

Biochemical characterization of midgut, salivary glands and haemolymph α -amylases of *Naranga aenescens*

Ameneh ASADI¹, Mohammad GHADAMYARI¹, Reza Hassan SAJEDI², Jalal JALALI SENDI¹, Mehrdad TABARI³

¹Department of Plant Protection, Faculty of Agriculture, University of Guilan, Rasht, Iran

²Department of Biology, Faculty of Science, University of Guilan, Rasht, Iran

³Institute of Rice, Amol, Iran

Abstract

Naranga aenescens L. (Lepidoptera Noctuidae), commonly known as the rice green caterpillar, is one of the most damaging pests of the rice crop in Iran causing extensive defoliation. Study of digestive enzymes is so imperative to reach and apply new pest management technologies. Amylases have an important role in the final stages of carbohydrate digestion. α -amylases activities were determined in midgut, haemolymph and salivary glands homogenates of *N. aenescens* larvae. The activities of α -amylases in the midgut, salivary glands and haemolymph of 5th instar larvae were 2.4 ± 0.04 , 2 ± 0.06 and 1.2 ± 0.06 $\mu\text{mol}/\text{min}/\text{mg}$ proteins, respectively. Zymogram pattern in the native gel revealed that *N. aenescens* α -amylases in midgut, salivary glands and haemolymph had two isoform. Results showed that the optimal pH for α -amylases in midgut, salivary glands and haemolymph were 8, 9 and 9, respectively. Also optimal temperature for α -amylases activity in midgut was 60 °C and salivary glands and haemolymph were 50 °C. As calculated from Lineweaver-Burk plots, the K_m for midgut, salivary glands and haemolymph were about 0.07, 0.14 and 0.14 mg/ml and the V_{max} were 0.2, 0.08 and 1.32 $\mu\text{mol}/\text{min}$. Hg^+ , Hg^{2+} and Mn^{2+} decreased enzyme activity even completely in all three tissue of *N. aenescens* α -amylases, whereas the addition of Na^+ , K^+ , Co^{2+} , Mg^{2+} , Ca^{2+} and Fe^{2+} increased their activity. α -amylase activity in *N. aenescens* is essential for developing a method for insect control, such as the use of an α -amylase inhibitor as a biocontrol agent and over expression of α -amylase inhibitor in transgenic plant.

Key words: amylases, digestive enzyme, rice green caterpillar, *Naranga aenescens*.

Introduction

Most living organisms are able to exploit environmental polysaccharides. α -amylases (α -1,4-glucan-4-glucanohydrolases, EC 3.2.1.1), which hydrolyze starch and other polysaccharides in maltose, maltotriose and maltodextrines, are key enzymes in this process (Henrissat *et al.*, 2002). These enzymes constitute a family of endo-amylases that catalyze the hydrolysis of α -D-(1,4)-glucan linkages in starch components, glycogen and other carbohydrates. In many cases, they form multigene families. The presence of several, sometimes divergent amylase molecules enables organisms to digest a broad range of substrates, in a broad range of environmental conditions (Da Lage *et al.*, 2007). The enzymes play a key role in carbohydrate metabolism of microorganisms, plants and animals (Franco *et al.*, 2002). α -amylases have been found in several insect orders including Lepidoptera, where they are usually reported in the caterpillar gut (Zibae *et al.*, 2008; Abraham, 1992; Dow, 1984; Somadder and Shrivastava, 1980; Kanekatysa, 1978). Moreover, several insects, especially those feed on starchy food during larval stage, depend on their α -amylases for survival (Strobl *et al.*, 1998; Franco *et al.*, 2000). Plant α -amylase inhibitors are proteins found in several plants, and play a key role in natural defenses especially those that feed on starchy food. These inhibitors, which are particularly abundant in legumes (Franco *et al.*, 2002) and cereals (Iulek *et al.*, 2000), represent a potent tool in engineering crop plants due to their role as defense factors against insect pests and pathogens (Svensson *et al.*, 2004). Search on starch digestion as a target for control of starch-dependent insects was stimu-

lated in recent years after results showed that α -amylase inhibitors from *Phaseolus vulgaris* L. seeds are detrimental to the development of cowpea weevil *Callosobruchus maculatus* (F.) (Coleoptera Bruchidae) and Azuki bean weevil *Callosobruchus chinensis* (L.). Pea and azuki transgenic plants expressing α -amylase inhibitors from common beans were completely resistant to the *Bruchus pisorum* (L.) and *C. chinensis* weevils (Ishimoto and Kitamura, 1989; Shade *et al.*, 1994).

Naranga aenescens L. (Lepidoptera Noctuidae), commonly known as the rice green caterpillar, is one of the most damaging pests of the rice crop in Iran causing extensive defoliation but, at times of severe infestation, it also feeds, on developing panicle resulting in massive loss of the crop. This insect was reported in northern Iran in 1986 and was widely distributed in all paddy fields. This pest outbreak in paddy fields in Guilan province and 30,000 hectares of paddy fields were sprayed with insecticides against this pest annually (Abivardi, 2001). Application of pesticide against *N. aenescens* is extremely hazardous not only to farmers and consumers, but also to the environment.

Digestion is a process of splitting un-absorbable macromolecules of food by digestive enzymes into utilizable and absorbable molecules by addition of water. The study of the digestive enzymes forms an important base for the study of digestive physiology. The carbohydrate digestion of occurs mainly in the lumen of the midgut and 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, 2002). Another source of α -amylase in insect is haemolymph. The present paper reports the α -amylases

of salivary glands, midgut and haemolymph of *N. aenescens* larvae.

