SOME PROPERTIES OF α -AMYLASE IN THE SALIVARY GLAND OF *EURYGASTER INTEGRICEPS* (PUT) (HET.: SCUTELLERIDAE)

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[Sa'adati Bezdi, M. S., Farshbaf Pur Abad, R. F. P., Sadeghi, H. & Golmohammadi, G. 2008. Some Properties of α-Amylase in the salivary gland of *Eurygaster integriceps* (Put) (Het.: Scutelleridae). Munis Entomology & Zoology, 3 (2): 733-744**]**

ABSTRACT: Studying insect digestive enzyme is important for understanding the function of their digestive systems. Today, many attentions are focused on plant enzyme inhibitors and their application in producing transgenic plants. Determining the properties of all digestive enzyme is the first step that should be observed. Enzyme was active at 25 to 50°C and its considerable activities were observed at 35 to 50°C. However, the highest level of activity occurred at 45°C. So, the optimum temperature for the enzyme activity was 45°C. Optimum pH for enzyme activity was 5. The amylase activity in anterior and posterior lobes and accessory gland of the salivary glands complex were 0.578, 1.545 and 0.405 U/mg protein, respectively, and were significantly different at $P<0.01$. The effects of the mineral compounds on adults' α-amylase activity were significant ($P<0.01$). Copper chloride, ammonium sulfate, sodium nitrate, magnesium chloride, magnesium nitrate (all at concentrations of 1 and 3 mM) and ammonium phosphate (1 mM) significantly inhibited the α-amylase activity in *E. integriceps*.

KEY WORDS: Digestive enzymes, Salivary gland, pH optimum, α-amylase, *Eurygaster integriceps*.

INTRODUCTION

The sunn pest, *Eurygaster integriceps* (Put.) (Het.: Scutelleridae) a serious pest of wheat and barley is distributed in the Palearctic Region, which covers The Near and Middle East, Southern Asia and North Africa (Brown Erlap, 1962). In Iran, sunn pest is a key pest that cause serious damages to wheat and barley (Amir-Maafi Parker, 2003). Because of mounting concerns about creature environmental pollution and destruction of important natural enemies by conventional insecticide, alternative strategies such as biological control by natural enemies, host plant resistance, development of transformed plants and other control methods are needed in the struggle to manage *E. integriceps*. For nearly all these strategies, it is important to have a strong understanding of the biology of feeding of the target pest. It is also important to understand the biochemical and physiological feeding adaption to help explain the ecology and evolution of heteropterous insects, which remain unclear (Zeng Cohen, 2000a). Amylase is an important member of a complex of

digestive enzymes that attack macromolecules. It converts starch to maltose, which is than hydrolyzed to glucose by an α-glucosidase (Applebaum,1985; Strobel et al., 1998). Because α-amylases play a major role in carbohydrate metabolism, organisms with a starch-rich diet depend on the effectiveness of their amylases for survival (Applebaum, 1985; Barbosa Pereira et al., 1999; Carlini and Grossi-de-Sa, 2002; D'Amico et al., 2000; Franco et al., 2002; Iulek et al., 2000; Oliveira-Neto et al., 2003; Strobl et al., 1998). Several insect α-amylases have already been described, some of which occur as mixture of different isozymes. For instance, in eight *Amy* strains of *Drosophila melanogaster* (Meig.) (Diptera: Drosophilidae), at least two major α-amylase isozymes were found (Doane, 1969). Conversely, single molecular forms of α-amylases have been reported in *Callosobruchus chinensis* (L.) (Col.: Bruchidae) (Podoler Applebaum, 1971), *Tenebrio molitor* (L.) (Col.: Tenebrionidae) (Applebaum et al., 1961), *Lygus hesperus* (Knight) (Het.: Miridae) (Zeng Cohen, 2000b), and *Lethocerus uhleri* (Montandon) (Het.: Belostomatidae) and *Belostoma lutarium* (Stal.) (Het.: Belostomatidae) (Swart et al., 2006). Digestive enzyme inhibitors are proteinacious or nonproteinacious compounds which reduce an enzyme activity through attaching to its active site and/or its substrate (Farias et al., 2006; Zeng Cohan.,2001). Nowadays, plant enzyme inhibitors are of great importance because these have considerable effects on insect digestive enzymes and as a result on their development (Ishimoto Kitamura, 1989; Silva et al., 1999).

