SALIVARY ALPHA-AMYLASE ACTIVITY OF THE STRIPPED BUG, *GRAPHOSOMA LINEATUM*: CHANGES DURING DEVELOPMENT

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ABSTRACT: Studying the digestive enzymes of true bugs is important in understanding the physiology of the digestive system and manner of injury to plants by these insects. In this study, the salivary α-amylase activity of the adults and nymphs was measured by using a special diagnostic kit and an autoanalyzer. Protein concentrations in all enzyme samples were determined by using bicinchoninic acid method and bovine serum albumin as the standard. The method of Yazdanian et al. used for removing the salivary glands under a stereomicroscope, and enzyme samples were prepared by the method of Cohen. In eggs (newly oviposited and 1- to 4-days old ones), no enzyme activity was observed. In different nymphal stages, enzyme activity in third instars (12.45 U/mg protein) had the highest mean that differed significantly from other means. Enzyme activity did not observe in enzyme samples of first instars. Age and sex of adult insects had a significant effect on enzyme activity, so that it was higher in females than males, and in 15- and 30-days old adults it was higher than those of other ages, especially in 20-days old adult females which had the highest value (44.57 U/mg protein). In addition, it found that salivary α-amylase activity in adults was correlated with their reproductive activities.

KEY WORDS: *Graphosoma lineatum*, salivary α-amylase, activity, developmental changes.

Insect diets and their feeding behaviors are very different and their digestive enzymes have evolved with respect to the foods they consume (Takanona & Hori, 1974; Hori, 1975). In true bugs, one of the most important aspects of feeding is the injection of salivary enzymes into the host plant tissues (Miles, 1968; Miles, 1972; Hori, 1973b). The survival of phytophagous insects depends on digestive enzymes, especially α-amylases that are produced by the insects' guts and especially salivary glands. These enzymes are also present and active in the gut but they are less active than salivary enzymes. Studying the insect digestive enzymes would lead to recognition of their alimentary canal physiology (Hori, 1970a,b, 1972, 1973a, 1975; Takanona & Hori, 1974) and the damages caused by them on plants. Studying the insect digestive system also helps to recognize different enzymes and their characteristics including optimum pH, optimum temperature, and kinds of enzymes present in different species; and to evaluate the effects of various factors such as developmental stages, food regimes, geographical conditions, etc. on the

enzyme activity. In 1987, the results of studies on characteristics of digestive enzymes reached to the practical aspect of them. In this year, with producing transgenic plants (tobacco) containing the genes of trypsin inhibitors from broad bean and inducing resistance to the tobacco budworm *Heliothis virescens*, a new control method was introduced. This work continued in other plant species via using different toxic proteins such as lectins, protease inhibitors, α-amylase inhibitors, and δ-endotoxin of *Bacillus thuringiensis* (Botter & Jongsma, 1995; Schroeder et al, 1995; Hinks & Hupka, 1995; Morton et al. 2000; Silva et al. 2001). Introducing the transgenic plants containing the genes of enzyme inhibitors needs co-working of entomologists and plant breeders; and in the first step, entomologists begin to evaluate the biochemical characteristics of the enzymes and to recognize effective inhibitors.

α-amylases (α-1,4-glucan-4-glucanohydrolases; EC 3.2.1.1) are hydrolytic enzymes that are wide distributed and are found in microorganisms, plants and animals (Applebaum, 1985; Strobl et al., 1997; Strobl et al., 1998a,b; Barbosa Pereira et al., 1999; Titarenko & Chrispeels, 2000; Carlini & Grossi-de-Sa, 2002). These enzymes catalyze the hydrolysis of α-(1,4)-D-glucan bonds in starch, glycogen and other related carbohydrates (Franco et al., 2002; Strobl et al., 1998b). This enzyme converts starch to maltose, which is then hydrolyzed to glucose by an α-glucosidase. In insects, it has been shown that α-(1,4)-D-glucan bonds in long chain carbohydrates such as starch or glycogen are hydrolyzed only by α-amylases (Terra et al., 1996). Salivary and gut α-amylase activity has been described from several insect orders including Coleoptera, Hymenoptera, Diptera, Lepidoptera and Hemiptera (Hori; 1971; Baker & Woo, 1985; Strobl et al., 1998b; Zeng & Cohen, 2000b; Mendiola-Olaya et al., 2000; Oliveira-Neto et al., 2003; Mehrabadi & Bandani, 2009a).

