EXPRESSING OF DIGESTIVE AMYLASE IN VARIOUS DEVELOPMENTAL STAGES OF *EURYGASTER MAURA*, AN ENZYMATIC APPROACH

Mohammad Mehrabadi* and Ali R. Bandani*

* Plant Protection Department, Faculty of Agriculture, University of Tehran, Karaj, Ir. IRAN. e-mails: mmehrabadi@ut.ac.ir, abandani@ut.ac.ir

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ABSTRACT: In order to identify and determine of α-amylase expressing in various developmental stages of *Eurygaster maura,* a series of biochemical and enzymatic experiments was carried out. For this goal midgut α-amylase was isolated and characterized. Enzyme samples from midguts of adults were prepared by the method of Cohen with slight modifications. The α-amylase activity was assayed by the dinitrosalicylic acid (DNS) procedure, using 1% soluble starch as substrate. Then absorbance was read at 540 nm by spectrophotometer. Amylase activity was detected in the midgut of the insects which were collected from wheat fields during spring. Amylase activity in the midgut of feeding insects was 0.083 U/insect. α-amylase activity was detected in the various nymphal stage. The results show that α-amylase activity in the immature stages increase constantly up to the third-instar. There were no significant differences of enzyme activity between third, fourth, fifth nymphal stages and adults (0.0071 - 0.0083 (U/insect)). α-amylase activity in first-nymphal stage was 0.0046 U/insect. Optimum temperature for the enzyme activity was determined to be between 30 to 40 ˚C. Optimum pH value for amylase was 6.5-7.

KEY WORDS: *Eurygaster maura*, nymphal stages, amylase assay.

Wheat is one of the main crops planted over a wide area in Iran. Several biotic factors influence the yield of this economic crop; among mentioned biotic factors the most important factors are insects which in recent years decrease wheat yield dramatically. Wheat bugs have an important role in the decrease of wheat products. Among hemipteran insects in wheat farms, genera of *Eurygaster* sp. is more important than others and have several species such as E. *integriceps, E. maura* etc. As wheat bugs are piercing- sucking insects, they must introduce theirs salivary enzymes into the seed and after partial digestion, sucking digested material (e.g. flush-feeding insects). The entrance of mentioned bugs salivary enzymes into the feeding seeds, in addition of its direct injury to wheat seeds, it causes the decrease of feeding seeds quality, and has harmful medical effects on consumers including humans. *Eurygaster maura* is the dominant wheat bug in north Iran particularly in the Gorgan area, Golestan province. The insect is mainly found in wheat farm which causes severe damage to the vegetative growth stage of wheat in the early season. It also feeds on wheat grains in the late growth stage, thus damaged grains lose their bakery properties. In addition to the direct damage to wheat grain it also injects salivary enzymes into the feeding seeds causing damage to seed quality, too. Injection of salivary enzymes into the wheat also produces hygienic problem for consumers. The most important periods in the life cycle of *E. maura* are the period of late nymphal development and the intense feeding of the newly emerged adults. Nymphs in the early instars do not feed intensively. After the third instar, feeding is intensified and the damage to crops becomes obvious. The emerged adults start intense feeding on wheat grains. During feeding, this pest with its piercing-sucking mouthparts injects saliva from

the salivary gland complexes into the grains to liquefy food. Then the liquefied food is ingested and further digestion is made inside the gut. Because of injecting enzymes into the grain during feeding, the enzymes degrade gluten proteins and cause rapid relaxation of dough which results in the production of bread with poor volume and texture (Kazzazi et al., 2005).

α-Amylases (α-1,4-glucan-4-glucanohydrolases; EC 3.2.1.1) are hydrolytic enzymes that are widespread in nature, being found in microorganisms, plants, and animals. These enzymes catalyze the hydrolysis of α-D-(1,4)-glucan linkage in starch components, glycogen and various other related carbohydrates (Franco et al., 2000; Strobl et al., 1998).

