African Journal of Biotechnology Vol. 8 (15), pp. 3640-3648, 4 August, 2009
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB09.045
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Biochemical characterization of digestive amylase of wheat bug, *Eurygaster maura* (Hemiptera: Scutelleridae)

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Accepted 27 March, 2009

Biochemical characterization of α-amylase in the midgut and salivary glands of *Eurygaster maura* was conducted. Results showed that α-amylase activities were present in the salivary glands and gut. The activity of α-amylase in the midgut and in the salivary glands was 0.098 and 0.057 U/ml, respectively. The pH of salivary glands and the gut was determined to be in the range of 5- 5.5 (for the salivary glands) and in the range of 6-6.5 (for the gut), using staining indicator. The optimum pH and temperature for salivary glands and midgut amylase activity was 6-7 and 35-40ºC, respectively. The stability of amylase was highest in the acidic pH (4-5). Ethylenediamine tetraacetic acid (EDTA), urea, sodium dodecyl sulfate (SDS) and Mg^{2+} inhibited the enzyme activity but, NaCl and KCl enhanced enzyme activity. Based on linear regression analysis of reciprocal starch concentration versus reciprocal amylase activity K_m and V_{max} were 0.11% and 0.04 mM maltose/min for midgut amylase and 0.298% and 0.071 mM maltose/min for salivary amylase, respectively. Sodium dodecyl sulfate electrophoresis (SDS-PAGE) showed that both midgut and salivary glands contain isozymes.

Key words: *Eurygaster maura*, digestive amylase, biochemical characterization, kinetic study.

INTRODUCTION

α-Amylases (α-1,4-glucan-4-glucanohydrolases; EC 3.2.1.1) are one of the most widely enzyme complexes encountered in animals, higher and lower plants, and microbes. Because of α-amylases important biochemical roles in organism growth and development, these enzymes from different origins including bacteria, nematodes, mammals and insects have been purified and their physical and chemical properties characterized (Nagaraju and Abraham, 1995; Zoltowska, 2001; Mohammed, 2004; Rao et al., 2002; Baker, 1991; Baker, 1985; Valencia et al., 2000; Mendiola-Olaya et al., 2000; Oliveira-Nato et al., 2003).

Wheat bugs (*Eurygaster* spp.) (Hemiptera: Scutelleridae), are the most serious pests of wheat and barley in the wide area of the Near and Middle East, West Asia, and many of the new independent states of central Asia. It also is found in Eastern and South Europe and North Africa (Kazzazi et al., 2005; Radjabi, 2000; Javaheri 1995). *Eurygaster maura* species is dominant in north of Iran particularly in Gorgan area, Golestan province and it has not been seen in other area of Iran (Rajabi, 2000). The insect causes severe quantitative and qualitative damage to crops by feeding on leaves, stems and grains. Its feeding is typical of heteropteran, piercing and cutting tissues with their stylets while injecting digestive enzymes, amylases and proteases through salivary canal to liquefy food into nutrient-rich slurry. The food slurry is ingested through the food canal and passed into the alimentary canal where it is further digested and absorbed (Cohen, 2000; Boyd et al., 2002). Wheat bug also feeds on different stage of developing grains. This

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insect sucks the milky nutrients from the immature grain by piercing it with their mouthparts and injecting their salivary juices, which contain very potent enzymes (Lorenz and Meredich, 1988). By injecting enzymes into the grain during feeding, Wheat bug enzymes degrade gluten proteins and cause rapid relaxation of dough resulting in the production of bread with poor volume and texture (Radjabi, 2000). Many insects including wheat bug constitute serious pests of grains live on a polysaccharide-rich diet and are depen-dent on their α-amylases for survival (Mendola- Olaya et al., 2000; Boyd et al., 2002). It converts starch to mal-tose, which is then hydrolyzed to glucose by an α-glucosidase. In insects only α-amylases has been found that hydrolyse long α-1,4-glucan chains such as native starch or glycogen (Terra et al., 1996).

The study of insect digestive enzymes seems to make sense in the realization that the gut is the major interface between the insect and its environment. Hence, an understanding of digestive enzyme function is essential when developing methods of insect control such as the use of enzyme inhibitors and transgenic plants to control insect pests (Morton et al., 2000; Bandani et al. 2001; Ghoshal et al., 2001; Maqbool et al., 2001). As strategies of control, inhibitors to insect α- amylase have been already demonstrated to be an important biotechnology system in the control of insect pests. Pea and azuki transgenic plants expressing α-amylase inhibitors from common beans (α-AI) were completely resistant to the *Bruchus pisorum* and *Callosobruchus chinensis* weevils (Morton et al., 2000). Considering the importance of carbohydrate digestion as a target for wheat bug control, it is clear that α-amylase needs more attention. So, the aim of the present study was to characterize the α-amylase of *E. maura* to gain a better understanding of the digestive physiology of the insect. The gained knowledge will lead to new management strategies for this pest.