In order to develop new strategies for controlling the herbivorous insects, such as the use of enzyme inhibitors and transgenic plants that express these inhibitors, we need to understand how digestive enzymes work (Bandani et al., 2001; Ghoshal et al., 2001; Maqbool et al., 2001). For nearly all of these strategies, having a strong understanding of the target pest's feeding is important (Mehrabadi & Bandani, 2009b). Furthermore, having knowledge about the biochemistry and physiology of feeding adaptation is of great importance (Mehrabadi & Bandani, 2009b).

In this study, we evaluated the effect of developmental changes on α-amylase activity of the stripped bug *Graphosoma lineatum* (L.) in order to approach a better understanding of the digestive physiology of this insect.

MATERIALS AND METHODS

Insects

All developmental stages of *G. lineatum* were reared at 28 ± 2 °C Temp., 60-70% R.H., and 16L: 8D. Parsley seeds were used for rearing the nymphs and adults. Water provided from dishes containing distilled water, through filter papers.

Preparing the Enzyme Solution

In the case of eggs, newly oviposited and 1- to 4-days old ones were selected. 40 eggs of each group were homogenized in a 1.5 ml microtube containing 1 ml of cold phosphate buffer (4 °C, pH = 6.95). The method of Yazdanian et al. (2006) was used for removing the salivary glands under a stereomicroscope (except for the first instars that head and thorax were homogenized). First to fifth instars and adult insects sampled randomly and starved for 24 hours before dissection to

accumulate enzymes in the salivary glands (Boyd et al., 2002; Zeng & Cohen, 2000a, 2000b; Cohen 1993). Adults and nymphs placed at –20 °C for 4 minutes to become motionless before dissection and then transferred to ice-cold phosphate buffer. At the next step, the 10 pairs of exposed salivary gland complex (including principal and accessory glands and principal and accessory ducts) homogenized in a 1.5 ml microtube containing 1 ml of cold phosphate buffer. The homogenates centrifuged at 15000 rpm for 20 min at 4 °C. The supernatants (or the enzyme solutions) were stored at –20 °C for subsequent analyses.

Enzyme Activity Assay

α-amylase activity in salivary glands of the adults and nymphs was measured by a special diagnostic kit (Amylase kit, Pars Azmoon Co., IRAN), using an autoanalyzer (Alcyon 300, Abbott, USA). Protein concentration in all enzyme samples was determined by using bicinchoninic acid method and bovine serum albumin (BSA) (Sigma-Aldrich, USA) as the standard. Finally, the specific activity of enzyme calculated as U/mg protein. The experiments repeated four times.

Statistical Analysis

Data were statistically analyzed by one-way analysis of variance (ANOVA). Means were separated by Duncan's multiple range test when significant differences were found at $p < 0.01$.

RESULTS

1. Activity of α-Amylase in Embryonic Stage

In any of enzyme solutions obtained from eggs (newly oviposited and 1- to 4-days old ones) no enzyme activity was observed.

2. Activity of α-Amylase in Different Instars

Average enzyme activity in different nymphal stages significantly differed from each other ($df = 4$, $F = 12.38$, $P = 0.00001$) (Fig. 1). Enzyme activity in first instars did not observe. Enzyme activity in third instars had the highest value (12.45 U/mg protein) and differed significantly from other means. After third instar, enzyme activities in forth and fifth instars had the highest amounts (6.71 and 4.45 U/mg protein, respectively) with no significant difference from each other but significantly differed from third instar. Mean of enzyme activity in second instar was 1.85 U/mg protein and had no significant difference with first and fifth instars but differed significantly from means of third and forth instars.