As many insect species depend on the effectiveness of their amylases for survival, characterization studies of insect amylases are not only of interest for comparative investigations, but they can also contribute to clarifying the compatibility of some natural diets with insect development. This is particularly true for an insect species such as *N. aenescens* that feeds on rice leaf during larval stage.

At present there is no information available on the α -amylases of *N. aenescens* and a detailed knowledge of *N. aenescens* digestive physiology will provide new opportunities for a sustainable pest management to control this insect pest. The goal of the present study is to characterize midgut, salivary glands and haemolymph α -amylases from *N. aenescens*.

Materials and methods

Insect

Insects were collected from rice seedling *Oryza sativa* L. 'variety of Hashemi' in the northern provinces of Iran. 5th instar larvae were randomly selected for measuring of enzyme activity.

Sample preparation

Enzyme samples from the midguts and salivary glands of the last instar larvae were prepared by the method of Cohen (1993). Last instar larvae were immobilized on ice and dissected under a stereo microscope in ice-cold saline buffer (6 μ mol/l NaCl). Whole of the midguts and salivary glands were removed and thoroughly rinsed in ice-cold distilled water. The haemolymph was collected by cutting their prolegs or making a few incisions in the cuticle. Each midgut and salivary glands was homogenized in 20 and 3 μ l of cold double-distilled water and/or buffer using a hand-held glass homogenizer, respectively. The homogenate was centrifuged at 13000 \times g for 15 min at 4 °C and supernatants were collected and stored at -20 °C for subsequent analyses. Total and specific activities of midgut digestive enzymes and salivary glands were determined in the homogenates.

Chemical

3,5-Dinitrosalicylic acid (DNS), triton X-100 and EDTA were purchased from Sigma (St. Louis, MO, USA). All other chemicals (reagent grade) were from Merck (Merck, Darmstadt, Germany).

Determination of α -amylase activity and protein concentration

α -amylase activity was determined at room temperature in 20 mM Tris-HCl, 10 mM Ca²⁺, pH 9.0. Supernatant (10 μ l) was added to a tube containing 40 μ l of the buffer and 50 μ l of 1% (w/v) starch. The concentration of reducing sugars obtained from the catalyzed reaction for 15 min (midguts α -amylases) and 30 min (salivary glands and haemolymph α -amylases) was measured by the dinitrosalicylic acid method according to Bernfeld (1955). Absorbance was measured at 545 nm with a Microplate

Reader Model Stat Fax[®] 3200 (Awareness Technology Inc.). One unit of α -amylase is defined as the amount of the enzyme that liberates 1.0 μ mol of reducing sugar/min with maltose as a standard. Protein concentration was determined by the Bradford method (1976).

Polyacrylamide gel electrophoresis and zymogram analysis

Non-denaturing polyacrylamide gel electrophoresis (PAGE) (8%) was carried out as described by Davis (1964) and electrophoresis was performed with 100 V at 4°C. Afterward, the gel was incubated in 2.5% (v/v) Triton X-100 for 30 min at room temperature with gentle agitation. Then, the gel was rinsed with deionized water and washed in 25 mM of Tris-HCl pH 7.4. The washed gel was incubated in fresh buffer containing 1% (w/v) soluble starch at 30 °C for 60 min. After being washed with distilled water, the gel was subjected to staining with Lugol solution (I₂ 1.3% and KI 3%) at ambient temperature until the appearance of clear zones in protein bands with α -amylase activity against a dark blue background. Quantification of enzyme activity was performed from zymogram, using the Image Master Total-Lab v1.10 (Life Science Analysis Essential) program.

Effect of pH and temperature on enzyme activity

The pH profiles of the α -amylases were determined at room temperature in a mixed buffer containing phosphate, glycine and acetate (25 mM of each) adjusted to various pHs (pH 3 to 12) by adding HCl or NaOH for acidic and basic pH values, respectively. Before determining activity, the reaction mixtures were incubated at different pHs at room temperature for 5 min. The activities of the enzymes were determined by incubating the reaction mixture at different temperatures ranging from 20 to 80 °C in 20 mM Tris-HCl, pH 9 for 5 min. Enzyme activity was measured by the standard assay method mentioned above.

Effect of metal ions and EDTA

The effects of various metal ions including Na⁺, K⁺, Mg²⁺, Co²⁺, Ba²⁺, Zn²⁺, Ca²⁺, Fe²⁺, Hg⁺, Hg²⁺, Mn²⁺ and ethylene diamine tetraacetic acid (EDTA) (20 mM for all the ions) were investigated. After 30 min of pre-incubation of ions or EDTA with enzymes at room temperature, residual activity was measured by the standard assay method.

Kinetic parameters of α -amylases

Catalytic activity of the enzymes were investigated at different concentrations of starch over the range 0.05-1.5% (w/v), in 20 mM Tris-HCl, pH=8 and Michaelis-Menten constant (K_m) and maximal velocity (V_{max}) were estimated from the Lineweaver-Burk plots. The kinetic values are the averages of three experiments and standard errors are less than 10%.

Statistical analysis

The data were compared by one-way analysis of variance (ANOVA) followed by Tukey's test when significant differences were found at $P = 0.05$ using SAS program (SAS, 1997).

