae whose pH is alkaline to overcome secondary metabolites of plants. So, salivary secretion of the bug must be alkaline adapting it to feed on these preys, a phenomenon that we have already found on salivary proteases and lipases (Zibaee *et al.,* 2012a, b). The optimal temperature of the purified α-amylase was found to be 35 - 40 °C (Fig. 4a). Also, it was found that the purified enzyme was stable for 96 h (3 days) at the optimal temperatures (Fig. 4b). This value is lower than that observed for the α-amylase activity in *Blatella germanica* (Blatodea: Blatellidae) 50 °C (Applebaum, 1985) and *Bombyx mori* (Lepidoptera: Bombycidae) 60 °C (Kanekatsu,1978). Also, it is more or less equal to that of the α-amylase activity in *L. disponsi* 37 °C (Hori,1970), *Dolycoris baccarum* (Hemiptera: Pentatomidae) 40 °C (Hori,1969), *Cerambyx cerdo* L. (Hemiptera: Cerambycidae) 35 °C (Applebaum, 1985), *E. maura* L. 30 - 35 °C (Mehrabadi and Bandani, 2009) and higher than *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) 25 °C (Barbosa *et al*., 1999). Optimal temperature of salivary enzymes surprisingly corresponds to field temperature during June to September when *A. spinidens* is active on rice fields of Iran. Higher activity of the enzymes in a specific temperature of *in vitro* assays generally reflects the temperature of the environment where the organism feeds on the hosts. Extremes in temperatures can also disrupt the hydrogen bonds that hold the enzyme in its three-dimensional structure leading denaturation of the protein (Zeng and Cohen, 2000). Meanwhile, biological reactions are catalyzed by proteinaceous enzymes, and each enzyme has a temperature above which its three dimensional structure is disrupted by heat. Therefore, biological

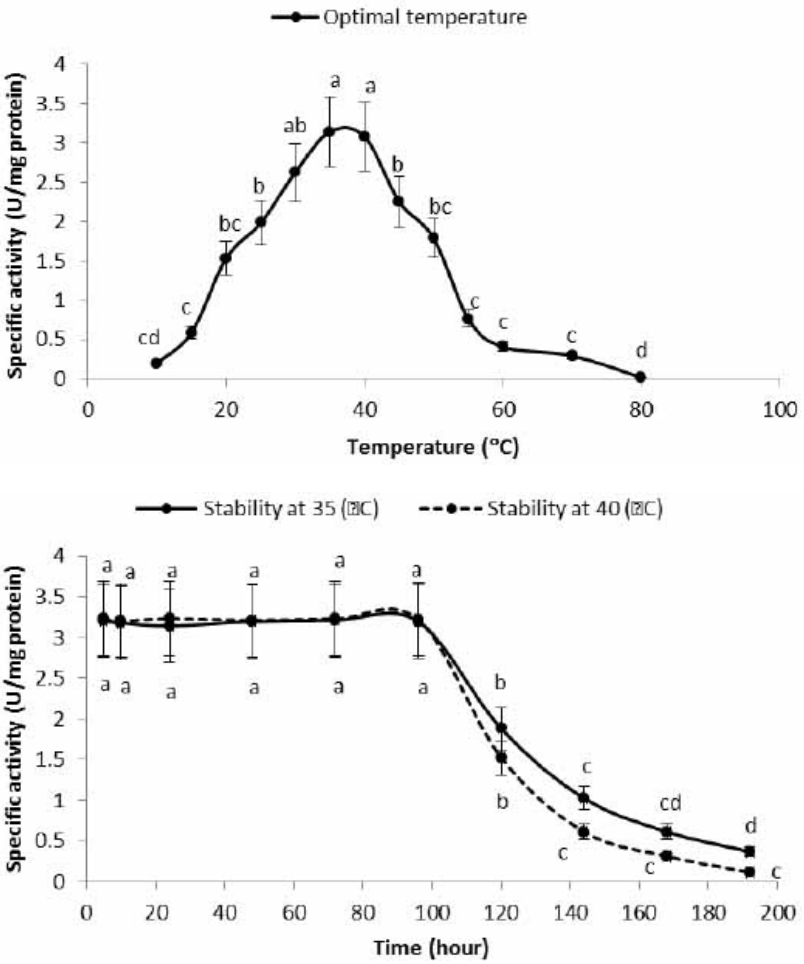

Fig. 4 Optimal temperature and stability of the salivary purified α-amylase from salivary glands of *A. spinidens* adults. Statistical differences have been shown by different letters for optimal temperature and for stability (Tukey's test, $p \leq 0.05$).

reactions occur faster with increasing temperature up to the point of enzyme denaturation, above which temperature enzyme activity and the rate of the reaction decreases sharply (Agblor *et al.*, 1994).