3. Activity of α-Amylase in Adults with Different longevities

Age and sex of adult insects and interaction between them affected the enzyme activity (age: $df = 6$, $F = 18.24$, $P = 0.0001$; sex: $df = 1$, $F = 18.51$, $P = 0.0$; interaction: $df = 6$, $F = 8.92$, $P = 0.00001$). Results of means comparison are shown in Fig. 2. Enzyme activities in females were higher than males in most cases with significant differences. Age of adult insects had a significant effect on enzyme activity. Enzyme activities in 15-, 20- and 25-days old males and females were considerable that are correlated with maximum reproductive activities of adults (Fig. 3). Increasing of the enzyme activity in female adult insects was more than that in adult males and the amount of the increase in enzyme activity at the maximum rate were 22.7 units in males and 43.29 units in females. Figs. 4 and 5 show the comparisons of means between nymphs and adults. Results obtained from some orthogonal comparisons are shown in Table 1.

DISCUSSION

It has reported that salivary enzyme activity in Heteroptera differs in different developmental stages (Nuorteva, 1954, 1956a,b; Saxena, 1955; Hori, 1968, 1970c, 1973a). Hori (1968) in a study on the effect of developmental stages of *Eurydema rugosa* on the salivary α-amylase activity showed that enzyme activity in second instars was low but in third, forth and fifth instars was higher and in forth instar was at maximum level. Enzyme activity in third instars was more than that of fifth instar. Variation in enzyme activity at different developmental stages attributed to the variations between these stages. Hori (1970c) reported that in *L. disponsi*, in each developmental stage, the salivary α-amylase activity in younger nymphs was lower but with ageing of nymphs and just before molting was at maximum rate. After molting, enzyme activity reduced considerably although its amount was higher than that in previous instars joust before molting. Comparison of enzyme activities in different instars just before molting showed that the enzyme activity in second instar was equal to that in third instar while the enzyme activities of forth and fifth instars were three and ten times greater than second instar, respectively. Activity of α-amylase in newly emerged adult insects were $^1/_{10}$ activity of that in fifth instar, then the activity of enzyme in adults rapidly increased and reached to the maximum level in 10-days old adults. Activity of enzyme in 30-days old adults was more than that of newly emerged insects but it was less than enzyme activities in 3- and 5-days old adults. Considerable enzyme activity just before molting may be resulted from high feeding of insects for getting energy for molting (Hori, 1970c, 1973a). Hori (1970c) reported that the reason for high enzyme activity in 10-days old female adults was their need for high amounts of proteins to produce eggs. Such reasons suggested in the case of crop proteases of *Miris dolabratus* (Nuorteva, 1956a,b) and *Calliphora erythrocephala* (Hori, 1973a). However, there must probably be other reasons for this phenomenon because enzyme activity was high in 10-days old adult males, too (Hori, 1970c, 1973a). No enzyme activity in first instars of the stripped bug can explained by the fact that they do not feed.

In *Prostephanus truncatus*, activity of crop α-amylase in adult insects and first to third instars were similar. Maximum and minimum enzyme activities were observed in second instars and pupae, respectively. Enzyme activity reduced with ageing of adult insects (Mendiola-Olaya et al., 2000). Hori (1973a) reported that activity of crop α-amylase in *B. mori* changed due to the growth of insects. Activity of crop α-amylase in *B. mori* gradually increased from first instar to fifth instar. Activity of crop proteases in this species increased from forth to fifth instars and in the early of fifth instar had the maximum activity. Enzyme activity decreased after few days then increased. Crop protease activity in *Galleria mellonella* increased at successive instars, but decreased at the time of molting (Hori, 1973a). Mehrabadi & Bandani (2009b) reported that the midgut α-amylase activity in the immature stages of the *Eurygaster maura* increased constantly up to the third instar. There were no significant differences of enzyme activity between third, fourth, and fifth instars and adults (0.071-0.083 U/insect). α-amylase activity in first instars was observed but it was very low (0.0046 U/insect). Enzyme activity in second instars was 0.030 U/insect and had a significant difference with other means. Comparing these results with our results shows that α-amylase activity in different instars of true bugs could be different depending on the instar and the site of enzyme production.