MATERIALS AND METHODS

Insects

The insects were collected from the Gorgan wheat farm of Golestan Province, Iran and maintained on wheat plants in the laboratory at 27 ± 2°C with 14 h light : 10 h dark cycle. Voucher specimens are kept in the Entomological Laboratory, Plant Protection Department, Tehran University.

Sample preparation

Enzyme samples from midguts and salivary glands of adults were prepared by the method of Cohen (1993) with slight modifications. Briefly, adults were randomly selected and midgut and salivary gland complexes (SGC) from these individuals were removed by dissection under a light microscope in ice-cold saline buffer (0.006 M NaCl). The SGC was separated from insect's body, rinsed in ice-cold buffer, placed in a pre-cooled homogenizer and ground in one ml of universal buffer containing succinate, glycine, 2-morpholino-ethanesulfonic acid at pH 6.5 (Hosseinkhani and Nemat-Gorgani, 2003).

The midgut was separated from the insect body, rinsed in ice-cold saline buffer, placed in a pre-cooled homogenizer and ground in one ml of universal buffer. The homogenates from both preparations (midgut and SGC) were separately transferred to 1.5 ml centrifuge tubes and centrifuged at 15000 × g for 20 min at 4˚C. The supernatants were pooled and stored at -20˚C for subsequent analyses.

Determination of gut and salivary glands pH

To have a clear understanding of the process of digestion in the bug and to determine the pH in the alimentary canal, the adult insects were dissected under light microscope and their alimentary canal was removed. Gut pH was determined according to the methods of Bignell and Anderson (1980) and Silva et al. (1999).

Each section of gut was cut and mounted on a microscopic slide and then five micro-liter of pH indicator solutions were added to each gut sections. Indicator used were 0.1% bromophenol blue (pH 3.0 – 4.6), 0.1% methyl red (pH 4.4 – 6.2), 0.1% bromcresol purple (pH 5.2 – 6.8), 0.1% bromophenol blue (pH 6.2 – 7.6), 0.1% natural red (pH 6.8 – 8.0), 0.1% cresol red (pH 7.2 – 8.8), 0.1% thymol blue (8.0 – 9.6) and 0.1% Alizarin yellow (pH 10 – 12).

Amylase assay

The α-amylase activity was assayed by the dinitrosalicylic acid (DNS) procedure (Bernfeld 1955), using 1% soluble starch (Merck, product number 1257, Darmstadt, Germany) as substrate. Ten microliters of the enzyme was incubated for 30 min at 35˚C with 500 µl universal buffer and 40 µl soluble starch. The reaction was stopped by addition of 100 µl DNS and heated in boiling water for 10 min. 3,5-Dinitrosalicylic acid is a color reagent that the reducing groups released from starch by α-amylase action are measured by the reduction of 3,5-dinitrosalicylic acid. The boiling water is for stopping the α-amylase activity and catalyzing the reaction between DNS and reducing groups of starch.

Then absorbance was read at 540 nm after cooling in ice for 5 min. 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 standard curve of absorbance against amount of maltose released was constructed to enable calculation of the amount of maltose released during α-amylase assays. Serial dilutions of maltose (Merck, Product Number 105911, Mr 360.32 mg mol^{-1}) in the universal buffer at pH 6.5 were made to give the following range of concentrations of 2, 1, 0.5, 0.25, 0.125 mg ml^{-1} (Figure 1).

A blank without substrate but with α-amylase extract and a control containing no α-amylase extract but with substrate were run simultaneously with the reaction mixture. All assays were performed in duplicate and each assay repeated at least three times.

Effect of pH on enzyme activity and stability

The effect of temperature and pH on α-amylase activity was examined using α-amylase extracted from adult midgut and salivary glands. Optimal pH for amylase activity was determined using