Table 1. α -amylases activity in different sources of last instar larvae of *N. aenes-cens**.

Parameter (Mean \pm SE)	Midgut	Salivary glands	Haemolymph
Specific activity ($\mu\text{mol}/\text{min}/\text{mg}$ proteins)	$2.4 \pm 0.04\text{a}$	$2 \pm 0.06\text{b}$	$1.2 \pm 0.06\text{c}$
Activity ($\text{nmol}/\text{min}/\text{mg}$ tissue)	$8.96 \pm 0.15\text{a}$	$4.75 \pm 0.14\text{b}$	$0.33 \pm 0.03^{**}\text{c}$

*Results are the mean \pm SE of nine individual insect tissues ($n = 3$), each with triplicate analysis.

**Activity of enzyme expressed as $\text{nmol}/\text{min}/\mu\text{l}$ haemolymph.

Different letters indicate that the activity of enzymes in different tissue is significantly different from each other by Tukey's test ($p < 0.05$).

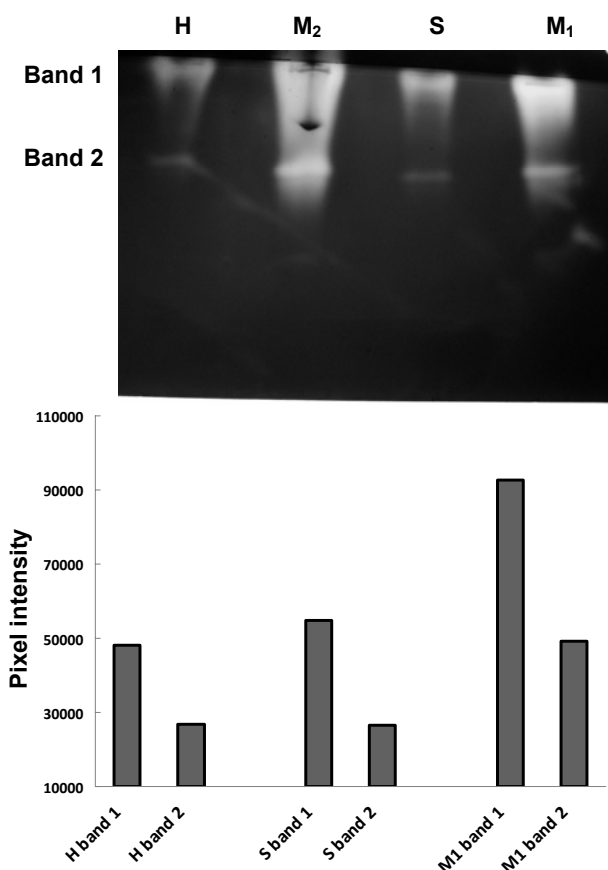


Figure 1. Zymogram of α -amylases from *N. aenes-cens*. H, Haemolymph (equivalent 30 μl); M₁ (equivalent 1 midgut) and M₂ (equivalent 2 midgut) and S, salivary glands (equivalent 8 salivary glands). For more details see materials and methods. The pixel intensity was determined from the scanned gel image using TotalLab v1.10 imaging software.

Results

Amylase activity

The activity of α -amylases was characterized in crude extracts. The data revealed that α -amylase is present in the midgut, salivary glands and haemolymph at last instar larval stage of *N. aenes-cens*. α -amylases activity in midgut, salivary glands and haemolymph of 5th larval instar were 8.94 ± 0.15 , 4.75 ± 0.14 $\text{nmol}/\text{min}/\text{mg}$ tissue and 0.33 ± 0.03 $\text{nmol}/\text{min}/\mu\text{l}$, respectively (table 1). Also, amylases specific activities in the midgut, salivary glands and haemolymph of 5th instar larvae were 2.4 ± 0.04 , 2 ± 0.06 and 1.2 ± 0.06 $\mu\text{mol}/\text{min}/\text{mg}$ proteins,

respectively (table 1). The results showed that there was significant difference between the amylase activities in tissues and highest specific activity was detected in the midgut and lowest in haemolymph.

Zymogram analysis

The crude *N. aenes-cens* larval extracts was analysed by native PAGE. After amylase activity staining, two major isoforms of α -amylase were clearly detected in any three tissues with the same electrophoretic pattern. However, as depicted in figure 1, intensity of both bands concerning α -amylase activity in salivary glands and haemolymph were less than in midgut.

Effect of pH and temperature

The influence of pH (on the activity of midgut, salivary glands and haemolymph α -amylases is shown in figure 2. The optimum pH for midgut, salivary glands and haemolymph α -amylases were 8, 9 and 9, respectively. The pH activity profile of salivary glands α -amylases showed a very broad pH range. The enzyme activity retained over than 60% of its maximal activity in the pH range of 3 to 11. This enzyme showed higher activity in acidic pH when compared to the amylases found in the two other tissues.