The use of genes that encode insecticidal proteins in transgenic crops has the potential to be beneficial for agricultural crop production, the environment, and consumers (Farias et al., 2006; Bahagiawati et al., 2007; Barbosa Pereira et al., 1999).

We undertook the study of the α-amylase of the sunn pest to gain a better understanding of its digestive physiology, which we hope would lead to new strategies for its control.

MATERIALS AND METHODS

1. Insects

Adult insects were collected from wheat farms in around Tabriz, Iran, during the summer of 2004.

2. Sample Preparation

Adult insects were dissected by the method of Yazdanian et al. (2006) and starved for 24h before dissecting (Cohen, 1993). This was based on the observations which had showed that the accumulation of the enzyme in the lumen of the true bugs salivary glands lasted 24 to 48 hrs (Cohen, 1993; Baptist, 1941). Enzyme samples were prepared by the methods of Cohen (1993) and Yazdanian et al. (2006) with slight modifications. All insects were dissected under a stereomicroscope in ice-cold phosphate buffer (4 ºC, pH=6.9). The salivary gland complex (SGC) (including

anterior and posterior lobes, accessory glands and principal and accessory ducts) was exposed by breaking the junction point of the prothorax and mesothorax located between the coxal bases of front and mid legs and removing it from the abdomen with fine forceps and application of gentle traction to remove the midguts (Yazdanian et al., 2006). The SGCs were separated from the insect bodies, rinsed in ice-cold phosphate buffer and 10 pairs placed in a microtube containing 1 ml of cold phosphate buffer. The SGCs were homogenized by using a homogenizer (Ultra-Turrax T8, IKA Labortechnik, Germany) immediately after dissection. The homogenates were centrifuged at 12000 rpm for 10 minutes at 4 ºC. The supernatants were stored at -20 ºC for later analyses. Protein concentrations of all of the enzyme samples were determined by the bicinchoninic acid (BCA) method using bovine serum albumin (BSA) as the standard (Yazdanian et al., 2006).

3. α-amylase Activity Assay

Amylase activity in the salivary gland was determined by using a diagnostic kit (Amylase kit®, Pars Azmoon Co., Iran). The substrate was ethylidene-*p*-nitrophenyl maltoheptaoside (EPS-G_7). Absorbance, which is directly related to α-amylase activity, was measured at 405 nm and 37 ºC using an auto analyzer (Alcyon 300® Plus, Molecular Devices Corporation, Sunnyvale, CA). Before application, the auto analyzer calibrated with the control sera N and P (TrueLab N® and TrueLab P®, respectively; Pars Azmoon Co., Iran) and a calibrator solution (TrueCal U®, Pars Azmoon Co., Iran). After calibration, the auto analyzer mixes 6 µl of enzyme sample with 300 µl of substrate solution, automatically, and calculates the enzyme activity (IU/L) after a reaction delay of 1 minute and 36 seconds. The assays were replicated three times. Finally, the specific α-amylase activity calculated as U/mg protein (Cohen, 1993).

4. Optimum temperature and pH

In all the determination, α-amylase activity was measured using by diagnostic kit (mentioned above). For the estimation of optimum temprature, the enzyme was incubated with substrates for 30 min at various temperature at 25, 30, 35, 37, 40, 45 and 50 °C.

For the determination of the optimum pH, α-amylase activities at various pH values ranging from 4.5 to 10 (adjusted by citric acid and sodium hydroxide and at 0.5 pH unit increments) were assayed to determine the optimum pH of α-amylase in the salivary gland of the *E. integriceps*. Measurements were repeated three times for each pH value.