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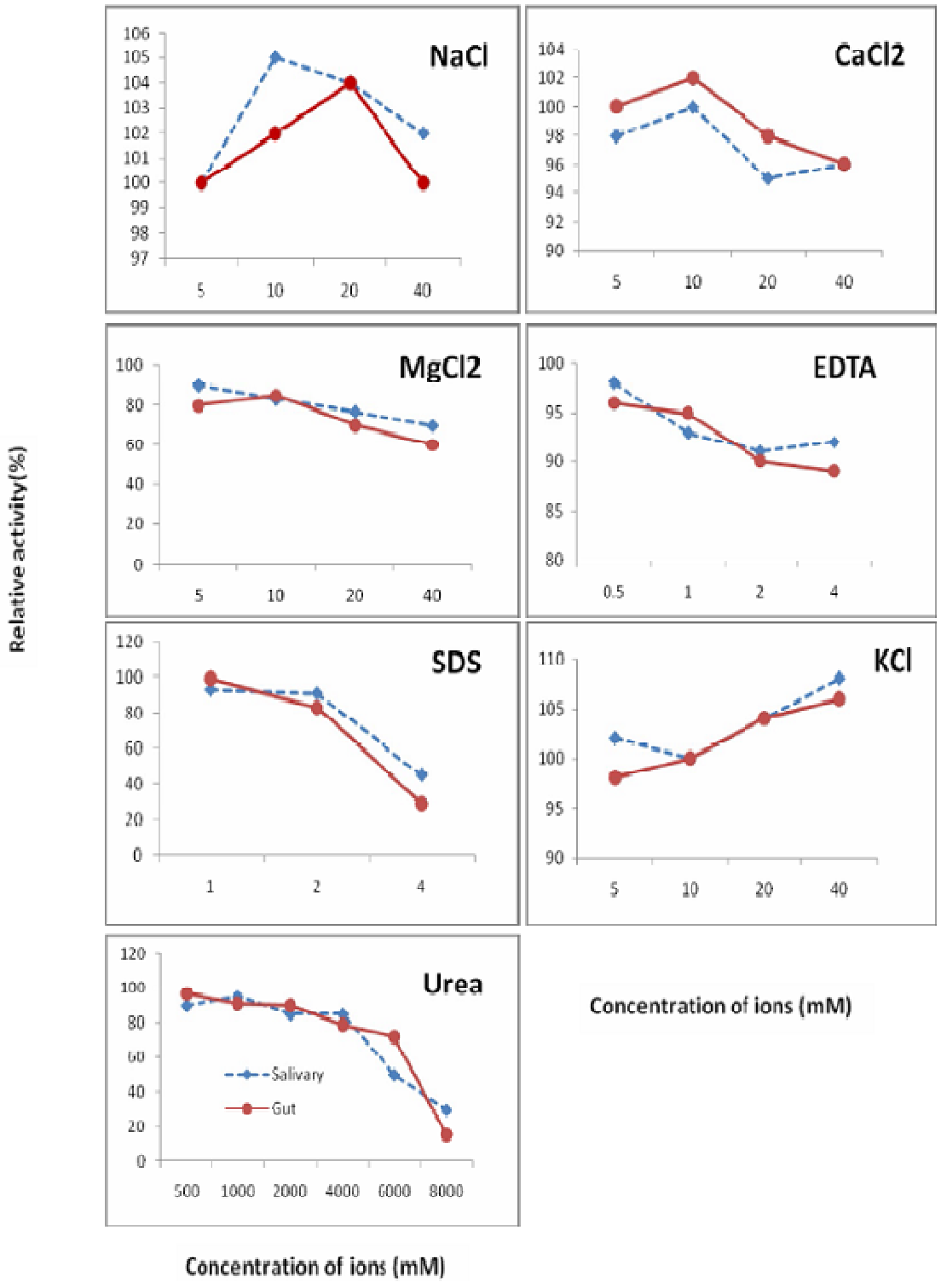

Figure 1. Relative activity of *E. maura* salivary and gut α-amylase toward different compounds. The enzyme was pre-incubated for 10 min at 35ºC with listed compounds at the final concentration indicated prior to substrate addition. Activity in absence of compounds was taken as 100%. Each value represents the average of three independent experiments, n=3.

Table 1. α-Amylase activities in the salivary glands and gut of *Eurygaster maura*.

Organ	Protein (mg/ml)	Unite activity (µM/min/ml enzyme)	Specific activity (µM/min/mg protein)
Salivary glands	0.996 ± 0.076	0.00574 ±0.0016	0.00576 ±0.001
Gut	1.66 ± 0.054	0.00987 ±0.0009	0.00593 ±0.0015

universal buffer with pH set at 2, 3, 4, 5, 5.5, 6, 6.5, 7, 7.5, 8, 9, and 10. Also, the effect of pH on stability of α-amylase was determined by pre-incubation of enzyme at mentioned pH for 60 min prior to the assay.