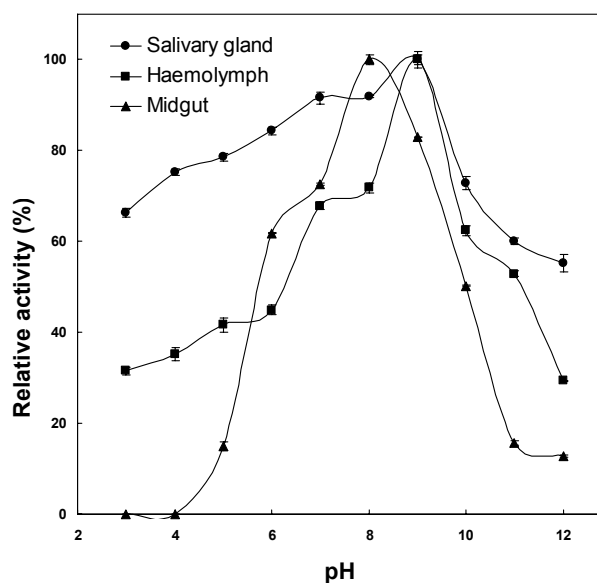


Figure 2. Effect of pH on the activity of midgut, salivary glands and haemolymph *N. aenes-cens* α -amylases. Mass homogenates were prepared in distilled water. Results are the means of eight replications. Vertical bars indicate standard error of means.

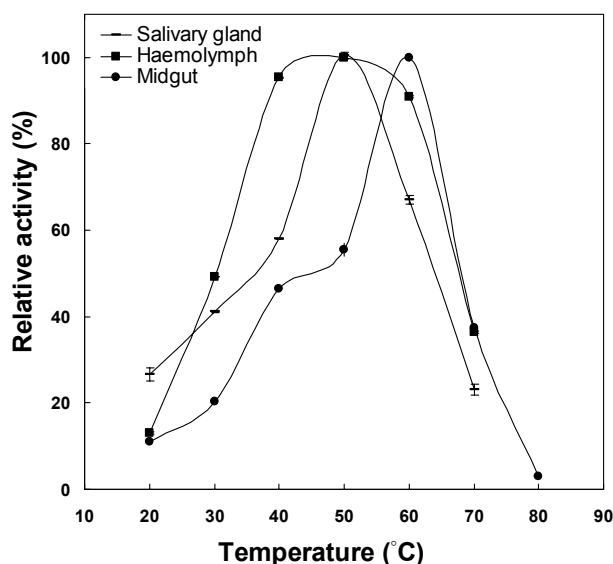


Figure 3. Effect of temperature on the activity of midgut, salivary glands and haemolymph *N. aenesecens* α -amylases. Results are the means of eight replications. Vertical bars indicate standard error of means.

The effect of temperature on the activity of the enzymes is shown in figure 3. The optimum temperatures for midgut, salivary glands and haemolymph α -amylases of the last instar larvae were 60, 50 and 50 °C respectively.

A broad temperature activity profile was also observed for haemolymph α -amylase compared with that of two other *N. aenesecens* α -amylases from midgut and salivary glands. A sharp decrease in activity was observed over 60°C for these three α -amylases.

Effect of metal ions and EDTA on α -amylase activity

The α -amylases activity was measured at optimum pH of any α -amylases in the presence of various metal ions and EDTA (20 mM). As shown in table 2, the addition of Hg^+ , Hg^{2+} and Mn^{2+} decreased enzyme activity completely in all three *N. aenesecens* α -amylases, whereas the addition of Na^+ , K^+ , Co^{2+} , Mg^{2+} , Ca^{2+} and Fe^{2+} increased their activities in all three tissues. Furthermore, Ba^{2+} increased enzyme activity in the midgut and salivary glands and decreased enzyme activity in the haemolymph. Zn^{2+} increased enzyme activity in the haemolymph and salivary glands and decreased enzyme activity in the midgut. EDTA increased α -amylases activity in the midgut and decreased activity of α -amylases in the salivary glands and haemolymph from *N. aenesecens*.

Catalytic properties

All three α -amylases revealed a Michaelis-Menten type kinetics when hydrolyzing soluble starch at their optimum pH. As calculated from Lineweaver-Burk plots, the K_m and V_{max} values for soluble starch at 37 °C are presented in table 3.

Table 2. Effect of various metal ions (20 mM) and EDTA (20 mM) on α -amylases from *N. aenesecens*. Mass homogenates were prepared in 20 mM Tris-HCl, pH 9.0. Results are the means of five replications. All the metal ions were added as chloride salts.

Metal ions	Relative activity (%) (Mean \pm SE)		
	Midgut	Salivary glands	Haemolymph
Control (no addition)	100	100	100
Na^+	187.2 \pm 0.44	194.8 \pm 0.5	192.6 \pm 1.48
K^+	161.2 \pm 1.63	167.2 \pm 0.5	154.1 \pm 0.68
Mg^{2+}	133.3 \pm 0.66	171.8 \pm 0.5	140.6 \pm 2.36
Co^{2+}	182.2 \pm 0.25	146.5 \pm 0.5	149.9 \pm 0.68
Ba^{2+}	110.1 \pm 0.29	120.1 \pm 0.5	82.1 \pm 1.88
Zn^{2+}	56.1 \pm 1.14	117.8 \pm 1.7	182.3 \pm 1.93
Ca^{2+}	133.9 \pm 0.70	148.2 \pm 0.5	186.2 \pm 0.68
Fe^{2+}	108.4 \pm 0.90	121.2 \pm 1.7	168.4 \pm 1.34
Hg^+	95.3 \pm 0.21	0	68.9 \pm 2.36
Hg^{2+}	84.0 \pm 0.66	16.1 \pm 0.5	65.4 \pm 0.89
Mn^{2+}	99.4 \pm 0.7	0	0
EDTA	122.9 \pm 0.86	64.3 \pm 0.5	170.5 \pm 0.68

Table 3. Kinetic parameters in different tissue α -amylases from *N. aenesecens*. The homogenates were prepared from 600 individuals in 20 mM Tris-HCl, pH 9.0. Each homogenate was diluted in the buffer. Equivalent to half a midgut, 3.33 salivary glands and 7.5 μ l of haemolymph was used for a homogenate for each assay. Results are the means of four replicates.