Female insects need more foods for reproduction. It is one of the reasons for more activity of α-amylase in adult females compared to the adult males. In

addition, considerable activity of the enzyme in 15-, 20- and 20-days old adults (Fig. 3, top) can explained with attention to the pre-oviposition and oviposition periods in adult females (Fig. 3, bottom) and the reproductive activities of both sexes. The more reproductive activities, the more feeding activities; and this may be an important reason for increasing the α-amylase activity in mature adults. As Hori (1968c) has stated, variation in α-amylase activity in different developmental stages can attributed to the variations in the stages. This is the first report on the effects of reproductive activities on the α-amylase activity in insects.

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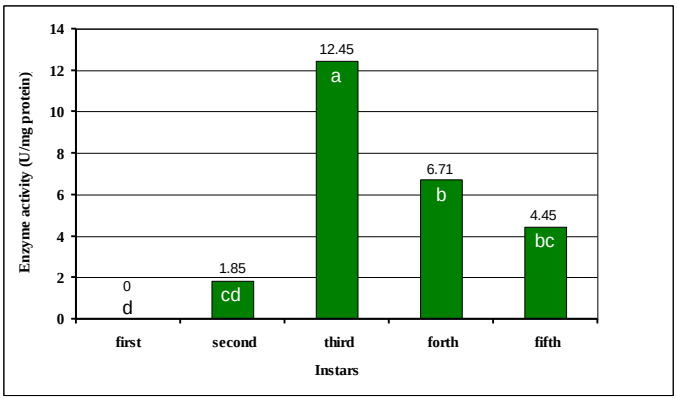

Figure 1. Comparison of means of salivary α-amylase activity in different instars of the stripped bug, *Graphosoma lineatum* (37 ºC, pH = 6.95).

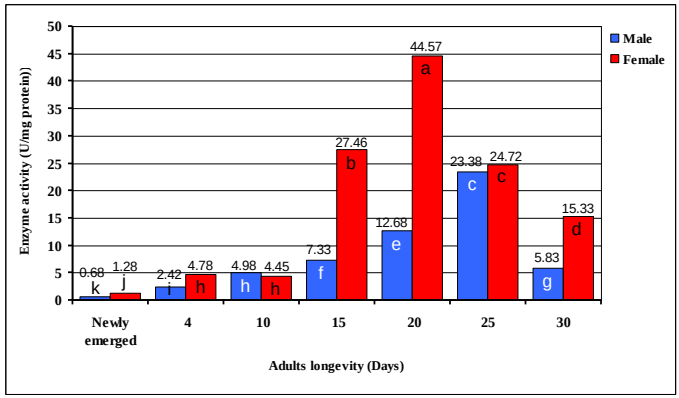

Figure 2. Comparison of means of salivary α-amylase activity in male and female adults of the stripped bug, *Graphosoma lineatum*, with different longevities (37 °C, pH = 6.95).