E. maura like other insect pests of wheat lives on a polysaccharide-rich diet and depends to a large extent on the effectiveness of its α-amylases for survival (Mendiola-Olaya et al., 2000). It converts starch to maltose, which is then hydrolyzed to glucose by an α-glucosidase. In insects only α-amylases has been found to hydrolyze long α-1,4-glucan chains such as starch or glycogen. Amylase activity has been described from several insect orders including Coleoptera, Hymenoptera, Diptra, Lepidoptera and Hemiptera (Terra et al., 1988; Mendiola-Olaya et al., 2000; Zeng, and Cohen 2000; Oliveira-Neto et al., 2003).

An understanding of how digestive enzymes function is essential when developing methods of insect control, such as the use of enzyme inhibitors and transgenic plants to control phytophagous insects (Bandani et al., 2001; Maqbool et al., 2001). For nearly all these strategies, having a strong understanding of the target pest's feeding is important. Also, an understanding of the biochemistry and physiology of feeding adaptation is important.

Nothing is currently known about the properties of α-amylase of *E. maura*. The purpose of the present study is to identify and characterize the α-amylase activity of *E. maura* in order to gain a better understanding of the digestive physiology of wheat bug. This understanding will hopefully 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 from these individuals were removed by dissection under a light microscope in ice-cold saline buffer (0.006 M NaCl).

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 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.

Nymphs' α-amylase was prepared by the method of Mendiola-Olaya (2000) with slight modifications. The nymphs' weight was determined. Whole *E. maura* nymphs were homogenized in the above mentioned universal buffer and centrifugation carried out as before. The supernatants were pooled and stored at -20˚C for later use.

Amylase Activity Assay

The α- amylase activity was assayed by the dinitrosalicylic acid (DNS) procedure (Bernfeld 1995), 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 40µl 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 the 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 following range of concentrations of 2, 1, 0.5, 0.25, 0.125 mg ml-1 (Fig. 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 Temperature on Enzyme Activity

The effect of temperature on α-amylase activity was determined by incubating the reaction mixture at different temperatures including 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, and 70 ˚C for 30 min. Also thermo-stability of enzyme over 10 days at specified temperature was determined. Samples were maintained at 4, 24, 34, and 44˚C for 10 days followed by determination of residual activity by enzyme assay as described before.

Effect of pH on Enzyme Activity

The pH optima of amylase was determined using universal buffer (Hosseinkhani and Nemat-Gorgani, 2003). The pH was tested were 2, 3, 4, 5, 5.5, 6, 6.5, 7, 7.5, 8, 9, and 10.

Protein Determination

Protein concentration was measured according to the method of Bradford (1976), using bovine serum albumin (Bio-Rad, Munchen, Germany) as a standard (Fig. 1).

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

α- amylase Activity

Studies showed that α- amylase activity is present in midgut of adult *E. maura* and in whole body of nymphs (Table 1). the activity of midgut enzyme was 0.0507 U/insect.

Only trace amounts of enzyme activity were detected in the first-nymphal stage (0.0046 U/insect), whereas α- amylase activity reached its highest value (0.083 U/insect) in the fifth-nymphal stage (Table 1).

These results show that α-amylase expression and 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) (Table 1). Enzyme activities in these stages were 0.071, 0.078 and 0.083 U/insect, respectively (Fig.2)

Effect of Temperature on Amylase Activity

Optimum temperature for the enzyme activity was determined to be between 30 to 40 ˚C (Fig. 3). The rapid decrease in amylase activity observed above 40˚C and amylase activity reached zero at 70˚C. α-amylase thermal stability was monitored by measuring residual activity after incubation of enzyme at 4, 24, 34, and 44˚C over 10 days. The amylolytic activity decreased at high temperature. For instance at 4 and 44˚C loss of enzyme activity over 10 days were 2 and 50%, respectively (Fig 3).

Effect of pH on Amylase Activity

Optimum pH value for amylase was 6.5-7 (Fig. 4). Activity dropped rapidly below pH 4.0 and mildly above pH 7.0. However, there was considerable activity over a broad range of pH.

DISCUSSIONS

The results from this study demonstrate that midget of adults and nymphal hole body of *E. maura* have α- amylase activity, then expressing of amylase was demonstrated. The presence of the amylase activity in the gut of other phytophagous heteropterans has been reported. 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 2003). The complete breakdown of starch should take place in the midgut where large amounts of amylase exist.