Parameter (Mean \pm SE)	Midgut	Salivary glands	Haemolymph
K_m (mg/ml)	0.076 \pm 0.0002b	0.14 \pm 0.003a	0.14 \pm 0.004a
V_{max} (μ mol/min)*	0.2003 \pm 0.01b	0.08061 \pm 0.0005c	1.322 \pm 0.01a

Different letters indicate that the activity of enzymes in different tissue are significantly different from each other by Tukey's test ($p < 0.05$).

Discussion

The data revealed that α -amylase is present in the midgut, salivary glands and haemolymph at last instar larval stage of *N. aenescens*. The specific activity of α -amylases from midgut was 1.2 and 1.98-fold higher than that of salivary glands and haemolymph, respectively (table 1). Our results showed that there is a significant difference in the activity of α -amylases in midgut and salivary glands of 5th instar (table 1). The activities of α -amylase in the midgut and salivary glands of *Chilo suppressalis* Walker (Lepidoptera Pyralidae) were 0.06 and 0.036 $\mu\text{mol}/\text{min}/\text{mg}$ protein, respectively (Zibae et al., 2008). Also, Yezdani et al (2010) showed that the activity of α -amylase in the midgut and salivary glands of *Glyphodes pyloalis* Walker (Lepidoptera Pyralidae) were 0.011 and 0.0018 $\mu\text{mol}/\text{min}/\text{mg}$ protein, respectively. The specific activity of the α -amylases in the midgut of *N. aenescens* was 1.2-fold higher than in the salivary glands which is consistent with α -amylases activity in *G. pyloalis* (Yezdani et al., 2010) and *C. suppressalis* (Zibae et al., 2008). Also, the presence of α -amylase activity in the midgut and salivary glands of other phytophagous lepidopterans has been reported (Abraham, 1992; Dow, 1984; Somadder and Shrivastava, 1980; Kanekatsya, 1978).

The specific activity of amylases in haemolymph was 1.2 ± 0.06 nmol/min/ μl . Tanaka and Kusano (1980) studied change of amylase activity in haemolymph of the silkworm, *Bombyx mori* L. during the development from the third instar to the adult emergence. Their results showed that highest activity value was at the last period of the fourth instar (105 mg maltose/hr./ml haemolymph). Also Banno et al. (1984) reported that the amylase activity in female fifth instar larvae of *B. mori* was 325 μg maltose/min/ml haemolymph. Changes of the haemolymph amylase activity during development in the cabbage armyworm, *Mamestra brassicae* L. was studied and results showed that activity values differ between developmental stages from 0.6 to 1.35 μg maltose/min/ μl haemolymph (Tanabe and Kusano, 1984).

The activity of α -amylase was also characterized by zymogram analysis after native PAGE which allowed visualization of the enzyme activity in situ. The results indicated the presence of at least two α -amylase isoforms in crude midgut, salivary glands and haemolymph. The activity of α -amylase from midgut on gel was much higher than that of other tissues and compared with the midgut zymogram, weak light bands appeared in the case of haemolymph and salivary glands. These results correlated well with α -amylase specific activities in crude extracts. Wisessing et al. (2008) showed that *C. maculatus* α -amylase had one isoform with a molecular weight of 50 kDa. However, the number of α -amylases identified in different insect species varied from 1 to 8 isoforms e.g., *Helicoverpa armigera* (Hubner), *Spodoptera litura* (F.), *C. chinensis* and *Coryca cephalonica* (Stainton) exhibited more than five isoforms whereas *Sitophilus oryzae* (L.) and *Tribolium castaneum* (Herbst) possessed only one isoform (Sivakumar et al., 2006).

Also, the different forms of α -amylases in the insect

midgut lumen have been observed in *C. maculatus* and *Zabrotes subfasciatus* (Bohemann) and *Tenebrio molitor* L. (Coleoptera Tenebrionidae) (Campos et al., 1989; Silva et al., 1999).

The pH-activity profile of *N. aenescens* larval amylase is a bell-shaped curve with the maximum at pH 8, 9 and 9 for midgut, salivary glands and haemolymph, respectively, similar to that reported for a crude enzyme preparation from the rice steam borer by Zibae et al., (2008). In addition, salivary glands α -amylase in *N. aenescens* showed higher activity in lower pH range in comparison with two other α -amylases. α -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). Optimal pH for α -amylase activity was 12 for midgut lumen of *Acherontia atropos* (L.) (Lepidoptera Sphingidae), 10.8 for midgut lumen of *Lasiocampa quercus* (L.) (Lepidoptera Lasiocampidae), 10.8 for midgut lumen of *Lichnoptera felina* Druce (Lepidoptera Noctuidae) and 11.3 for midgut lumen of *Manduca sexta* (L.) (Lepidoptera Sphingidae) (Dow, 1980). Also, in other non lepidopteran insect, optimum pH were 6.5 in *Lygus hesperus* Knight and *Lygus lineolaris* (Palisot de Beauvois), 6 in *Erinnyis ello* L. (Lepidoptera Sphingidae) (Terra et al., 1996). Midgut and salivary glands α -amylases from a *C. suppressalis* exhibited optimum pH in the range of 3 to 8 and the suitable pH was observed at 9 in both midgut and salivary glands α -amylases (Zibae et al., 2008).