Lineweaver-Burk analysis to find kinetic parameters of the purified salivary α-amylase in presence of two substrates, starch and glycogen, revealed that glycogen is slightly more specific for the enzyme. By using starch, the maximal velocity (V_{max}) of the purified enzyme was observed 7.35 U/mg protein and K_m was 1.04 % in comparison with 7.14 U/mg protein and 0.57 % for glycogen (Fig. 5). Since K_m has an inverse relationship with the substrate concentration required saturating the active sites of the enzyme, this indicates decreased enzyme affinity for the substrate (Wilson and Goulding, 1986). In the other words, K_m is the measurement of the stability of the enzyme-substrate complex and a high Km would indicate weak binding while a low Km would indicate strong binding (Stryer, 1995).

Many fertilizers were used in agricultural ecosystems that might affect ecological levels in case of herbivores and carnivores. One of the side effects of these compounds could be on enzymes

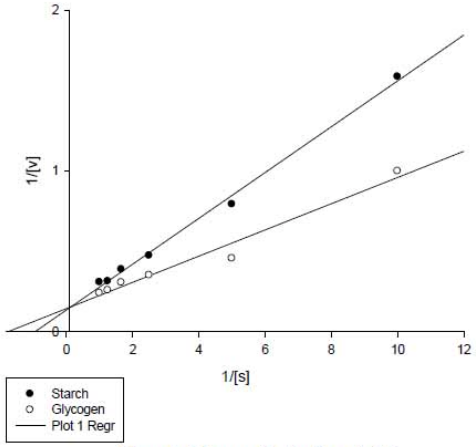

Fig. 5 Double reciprocal plot to show the kinetic parameters of the salivary purified α-amylase from the salivary glands of *A. spinidens* adults by using starch and glycogen (1 %) ($1/V_{max}$ = intercept on the $1/V_0$ ordinate, $-1/K_m$ = intercept on the negative side of the 1/[S] abscissa).