Amylases in insects are generally most active in neutral to slightly acid pH conditions. Optimal pH values for amylases in larvae of several coleopterans were 4-5.8 and in Lygus spp. (Heteroptera) was 6.5 (Zheng and Cohen 2000). Optimum pH generally corresponds to the pH prevailing in the midguts from which the amylases are isolated.

The first nymphal stage of the wheat bug does not feed, which may be one reason why they have very low expression of amylase and activity (0.0046 U/insect). 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.

The Wheat bug α-amylase has an optimum temperature activity of 30-400C, which is consistent with the other reports (Ishaaya et al. 1971; Mendiola-Olaya et al. 2000).

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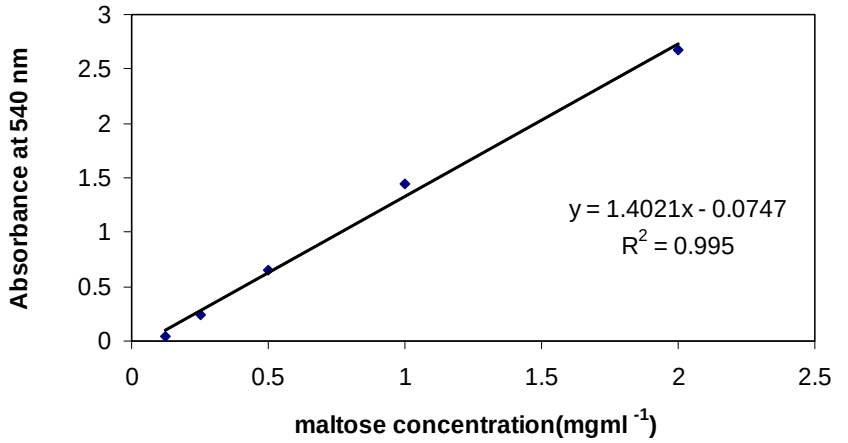

Fig. 1. Standart calibration curve for the determination of maltose released in the α-amylase assay.

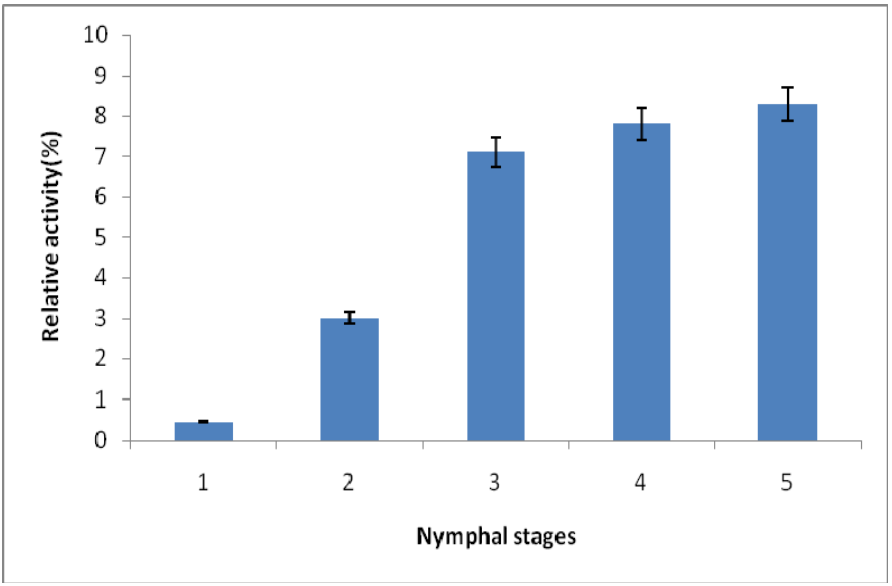

Fig. 2. Histogram of the activity of α-amylase in different nymphal stages of *E. maura*.