The α -amylase activity was determined at different temperatures ranging from 20 to 80 °C. The *N. aenescens* α -amylases in midgut, salivary glands and haemolymph had an optimum temperature activity at 60, 50 and 50 °C respectively, which is consistent with activity in *Bombyx mori* L. (60 °C) (Lepidoptera Bombycidae) (Kanekatsya, 1978) and *Blattella germanica* (L.) (50 °C) (Blattodea Blattellidae) (Applebaum, 1985). Zibae et al. (2008) showed that α -amylase of *C. suppressalis* has an optimal activity in 40 °C for both midgut and salivary glands. In midgut at temperatures ranging from 15 to 60 °C, the enzyme activity increased steadily, achieving the maximum at 60 °C (figure 3). At 70 °C, α -amylase activity dropped sharply to about 25% of the maximal value. On the other hand, in non lepidopteran insect optimum temperature was lower than that of the α -amylases from *N. aenescens*. Optimum temperature was determined 25 °C in *T. molitor* (Barbosa Perreira et al., 1999) and 40 °C in *Dolycoris baccarum* L. (Heteroptera Pentatomidae) (Hori, 1969). The enzyme activity gradually declined at temperatures beyond 60 °C (figure 3). This condition was different from these found in other insect α -amylases, such as α -amylases from *Z. subfasciatus*, *T. castaneum* and *T. molitor*, which showed higher activities at 37 °C (Sivakumar et al., 2006).

It is well known that some animal amylases are activated or inhibited by certain ions and inhibitory effect of some mineral compounds on the digestive enzymes may offer a disadvantageous condition for digestion of food (Hori, 1970; Cohen, 1993; Payan, 2004). The effect of several metal ions and EDTA on the amylase activity in the midgut, salivary glands and haemolymph of

N. aenescens last instar larvae was studied at their optimum pH. The results showed Mn^{2+} , Hg^{+} and Hg^{2+} ions decreased α -amylases activity, whereas the α -amylase activities were enhanced by the addition of Na^{+} , K^{+} , Mg^{2+} , Ca^{2+} , Co^{2+} and Fe^{2+} . NaCl decreased the α -amylases activity of *C. suppressalis* (Zibae *et al.*, 2008). We have also observed that the activity of α -amylases was increased by addition of Ca^{2+} to the assay mixture. However in some insect, it has been reported that α -amylases are metalloproteins that require calcium for maximum activity (Baker 1983). Calcium also afford stability for the amylases from a variety of sources, including insects, to both pH and temperature extremes (Baker 1983). Midgut α -amylase of *T. molitor* was slightly activated by Ca^{2+} and Cl (Applebaum, 1961).

Kinetic behaviour of *N. aenescens* α -amylase toward starch was not significantly different in salivary glands and haemolymph extract. As calculated from Lineweaver-Burk plots, the K_m for midgut, salivary glands and haemolymph were about 0.07, 0.14 and 0.14 mg/ml and the V_{max} were 0.2, 0.08 and 32 μ mol/min, respectively. K_m values calculated for haemolymph α -amylase of *M. brassica* and silkworm were 0.33 and 0.57 mg/ml, respectively (Tanabe and Kusano, 1984; Matsumura, 1934). Also, Ramzi and Hosseinaveh (2010) showed that K_m values of α -amylase in midgut and salivary glands of pistachio green stink bug, *Brachynema germari* Kolenati (Hemiptera Pentatomidae) were 0.77 and 0.41 mM, respectively. Our results showed that the amylase K_m value in midgut of *N. aenescens* was lower than salivary glands and haemolymph α -amylase. This condition was different from those found in other insect α -amylases, such as α -amylases from *G. pyloalis* and *B. germari*, which showed midgut K_m values higher than salivary glands α -amylase (Yeزدani *et al.*, 2010; Ramzi and Hosseinaveh, 2010).

Proper quality and quantity of necessary nutritional compounds are the factors that ensure the developmental success of specialized herbivores, thus their digestive functions change in parallel with their specific food source. This can be manifested in a synthesis of a limited set of digestive enzymes, the relative activities of which reflect food preferences of an insect (Applebaum, 1985; Ishaaya, 1986; Broadway, 1989). Insect-resistant crops have been one of the major successes of applying plant genetic engineering technology to agriculture. Plants secondary metabolites can act as protective agents in plant against insects either by repellence or by direct toxicity. Many different types of secondary metabolites including alkaloids, terpenes, steroids, iridoid glycosides, aliphatic molecules, phenolics (Hsiao, 1985), and other have been demonstrated to confer resistance to different plant species against insects. Among them, α -amylase inhibitors seem to play an important role in host plant resistance to insects. For example, Pea and azuki transgenic plants expressing α -amylase inhibitors from common beans (a-AI) were completely resistant to the *B. pisorum* and *C. chinensis* weevils (Ishimoto and Kitamura, 1989; Shade *et al.*, 1994).

Plants have developed many secondary metabolites involved in plant defense, which are collectively known

as antiherbivory compounds and study of carbohydrates in herbivorous insects is so important not only for understanding of digestion biochemistry but also for developing of insect pest management strategies.