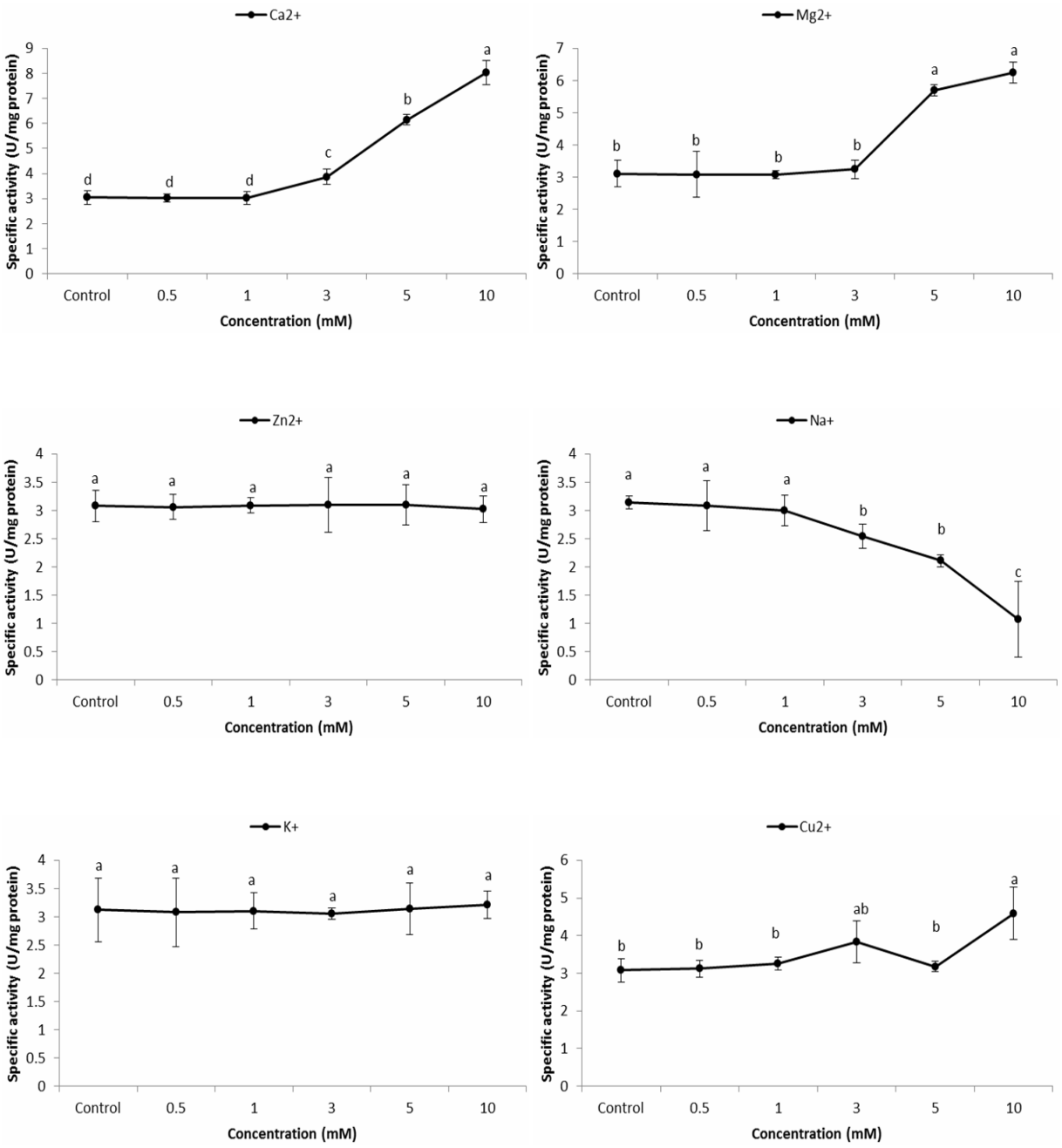

Fig. 6 Effects of different concentrations of cations on the salivary purified α-amylase from the salivary glands of *A. spinidens* adults. Statistical differences have been shown by different letters (Tukey's test, $p \leq 0.05$).

that recruit ions in their active site. In fact, ions, especially divalent ones, work as cofactors and increase or sometimes decrease the enzymatic activity. In our experiments, amylolytic activity in salivary secretion of *A. spinidens* was increased in the presence of the divalent ions like Mg^{2+}, Ca^{2+} and last concentration of Cu^{2+} (Fig. 6). But presence of Na^{+} decreased the enzymatic activity, K^{+} and Zn^{2+} showed no effects (Fig. 6). Saadati *et al*. (2008) observed inhibitory effects of Cu^{2+} on salivary α-amylase of *E. integriceps* but Na^{+} showed no effects. Mehrabadi and Bandani (2009) reported that Na^{+}, Ca^{2+} had no effects on salivary amylase of *E. maura* but Mg^{2+} decreased it. Hori (1969) stated that the polygalacturonase activity in the salivary gland of *L. rugulipennis* was greatly affected by salts in the medium. However, it has been reported that α-amylases are metalloproteins that require calcium for maximum activity. Calcium also affords stability for the amylases from a variety of sources, including insects, to both pH and temperature extremes (Zeng and Cohen, 2000).

SDS, EDTA, EGTA and TTHA significantly decreased the activity of the purified salivary α-amylase

Table 2 Effects of different concentrations of inhibitors on purified salivary α-amylase in *A. spinidens* adults

Inhibitor	Concentrations (mM)	Specific activity (U/mg protein)	IC_{50} (mM)
Control	0	3.41[a]	
SDS	0.5	3.12[a]	
	1	2.45[b]	4.33
	3	1.59[c]	
	5	1.21[c,d]	
	10	0.36[d]	
Control	0	3.24[b]	
Triton	0.5	3.38[b]	
	1	3.97[a]	*
	3	4.25[a]	
	5	4.33[a]	
	10	4.38[a]	
Control	0	3.22[a]	
EDTA	0.5	3.15[a]	
	1	3[a]	
	3	2.14[b]	4.88
	5	1.26[c]	
	10	0.14[d]	
Control	0	3.25[a]	
TTHA	0.5	3.14[a]	
	1	3.2[a]	
	3	2.52[a,b]	8.80
	5	2.04[b]	
	10	1.57[c]	
Control	0	3.18[a]	
DTC	0.5	3.18[a]	
	1	3.26[a]	[i]
	3	3.11[a]	
	5	3.05[a]	
	10	3.09[a]	
Control	0	3.23[a]	
EGTA	0.5	3[a,b]	
	1	2.18[b]	
	3	1.17[c]	3.76
	5	0.87[d]	
	10	0.16[e]	

*) Triton is an activator so no IC50 has been calculated.
[i]) No statistical differences were observed so no IC50 was calculated.
Statistical differences have been shown by different letters (Tukey's test, $p \leq 0.05$) for each compound seperately.