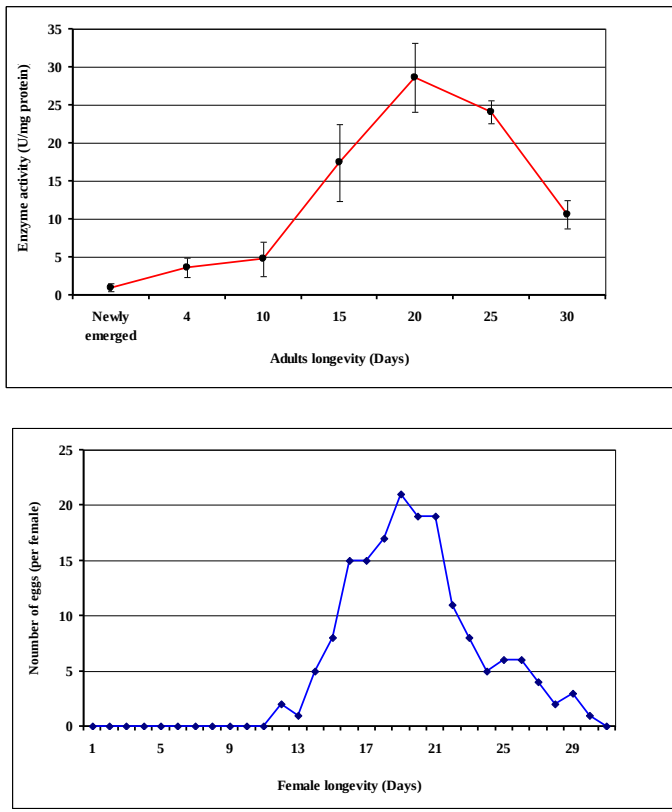

Figure 3. Total salivary α-amylase activity in adults of the stripped bug, *Graphosoma lineatum*, with different longevities (top) and oviposition trend in females (bottom) which show the correlation between the enzyme activity and reproductive activities (37 °C, pH = 6.95).

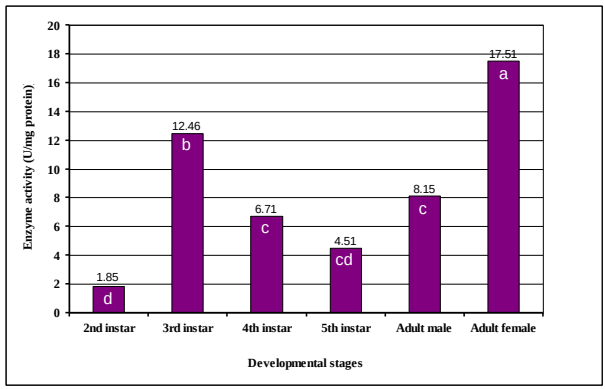

Figure 4. Comparisons of salivary α-amylase activity in nymphs, and male and female adults of the stripped bug, *Graphosoma lineatum* (37 °C, pH = 6.95).

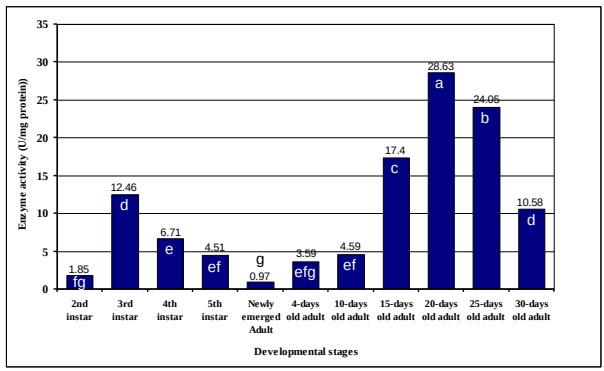

Figure 5. Comparisons of salivary α-amylase activity in nymphs and adults with different longevities in the stripped bug, *Graphosoma lineatum* (37 °C, pH = 6.95).

Table 1. Orthogonal comparisons of salivary α-amylase activities in nymphs and adults of the stripped bug, *Graphosoma lineatum*.

Kind of comparision			**MS**	**Mean**	
				Left group	**Right group**
Nymph	*vs*	Adults	221.772**	6.39^{b}	12.83^{a}
Nymph	*vs*	Female adult	396.463**	6.39^{b}	17.51^{a}
Nymph	*vs*	Male adult	9.981^{ns}	6.39^{a}	7.85^{a}
2nd instar	*vs*	Adults	321.429**	1.85^{b}	12.83^{a}
3rd instar	*vs*	Adults	0.366^{ns}	12.45^{a}	12.83^{a}
4th instar	*vs*	Adults	100.087**	6.71^{b}	12.83^{a}
5th instar	*vs*	Adults	184.508**	4.51^{b}	12.83^{a}

^{ns} and ** show non-significant, and significant differences at 1% level, respectively.