5. α-amylase activity in different parts of salivary gland

The glands extracted from adult insects were seprated in the anterior lobe (AN), posterior lobe (PL) and accessory gland (AG) by using microdissecting scissor, than, 10 pairs of each them placed in a microtube containing 1mM of cold phosphate buffer. α-amylase activity was assayed as mentioned in part 2.3.

6. Enzyme Inhibition and Activation

The effects of ammonium nitrate (AN), ammonium phosphate (AP), ammonium sulfate (AS), copper chloride (CC), magnesium chloride (MC), magnesium nitrate (MN), magnesium sulfate (MS), potassium nitrate (PN), sodium nitrate (SN) and sodium phosphate (SP) on salivary α-amylase activity of the sunn pest were determined. Two concentrations (1 and 3 mM) of each compound were prepared. The above mentioned concentrations were prepared in distilled water, and the pH was adjusted to 7 using citric acid and sodium hydroxide. Each solution (100 µl) was pre-incubated with 10 µl of enzyme solution at room temperature (25- 28 ºC) and the residual activities were determined after 30 minutes. The percentages of inhibition or activation were determined by comparing the enzyme activity in distilled water with its activities in the above mentioned solutions. The experiments were repeated three times, and data were analyzed by analysis of variance (ANOVA), and means of enzyme activity of *E. integriceps* in different solutions were compared by Fisher's protected least significant difference (FPLSD) (SAS Institute, 1988) at P= 0.01.

RESULTS

1. Enzyme activities in the principal and accessory glands of *E.integriceps*.

The α-amylase activity was detected in both lobes of the principal gland and accessory gland. The results showed that the α-amylase activity in different parts of salivary gland were significant (P<0.01).

α-amylase activity in posterior lobe (1.545 U/mg protein) was higher than that in anterior lobe (0.570 U/mg protein) and accessory gland (0.405 U/mg protein) (P<0.01). α-amylase activity was not significant between anterior lobe and accessory gland (Fig. 1).

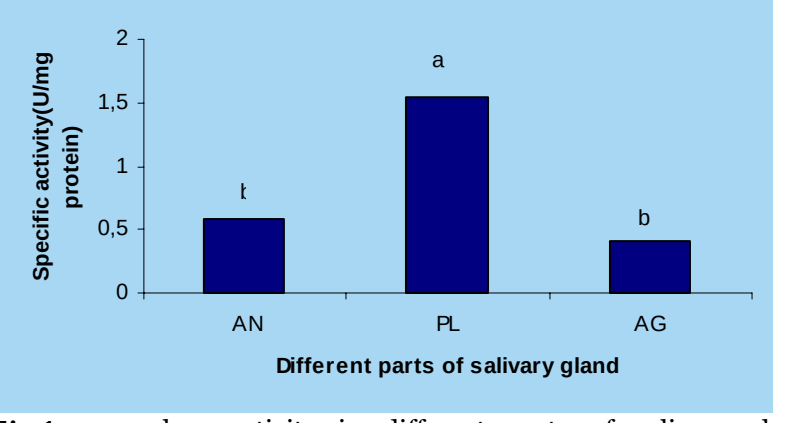

Fig.1. α-amylase activity in different parts of salivary gland of *Eurygaster integriceps* (37°C tempt., pH=7).

2. Optimum temperature of the α-amylase activity

Enzyme was active at 25 to 50°C and its considerable activities were observed at 35 to 50°C. However, the highest level of activity occurred at 45°C ($P<0.01$). So, the optimum temperature for the enzyme activity is 45°C (Fig.2).

The α-amylase activity at 35, 37, 40 and 50°C was not significant, and was lower than that at 45°C. The lowest level of activity occurred at 25 and 30°C, respectively.