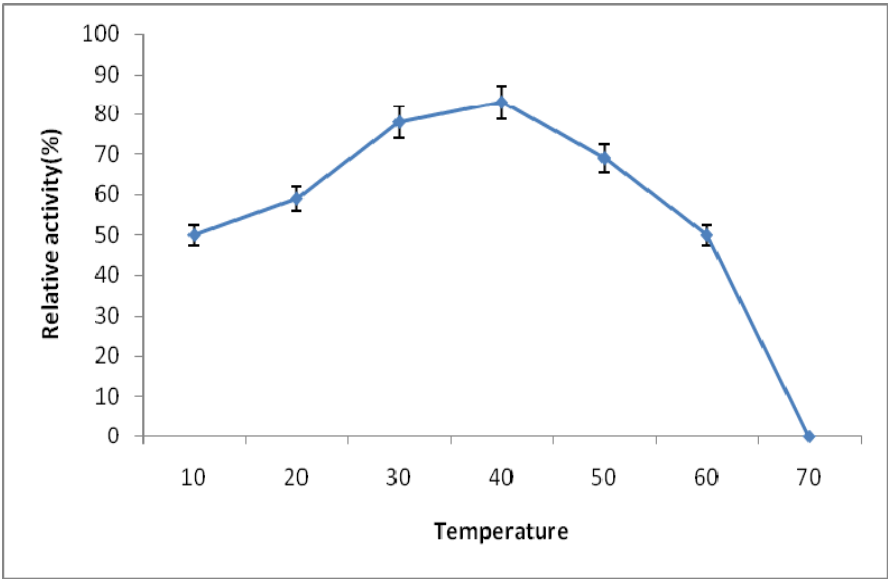

Fig 3. Effect of temperature on α-amylase activity of *E. maura*.

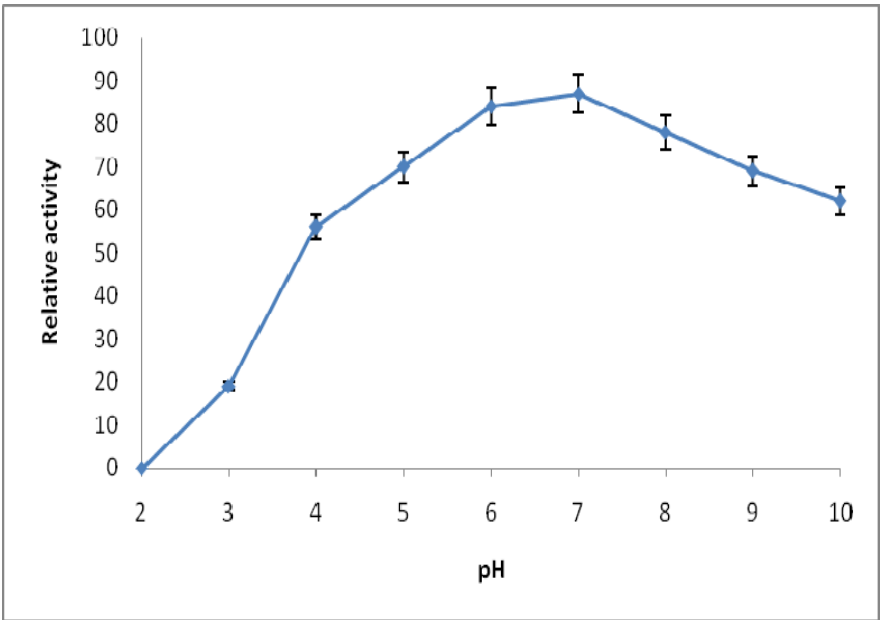

Fig 4. Effect of pH on α-amylase activity of *E. maura*.

Table 1. Comparison of the activity of α-amylase in different nymphal stages of *E. maura*.

Nymphal stage	Activity per ml enzyme (µmol/min/ml; Mean ± SE)	Unit Activity (µmol/min/u, Mean± SE)
1	0.00046 ± 0.020b	0.0046 ± 0.023b
2	0.0030 ± 0.025c	0.030 ± 0.034c
3	0.0071 ± 0.020a	0.071±0.05a
4	0.0078 ± 0.030a	0.078 ± 0.042a
5	0.0083 ± 0.026a	0. 083 ± 0.011a

Sample size for each nymphal stage, n = 3. Values with the same letter did not significantly differ.