Acknowledgements

The authors express their gratitude to the research council of the University of Guilan and Ministry of Science, Researches, and Technology for financial support during the course of this project.

References

- ABIVARDI S., 2001.- *Iranian Entomology: An Introduction, Vol. 1.*- Springer, Heidelberg, Germany.
- ABRAHAM E. G., NAGARAGU J., DATTA R. K., 1992.- Biochemical study of amylase in the silkworm, *Bombyx mori* L.: comparative analysis in diapausing and nondiapausing strain.- *Insect Biochemistry and Molecular Biology*, 22: 867-873.
- APPLEBAUM S. W., 1985.- Biochemistry of digestion, pp. 279-307. In: *Comprehensive insect physiology, biochemistry and pharmacology, Vol.4: regulation, digestion, excretion* (KERRUT G. A., GILBERT L. I., Eds).- Pergamon Press, Oxford, UK.
- APPLEBAUM S. W., JANKOVIC M., BIRY Y., 1961.- Studies on the midgut amylase activity of *Tenebrio molitor*.- *Journal of Insect Physiology*, 7: 100-108.
- BAKER J. E., 1983.- Properties of amylase from midguts of larvae of *Sitophilus zeamais* and *Sitophilus granarius*.- *Insect Biochemistry*, 13: 421-428.
- BANNO Y., KAWAGUCHI Y., DIOR H., 1984.- Sexual dimorphism and developmental changes in haemolymph amylase of *Bombyx mori*.- *Journal of Sericulture Science Japan*, 53 (4): 335-340.
- BARBOSA PEREIRA P. J., LOZANOV V., PATTHY A., HUBER R., BODE W., PONGOR S., STROBL S., 1999.- Specific inhibition of insect α -amylases: yellow meal worm α -amylase in complex with the Amaranth α -amylase inhibitor at 2.0 Å resolution.- *Structure*, 7: 1079-1088.
- BERNFELD P., 1955.- Amylase, α and β .- *Methods in Enzymology*, 1: 149-151.
- BOYD JR D. W., 2002.- Digestive enzymes and stylet morphology of *Deraeocoris nigrifolius* (Uhler) (Hemiptera: Miridae) reflect adaptations for predatory habits.- *Annals of the Entomological Society of America*, 96: 667-671.
- BRADFORD M., 1976.- A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.- *Analytical Biochemistry*, 72: 248-254.
- BROADWAY R. M., 1989.- Characterization and ecological implications of midgut proteolytic activity in larval *Pieris rapae* and *Trichoplusia ni*.- *Journal of Chemical Ecology*, 15: 2101-2113.
- CAMPOS F. A. P., XAVIER-FILHO J., SILVA C. P., ARY M. B., 1989.- Resolution and partial characterization of proteinases and α -amylases from midguts of larvae of the bruchid beetle *Callosobruchus maculatus* (F.).- *Comparative Biochemistry and Physiology*, 92: 51-57.
- COHEN A. C., 1993.- Organization of digestion and preliminary characterization of salivary trypsin like enzymes in a predaceous Heteropteran, *Zelus renardii*.- *Journal of Insect Physiology*, 39: 823-829.
- DA LAGE J., DANCHIN E., CASANE D., 2007.- Where do animal α -amylases come from? An interkingdom trip.- *FEBS Letters*, 581 (21): 3927-3935.