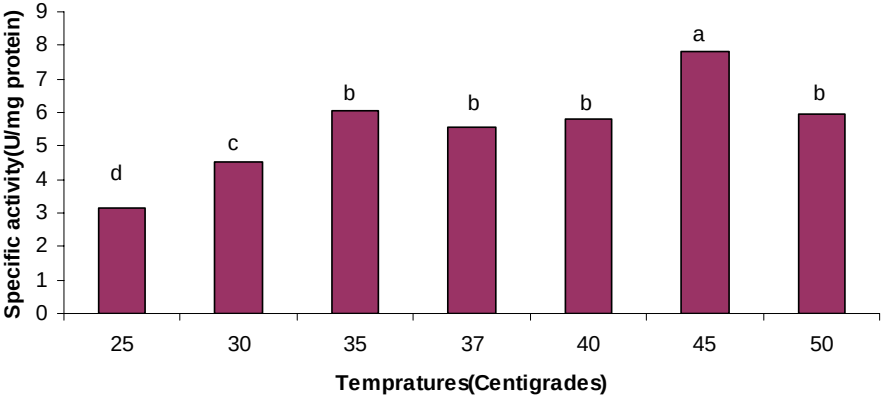

Fig.2. Effect of different tempratures on α-amylase activity of salivary gland of *Eurygaster integriceps* (pH=7).

3. Effect of Hydrogen Ion Concentration on the Enzyme Activity

The results showed that there were considerable activities over a broad range of pH (4.5-10) for the amylase of this species.There were not significant difference at any pH value at $P<0.01$. The activity of α-amylase in the salivary gland of *E. integriceps* was higher at pH=5 (Fig. 3).

4. Inhibition and Activation of the α-amylase

The results of this study showed that some mineral compounds reduced and some others increased the salivary α-amylase activity of *E. integriceps.*

As a whole, α-amylase activity was inhibited by CC, MN, SN, AS and MC. MC and MN had more inhibitory effects on the enzyme activity in compare to the other compounds (Fig. 4).

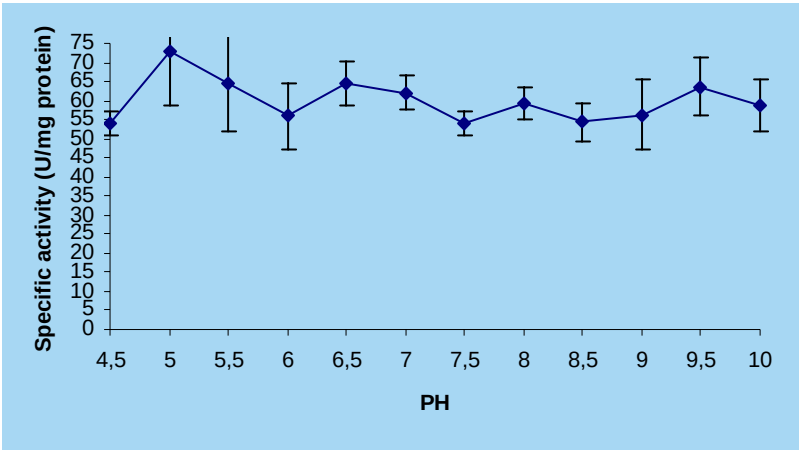

Fig.3. Salivary α-amylase activity of *E. integriceps* at different hydrogen ion concentrations (37 ºC).