Effect of temperature on enzyme activity and stability

The effect of temperature on α-amylase activity was determined either by incubating the reaction mixture at 10, 20, 30, 35, 40, 50, 60 and 70˚C for 30 min. The effect of temperature on stability of amylase activity was tested by pre-incubation of the enzyme at 10, 20, 30, 40, 50, 60, and 70˚C for 30 min, followed by measurement of activity as mentioned before.

Effect of activators and inhibitors on enzyme activity

To test the effect of different ions on the enzyme, midguts were dissected in distilled water. Enzyme assays were performed in the presence of different concentrations of chloride salts of Na^+ (5, 10, 20 and 40 mM), K^+ (5, 10, 20 and 40 mM), Ca^{+2} (5, 10, 20 and 40 mM), Mg^{+2} (5, 10, 20 and 40 mM) and EDTA (0.5, 1, 2 and 4 mM), SDS (1, 2 and 4 mM) and urea (0.5, 1, 2, 4, 6 and 8 M). These compounds were added to the assay mixture, and activity was measured after 30 min incubation period. Control was measured without adding any compounds.

Kinetic studies

The dependence of the hydrolytic activity of the amylase on substrate concentration was analyzed. The assay was performed in the presence of substrate concentration of 0.05, 0.1, 0.2, 0.4, 0.6 and 0.8% starch and the enzyme concentration was kept constant. Controls were run in parallel in which distilled water replaced the enzyme for each substrate concentration. Double reciprocal plot (Lineweaver-Burk plots) of enzyme activity versus substrate concentration were used to establish that Michaelis Menten kinetics were obeyed over this substrate concentration range and to obtain values for the maximum velocity (V_{max}) and Michaelis constant (K_m).

Electrophoresis

The visualization of activity of amylases present in crude homogenates of midgut and salivary glands after SDS-PAGE was carried out using the procedure described by Laemmli (1970) and Campos et al. (1989) with minor modification. SDS-PAGE was performed in 10% (w/v) gel with 0.05% SDS for separating gel and 5% for stacking gel with.0.05 % SDS.

The electrode buffer was prepared based on the method of Lammli (1970) but SDS was not used. The sample buffer contained 25% stacking buffer (0.5 M Tris-Hcl ,pH 6.8), 20% glycerol, 2% SDS, 0.005%(w/v) bromophenol blue, but without mercaptoethanol and heating. Electrophoresis was conducted at room temperature with a voltage of 120 V until the blue dye reached the bottom of the slab gel. To prepare gels for α-amylase assay, the gel was rinsed with water and washed by shaking gently with 1% (v/v) Triton X-100 in phosphate buffer containing 2 mM $CaCl_2$ and 10 mM NaCl for 1.5 h. Then, the gel was rinsed with water and treated with a solution of 1.3% I_2, 3% KI to stop the reaction and to stain the un-reacted starch background. Zones of α-amylase activities appeared at light band against dark background.

Protein determination

Protein concentration was measured according to the method of Bradford (1976), using bovine serum albumin (Bio-Rad, Munchen, Germany) as a standard.

Statistical analysis

Data were compared by one-way analysis of variance (ANOVA) followed by Duncan multiple range test when significant differences were found at $P = 0.05$.

RESULTS

Gut and salivary glands pH

Wheat bug alimentary canal consists of a short narrow oesophagus, a large midgut and a short hindgut (rectum). The midgut shows four distinct regions which according to Saxena (1954) are referred to 1st (V1), 2nd (V2), 3rd (V3), and 4th ventriculus (V4). Application of pH indicators showed that the first three regions of the midgut (1st, 2nd, and third regions) were acidic (pH 5.0 – 5.5), fourth region of the midgut (V4) was less acidic (pH 6.5 – 6.9) and rectum was slightly acidic to neutral (pH 6.7 – 7).

α- Amylase activity

Studies showed that α- amylase activity is present in midgut and salivary glands of adult *E. maura* and in whole body of nymphs (Table 1). The activity of the enzyme in the midgut was almost equal to that of the salivary glands: the specific activity of midgut enzyme was 0.0057 U mg $protein^{-1}$ and the specific activity of the salivary gland enzyme was 0.0059 U mg $protein^{-1}$.