- DAVIS B. J., 1964.- Disc electrophoresis II. Method and application to human serum proteins.- *Annals of the New York Academy of Sciences*, 12: 404-427.
- DOW J. A., 1984.- Extremely high pH in biological systems: a model for carbonate transport.- *American Journal of Physiology*, 246: R633-R635.
- FRANCO O. L., RIGDEN D. J., MELO JR F. R., BLOCH C., SILVA C. P., GROSSI-DE-SA M. F., 2000.- Activity of wheat α -amylase inhibitors towards bruchid α -amylases and structural explanation of observed specificities.- *European Journal of Biochemistry*, 267: 1466-1473.
- FRANCO O. L., RIGDEN D. J., MELO F. R., GROSSI-DE-SA M. F., 2002.- Plant α -amylase inhibitors and their interaction with insect α -amylases structure, function and potential for crop protection.- *European Journal of Biochemistry*, 269: 397-412.
- HENRISSAT B., DELEURY E., COUTINHO P. M., 2002.- Glycogen metabolism loss: a common marker of parasitic behaviour in bacteria?.- *Trends in Genetics*, 18: 437-440.
- HORI K., 1969.- Some properties of salivary amylases of *Adelphocoris suturalis* (Miridae), *Dolycoris baccarum* (Pentatomidae), and several other heteropteran species.- *Entomologia Experimentalis et Applicata*, 12: 454-466.
- HORI K., 1970.- Some properties of amylase in the salivary gland of *Lygus disponsi*.- *Journal of Insect Physiology*, 16: 373-386.
- HSIAO T. H., 1985.- Feeding behavior, pp. 471-512. In: *Comprehensive insect physiology, biochemistry and pharmacology*, Vol 9 (KERKUT G. A., GILBERT L. I., Eds).- Pergamon Press, Oxford, UK.
- ISHAAYA I., 1986.- Nutritional and allelochemic insect-plant interactions relating to digestion and food intake: some examples, pp 191-216. In: *Insect-plant interactions* (MILLER J. R., MILLER T. A., Eds).- Springer, New York, USA.
- ISHIMOTO M., KITAMURA K., 1989.- Growth inhibitory effects of an α -amylase inhibitor from kidney bean, *Phaseolus vulgaris* (L.) on three species of bruchids (Coleoptera: Bruchidae).- *Applied Entomology and Zoology*, 24: 281-286.
- IULEK J., FRANCO O. L., SILVA M., SLIVINSKI C. T., BLOCK JR C., RIGDEN D. J., GROSSI-DE-SA M. F., 2000.- Purification, biochemical characterization and partial primary structure of a new α -amylase inhibitor from *Secale cereale* (rye).- *International Journal of Biochemistry and Cell Biology*, 32: 1195-1204.
- KANEKATYSA U., 1978.- Studies on further properties for an alkaline amylase in the digestive juice of the silk worm, *Bombyx mori*.- *Journal of the Faculty of Textile Science and Technology*, 76: 1-21.
- MATSUMURA S., 1934.- Genetical and physiological studies on amylase activity in the digestive juice and haemolymph of the silkworm, *Bombyx mori* L.- *Bulletin Nagano-Ken Sericulture Experiment Station*, 28: 1-24.
- PAYAN F., 2004.- Structural basis for the inhibition of mammalian and insect α -amylases by plant protein inhibitors.- *Biochimica et Biophysica Acta*, 1696: 171-180.
- RAMZI S., HOSSEININAVEH V., 2010.- Biochemical characterization of digestive α -amylase, α -glucosidase and β -glucosidase in pistachio green stink bug, *Brachynema germari* Kolenati (Hemiptera: Pentatomidae).- *Journal of Asia-Pacific Entomology*, 13: 215-219.
- SAS, 1997.- *SAS/STAT user's guide, version 6.12*.- SAS Institute, Cary, NC, USA.
- SHADE R. E., SCHROEDER H. E., PUEYO J. J., TABE L. L., MURDOCK T. J. V., HIGGINS M. J., CHRISPEELS M. J., 1994.- Transgenic pea seeds expressing the α -amylase inhibitor of the common bean are resistant to bruchid beetles.- *Bio/Technology*, 12: 793-796.
- SILVA C. P., TERRA W. R., XAVIER-FILHO J., GROSSI-DE-SA M. F., LOPES A. R., PONTES E. G., 1999.- Digestion in larvae of *Callosobruchus maculatus* and *Zabrotes subfasciatus* (Coleoptera: Bruchidae) with emphasis on α -amylases and oligosaccharidases.- *Insect Biochemistry and Molecular Biology*, 29: 355-366.
- SIVAKUMAR S., MOHAN M., FRANCO O. L., THAYUMANAVAN B., 2006.- Inhibition of insect pest α -amylases by little and Winger millet inhibitors.- *Pesticide Biochemistry and Physiology*, 85: 155-160.
- SOMADDER K., SHRIVASTAVA M., 1980.- Digestive enzymes in the gut and salivary gland of the larvae of *Chilo auricilius* Ddgn.- *Cellular and Molecular Life Sciences*, 36: 218-223.
- STROBL S., MASKOS K., BETZ M., WIEGAND G., HUBER R., GOMIS-RUTH F. X., GLOCKSHUBER R., 1998.- Crystal structure of yellow mealworm α -amylase at 1.64 Å resolution.- *Journal of Molecular Biology*, 278: 617-628.
- SVENSSON B., FUKUDA K., NIELSEN P. K., BONSAGER B. C., 2004.- Proteinaceous α -amylase inhibitor.- *Biochimica et Biophysica Acta*, 1696: 145-156.
- TANABE S., KUSANO T., 1984.- Changes of the haemolymph amylase activity during development and its properties in the cabbage armyworm, *Mamestra brassicae* L.- *Kontyu*, 542 (4): 472-481.
- TANAKA Y., KUSANO T., 1980.- The haemolymph amylase activity during development of the silkworm, *Bombyx mori*, L.- *Journal of Sericulture Science Japan*, 49 (2): 95-99.
- TERRA W. R., FERREIRA C., JORDAO B. P., DILLON R. J., 1996.- Digestive enzymes, pp. 153-193. In: *Biology of the insect midgut* (LEHANCE M. J., BILLINGSLEY P. F., Eds).- Chapman and Hall, London, UK.
- WISESSING A., ENKAGUL A., WONGPIYASATID A., CHUWONGKOMON K., 2008.- Purification and characterization of *Callosobruchus maculatus* Kasetsart α -amylase.- *Journal of Natural Science*, 42: 240-244.
- YEZDANI E., JALALI SENDI J., ZIBAE A., GHADAMYARI M., 2010.- Enzymatic properties of α -amylase in the midgut and the salivary glands of Mulberry moth, *Glyphodes pyralis* Walker (Lepidoptera: Pyralidae).- *Comptes Rendus Biologies*, 333: 17-21.
- ZIBAE A., BANDANI A., KAFIL M., RAMZI S., 2008.- Characterization of α -amylase in the midgut and the salivary glands of rice striped stem borer, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae).- *Journal of Asia Pacific Entomology*, 11: 201-205.

Corresponding author: Mohammad GHADAMYARI (e-mail: ghadamyari@guilan.ac.ir, mghadamyari@gmail.com) Department of Plant Protection, Faculty of Agricultural Science, University of Guilan, Rasht, Iran.

Received December 17, 2009. Accepted May 31, 2010.