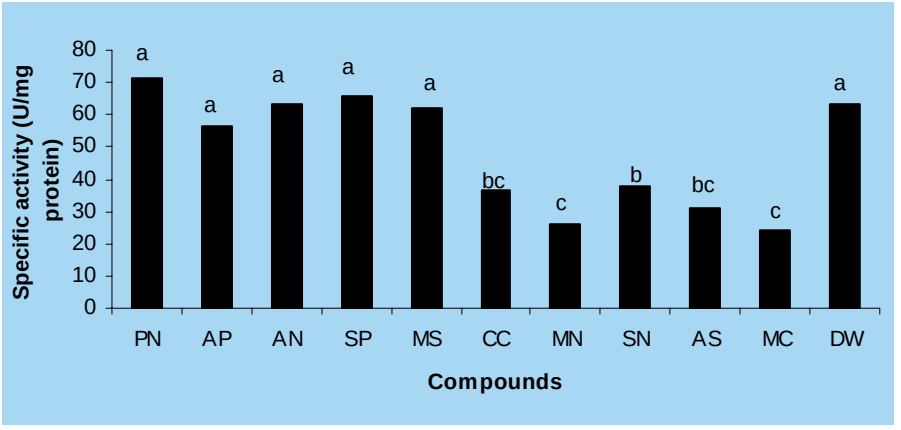

Fig.4. Total effects of some mineral compounds on salivary α-amylase activity of *Eurygaster integriceps* 30 minutes after incubation (37 ºC temp., pH=7).

Effects of two concentrations (1 and 3 mM) of different compounds are presented in Table 1. PN and SP at the concentration of 1 mM activated the salivary α-amylase of the *E. integriceps*, significantly (Tab. 1). CC, AS, SN, MC, MN (all at the concentrations of 1 and 3 mM) and AP (1 mM) significantly inhibited the α-amylase activity of *E. integriceps*. SP (3 mM), AN (1 mM), MS (1 and 3 mM), PN (3 mM) and AP (3 mM) had no effect on the α-amylase activity.

Table 1. Effects of two concentrations of some mineral compounds on the α-amylase activity in the salivary gland of *Eurygaster integriceps* 30 minutes after incubation.

Compound	Enzyme activity (% control)	
	1 mM	3mM
Ammonium nitrate	98.9 c	101.5 ab
Ammonium phosphate	75.1 d	103.1ab
Ammonium sulfate	20.6 g	77.7 c
Copper chloride	81.4 d	33.8 e
Magnesium chloride	38.6 f	38.6 e
Magnesium nitrate	29.1 fg	52.9 d
Magnesium sulfate	99.4 bc	96.8b
Potassium nitrate	119.1 a	100.6 ab
Sodium nitrate	62.9 e	57.6 d
Sodium phosphate	110.5 ab	97.3 b
Distilled water (Control)	100 bc	100 ab

DISCUSSION

The salivary gland is divided in to two functional component ,the principal and accessory gland (Miles,1969 and 1972).

The relative size of the lobes varies in different forms but the anterior lobe is always the amaller (Baptist, 1941). Our data showed that the amylase activity in anterior and posterior lobes and accessory gland of the salivary glands complex were 0.578, 1.545 and 0.405 U/mg protein, respectively.

These results showed that the posterior lobe of the salivary gland was the major source of α-amylase.

It was revealed that the enzyme activity in posterior lobe was significantly ($P<0.01$) higher than that in anterior lobe (nearly 2.6 times) and accessory gland (nearly 3.8 times).

In findings similar to ours, Hori (1972, 1975) reported that α-amylase activity from *lygus disponsi* and *L.rugulipennis* was higher than that other parts of the salivary gland. Miles (1972) stated that, in Lygaeidae,

the posterior lobe of the salivary gland was the major source of α-amylase activity.

The way enzymes work imposes constraints on the function (Silva et al.,2001). No single enzyme can function under all of the physical and chemical condition under which life is found (Applebaum,1985). In fact most enzyme work well only in narrow ranges of temperature and pH (Zeng et al., 2002a).