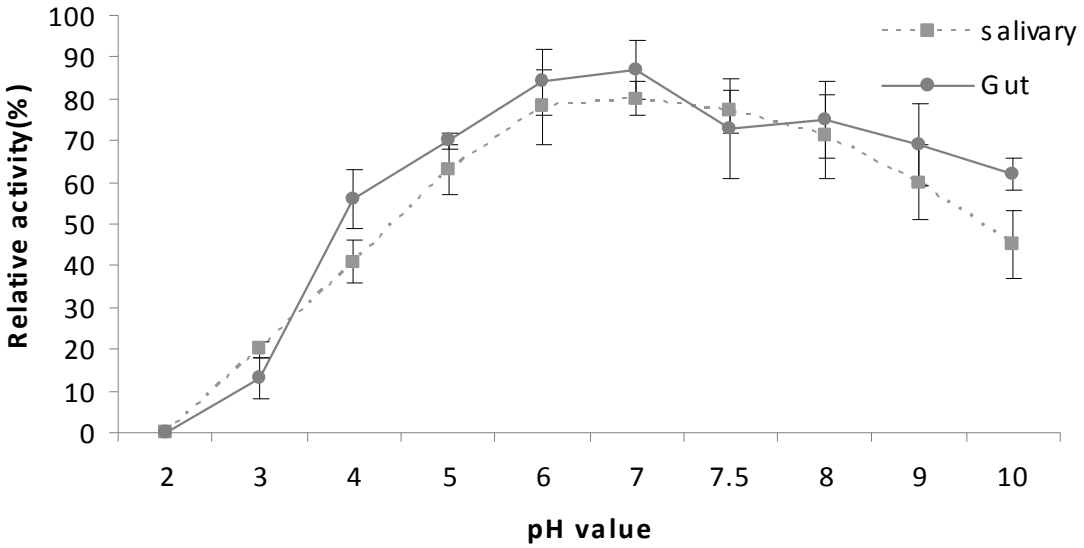

Figure 2. Effect of pH on relative activity of salivary glands and gut α-amylase of *E. maura.*

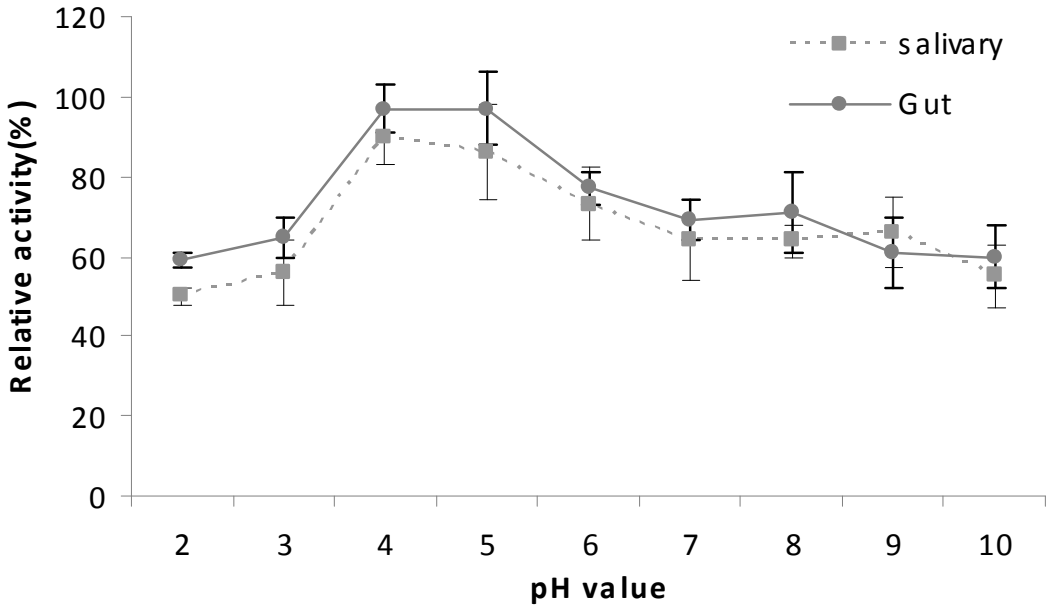

Figure 3. Effect of pH on stability of salivary (--■--) and gut (-●-) α-amylase of *E. maura.*

Only trace amounts of enzyme activity were detected in the first-nymphal stage (0.001 U mg protein^{-1}), whereas α- amylase activity reached its highest value (0.0063 U mg protein^{-1}) in the third-nymphal stage. When activity of α- amylase per mg insect (U mg insect^{-1}) was determined, data showed that the highest activity (0.0158 U mg insect^{-1}) was observed in the third nymphal stage and the lowest activity (0.0043 U mg insect^{-1}) in the first nymphal stage.

These results show that α-amylase specific activity in the immature stages increase constantly up to third-instar nymph. There was significant differences in amylase activity between first, second and third instars (d.f. = 4, F =57.41, P =0.0001). The amounts of α-amylase activity did not change significantly in the last nymphal stages (third, fourth and fifth instars). Enzyme activities in these stages were 0.0063, 0.0059 and 0.0056 U mg protein^{-1}, respectively.