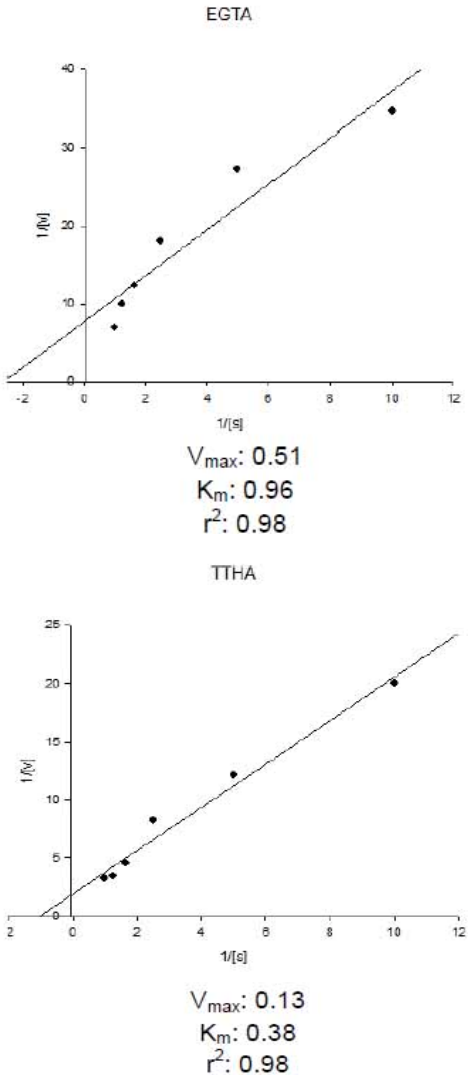

Fig. 7 Double reciprocal plot to show the kinetic parameters of purified α-amylase from the salivary glands of *A. spinidens* adults by using IC50 concentration of EGTA and TTHA (1 %) ($1/V_{max}$ = intercept on the $1/V_0$ ordinate, $-1/K_m$ = intercept on the negative side of the 1/[S] abscissa).

in *A. spinidens* (Table 2). DTC showed no effects but Triton increased the enzymatic activity (Table 2). These results clearly indicate that salivary α-amylase of *A. spinidens* is a metallo-enzyme that requires metal ions in its active site. EDTA is a general chelating agent that removes all metal ions from active site of the enzyme. EGTA and TTHA are specific chelating agents of Ca^{2+} and Mg^{2+}, respectively. Inhibitory effects of both EGTA and TTHA demonstrated the possible substitution of Ca^{2+} and Mg^{2+} in active site of the enzyme (Podoler and Applebaum, 1971; Baker, 1983; Andersson *et al.*, 1989; Kazzazi *et al.*, 2005; Feng *et al.*, 2008; Zibaee *et al.*, 2008). Using IC50 of EGTA and TTHA along with different concentrations of starch revealed lower V_{max} caused by TTHA and higher K_m value of EGTA (Fig. 7). Since, V_{max} of the purified enzyme by using IC_{50} concentrations of both inhibitors decreased in comparison with control, it can be concluded that both of these inhibitors can intervene in enzyme-substrate interaction. Meanwhile, calculated K_m value for EGTA was higher than that of TTHA so it can be inferred that EGTA bind to the active site of the enzyme to remove Ca^{2+} ion decreasing enzymatic activity. Results of Fig. 7 seems to verify statement of Andersson *et al.* (1989) and Feng *et al.* (2008) in replacing of Mg^{2+} and Ca^{2+} in active site of enzymes. Triton has been shown to be an activator on the majority of insect digestive enzymes like amylases, glycosidases and exopeptidases (Terra and Ferriera, 2005).

EDO in predacious bugs like *A. spinidens* facilitates their ability to utilize larger insects, but require a relatively longer time with their prey until they are completely satiated. This is because of their high investment in enzyme production and injection into the prey. This process requires time for the enzymes to act, and the subsequent necessity of recovering these enzymes (Oliveira *et al*. 2006). Short handling time and facultative phytophagy are important traits in biocontrol agents and largely based on the EOD of predator on Heteroptera (Cohen, 1998). Amylases might have crucial role in digestion of prey tissues, especially on glycogen which is the major carbohydrate stored in their body.

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