In this study, the optimum temperature of the enzyme was found to be 45 °C. This value is lower than that of the α-amylase activity in *Blatella germanica* 50°C (Applebaum, 1985) and *Bombyx mori* 60°C (Kanekatsu,1978). On the other hand, this value is higher than that of the α-amylase activity in *L. disponsi* 37°C (Hori,1970), *Dolycoris baccarum* 40°C (Hori,1969), *Cerambyx cerdo* 35°C (Applebaum, 1985) and *Tenebrio molitor* 25°C (Barbosa Prreira et al., 1999). Increased temperature speeds reactions. However, biological reactions are catalyzed by proteinaceous enzymes, and each enzyme has a temprature above which its three dimensional structure is disrupted by heat. Therefore, biological 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; Applebaum, 1985; Zeng et al., 2002b).

Results from this study showed that α-amylase activity of *E. integriceps* has a broad pH range between 4.5 to 10. Zeng & Cohen (2000) reported that optimal pH for α-amylase from *L. herperus* and *L. lineolaris* was 6.5, similarly. Ferreria et al. (1994) reported the optimum pH for α-amylase in *Erinnyis ello* larva as 6. The optimum pH value of α-amylase from *E. integriceps* was higher compared with that of *Sitophilus oryzae*, *S. granareis*, *Rhyzopertha dominica*, *T. molitor* and *Anagasta kuhenilla*, which ranged from 3.7 to 4.5 (Buonocore et al., 1976; Baker Woo, 1985; Baker, 1987, 1989). These differences may reflect the phylogenetic relationship, or response to different food sources. Also there may be a difference attributable to the origin of the enzyme, i.e. gut or salivary glands. The pH optimum generally reflects the pH of the enviroment in which the enzyme normally function. One way in which pH affects reactions rates is by altering the charge state of the substrate or of the active site of the enzyme. Extremes in pH can also disrupt the hydrogen bonds that hold the enzyme in its three-dimensional structure, denaturing the protein (Da silva et al., 2004; Zeng et al., 2002a).

The salivary α-amylase activity proved to be activated strongly by PN in Hemiptera (Hori, 1969). The salivary α-amylases of *Adelphocoris suturalis* and *Lygus disponsi* were activated by PN (Hori, 1969, 1972). In agreement with observation of Hori (1969, 1972), our data showed that the salivary amylase of *E. integriceps* was activated by PN (1 mM) and not affected by PN (3 mM).

Hori (1969) stated that the polygalacturonase activity in the salivary gland of *Lygus rugulipennis* was greatly affected by salts in the incubation medium. Inhibitory salts were $CaCl_2$, $FeCl_2$, $FeCl_3$ and $MgCl_2$. He also reported that the salivary phosphatase activity was inhibited in alkaline solution. For example, addition of 0.01 M potassium phosphate caused 50% inhibition of the enzyme activity. In the present study, the salivary amylase of *E. integriceps* was inhibited by CC, AS, SN, MC, MN (all at the concentrations of 1 and 3 mM) and AP (1 mM), strongly. It is to be supposed that the inhibiting effect of some mineral compounds on the digestive enzymes may offer an disadvantageous condition for digestion of food (Cohen, 1993; Hori, 1970).

Successful results have in the past been obtained with inhibitors that completely inhibited their target enzymes but recent results show that even partial inhibition can give substantial control of insect pests (Ishimoto Kitamura, 1989). Nevertheless, the use of nonproteinacious inhibitors for production of insect-resistant transgenic plants is much more difficult. Hence, the presence of multiple expressed transgenes would be required in order to confer protection (Baker, 1989). The primary reason for producing insect-resistance transgenic crops is to reduce the use of chemical pesticides and, thereby, the cost to the farmer and the consumer and to reduce the insecticide loads on the environment (Da Silva et al., 2004). Making insect-resistant plants requires the characterization of α-amylase of the target insect and the identification of suitable inhibitors from plants or other sources (Strobl et al., 1998).

In our opinion, the purification and characterization of more insect α-amylases will greatly facilitate the understanding of the mechanisms responsible for this selectivity and will help to design new and more specific strategies for insect control.

ACKNOWLEDGEMENTS

We are grateful to Mohammad Vatankhah, Tanin Naderi and Fatemeh Zivary for technical help.

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