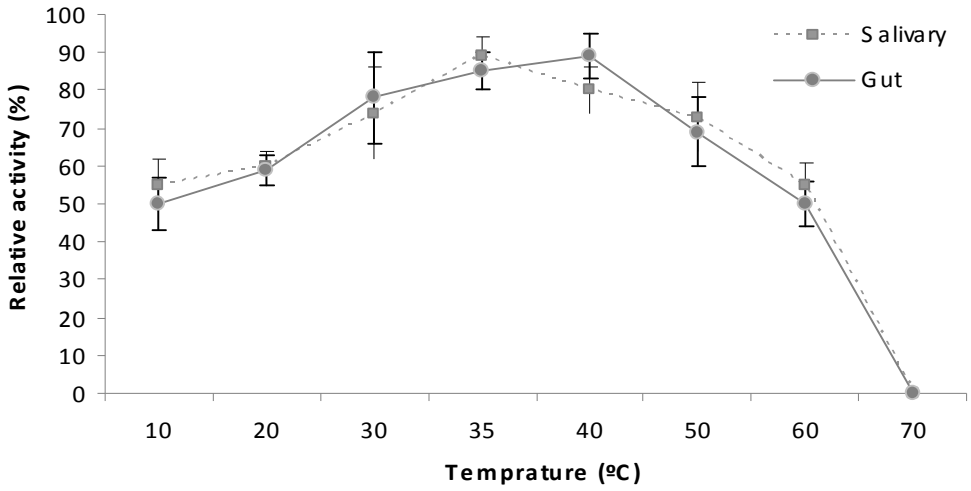

Figure 4. Effect of temperature on activity of salivary (--■--) and gut (-●-) α-amylase of *E. maura*.

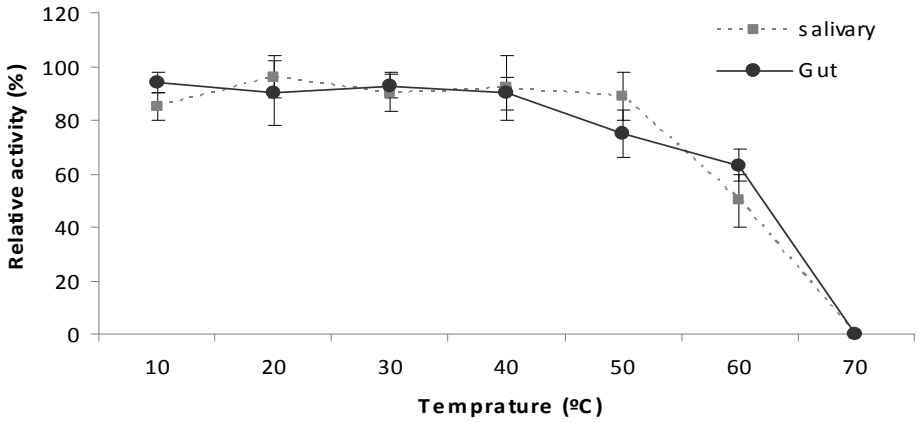

Figure 5. Effect of temperature on stability of salivary (--■--) and gut (-●-) α-amylase of *E. maura* .

Effect of pH and temperature on enzyme activity

Similar to most insect α-amylases, which have optimal activities at neutral or slightly acid pH values, α-amylase of wheat bug showed an optimal pH of 6 - 6.5 (Figure 2). The enzyme activity increased steadily from pH 2 to 6.5 and then decreased with increasing pH.

Pre-incubation of enzyme in different pHs for 1 h affected enzyme only in small scales (Figure 3), showing that both acidic and alkaline pHs have more or less the same effect on enzyme stability. For example, enzyme pre-incubated at pH 3 and 9 retained 77.8 and 77.1% activity, respectively. However, beyond this range (pH 2 or 10) more activity was lost.

Amylase was considerably active over a broad range of temperatures, with the optimum between 25 to 40°C (Figure 4). Sensitivity of amylase to pre-incubation did not change significantly at pre-incubation temperature of 10 to 50°C, but the greatest sensitivity was found at higher temperatures (Figure 5). About 24 and 100% of its activity was lost during 30 min pre-incubation at 60 and 70°C, respectively.

Effect of activators and inhibitors on enzyme activity

Na and K ions increased amylase activity only a little (Figure 1), with the highest activity obtained with 20 mM Na ion concentration and with 40 mM K ion (Figure 1). Other two ions (Ca and Mg) had inhibitory effects that increased with increasing ion concentration (Figure 1). The inhibitory effect of Mg ion was stronger than Ca ion.

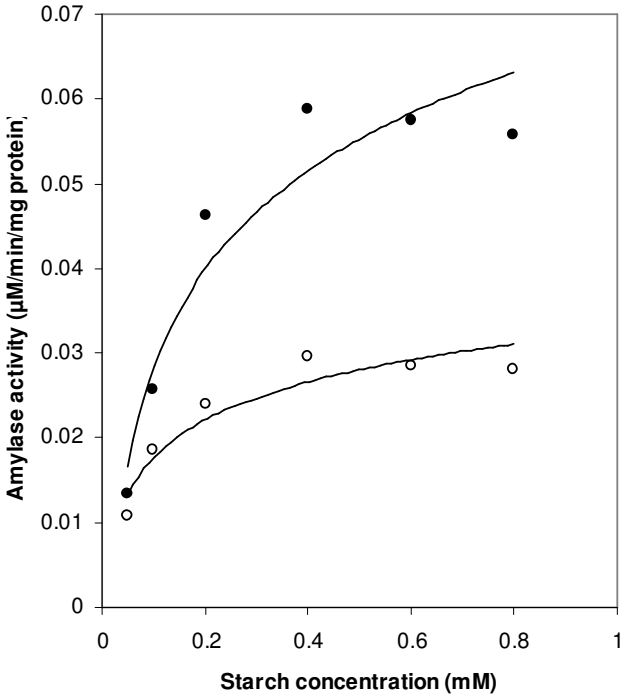

Figure 6. Michaelis-Menten plot of enzyme activity (velocity) versus starch concentration (%) to obtain values for the maximum velocity (V_{max}) and Michaelis constant (K_m) for gut amylase (blank circles) and salivary amylase (filled circles).

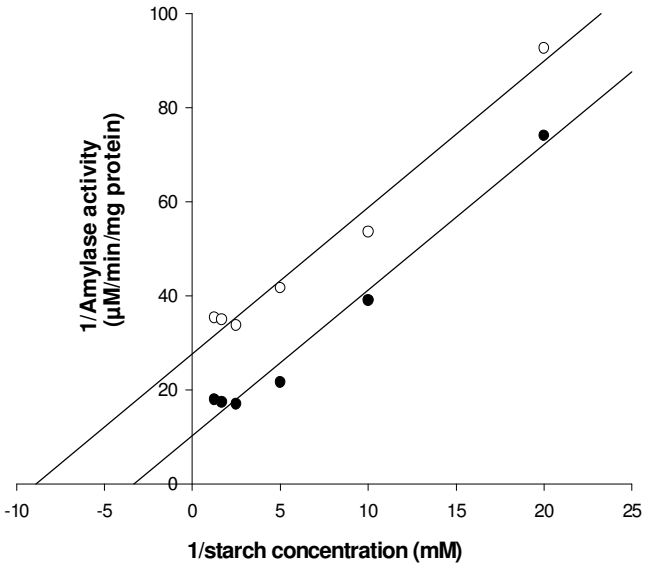

Figure 7. Double reciprocal plot (Lineweaver-Burk plots) of enzyme activity (velocity) versus starch concentration (%) to obtain values for the maximum velocity (V_{max}) and Michaelis constant (K_m) for gut amylase (blank circles) and salivary amylase (filled circles).

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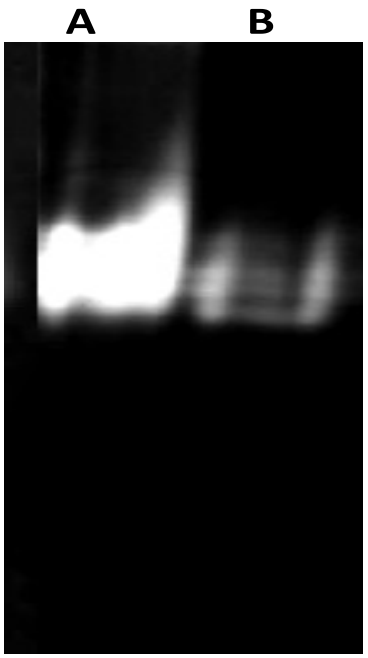

Figure 8. Polyacrylamide gel electrophoresis (Native Page) of the midgut regions (A) and the whole gut (B) of the adult *E. maura*. SDS-PAGE was performed in 12% (w/v) gel with 0.05% SDS for separating gel and 5% for stacking gel with 0.05 % SDS. The sample buffer contained 25% stacking buffer (0.5 M Tris-HCl, pH 6.8), 20% glycerol, 2% SDS, 0.005% (w/v) bromophenol blue.

Three other compounds, urea, SDS, and EDTA, had an inhibitory effect on enzyme activity (Figure 1). Inhibitory effects of SDS, urea, and EDTA at concentration of 1 mM were 2, 5 and 10%, respectively.

Kinetic studies

Based on linear regression analysis of reciprocal starch concentration versus reciprocal amylase activity K_m and V_{max} were 0.11% and 0.04 mM maltose/min for midgut amylase and 0.298% and 0.071 mM maltose/min for salivary amylase, respectively (Figures 6 and 7).

Electrophoresis

Analysis of midgut and salivary glands of *E. maura* homogenates by vertical slab electrophoresis on 10% polyacrylamide gel indicated 3 bands in midgut and 3 bands in

salivary glands (Figure 8). The mobility relative to that of bromophenol blue for midgut bands were 0.38, 0.43, and 0.5, and for salivary glands, 0.44, 0.54 and 0.64.

DISCUSSION

The present study showed that the α-amylase activity is present in both the salivary glands and midgut. The presence of the amylase activity in the salivary glands midguts of other phytophagous heteropterans has also been reported (Zeng and Cohen, 2000; Boyd et al., 2002; Boyd, 2003; Bandani et al., 2009; Mehrabadi et al., 2009). The insects can digest polysaccharides partially by salivary secretions, which would be ingested along with partially digested starches to be used in the midgut (Boyd et al., 2002). Complete breakdown of starch should take place in the midgut where large amounts of amylase exist.

The first nymphal stage of the wheat bug does not feed (Radjabi 2000), which may be one reason why they have very low amylase activity (0.001 U mg protein^{-1}). In the field, feeding is usually intensified at the third instar where damage to crops is obvious. The present study found the maximum α-amylase activity present in the third to fifth nymphal stages.

Amylases in insect are generally most active in the neutral to slightly acid pH condition (Baker 1983; Terra et al., 1996). Optimal pH values for amylases in larvae of several coleopterans were 4 - 5.8 (Baker, 1983) and in *Lygus* spp. (Heteroptera) was 6.5 (Zeng and Cohen, 2000). The optimum pH value for a-amylases of *E. amura* was also in the range of acidic condition. The wheat bug α-amylase has an optimum temperature activity of 30-40^{o}C, which is consistent with the other reports (Ishaaya et al., 1971; Mendiola-Olaya et al., 2000).

Inhibitors and activators used were chosen for comparison with reported values (Baker, 1983; Terra et al., 1996; Zeng and Cohen, 2000; Mohammed, 2004). Data showed that NaCl activated the enzyme. Similarly, in *Lygus hesperus* Knight and *L. lineolaris* (Palisot de Beauvois), α-amylases were activated by NaCl (Agblor et al., 1994; Zeng and Cohen, 2000). Cohen and Hendrix (1994) found that some homopterans' α-amylase is also Cl^{-} activated. Amylase activation by Cl^{-} has been reported in many mammals and bacteria (Shaw et al., 1995; Terra et al., 1996), nematodes (Mohammed, 2004), as well as other insects (Terra et al., 1996). However, the amylases in some insect species, e.g., *Callosobruchus chinensis* (Linaeus) (Coleoptera: Bruchidae), *Bombyx mori* (Linnaeus) (Lepidoptera: Bombycidae), are inhibited by Cl^{-} (Terra et al., 1996). Potassium ions have been shown to have more or less the same effect on α-amylase as Cl^{-} ions. Mg and Ca ions have inhibitory effects on the α-amylase activity of this insect. Also, there are reports that bacterial α-amylase (*Thermus* sp.) is not affected by Ca^{2+} (Shaw et al., 1995). 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 (Baker, 1983). The other features of this enzyme, such as sensitivities to chelating agent (EDTA), urea, and SDS, are typical to many animal amylases (Terra et al., 1996; Mohammed, 2004).

SDS-PAGE of midgut and salivary glands resulted in different bands for each gland, showing that they have different isozymes. Mixture of different isozymes of α-amylases has been reported in some other insect species, such as the fruit fly *Drosophila melanogaster* Meigen (Doani 1967), and coleopterans, *Sitophilus oryzae* (Linnaeus) (Terra et al., 1977), *Tribolium castaneum* (Herbst) (Chen et al., 1992), *Rhyzopertha dominica* (Fabric us) (Baker, 1991) and *Anthonomus grandis* Boheman (Oliveira-Neto et al., 2003).

ACKNOWLEDGMENTS

This research was founded by a grant (No. 86025.11) from the Iran National Science Foundation (INSF).

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