CHARACTERIZATION OF A-AMYLASE IN THE ALIMENTARY CANAL OF *NARANGA AENESCENS* MOORE (LEPIDOPTERA: NOCTUIDAE), THE RICE GREEN CATERPILLAR

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ABSTRACT: The α-amylases in whole gut of *Naranga aenescens* (Lepidoptera: Noctuidea) were isolated and characterized by using spectrophotometrically assays. The results showed the existence of the enzyme with a high activity in the gut so that the optimal pH and temperature were evaluated 10 and 30 °C, respectively. The activity of α-amylase was inhibited by NaCl and $CaCl_2$ and increased or remained no effect by other used compounds. Determination of the band on the Native-PAGE electrophoresis showed a single isozyme of α-amylase in the gut *N. aenescens*. Study on digestive enzymes of insects is one of the new and winning area to reach a safe and effective way to decrease the damages of the pest on agricultural products. Also, determination of different specific inhibitors are necessary to reach a real and reliant results.

KEY WORDS: α-amylases, Rice green caterpillar, gut.

The α-amylases (α-1,4-glucan-4-glucanohydrolases; EC3.2.1.1) are the hydrolytic enzymes that are found in microorganisms, plants and animals. These enzymes catalyse the hydrolysis of α-D-(1,4)-glucan linkage in starch and related carbohydrates (Strobl et al., 1998). They are the important enzymes involved in digestion and carbohydrate metabolism in insects (Daone et al., 1975; Buonocore et al., 1976; Horie & Watanabe, 1980). Amylases have been characterized from different origins in insect's body (Fisher & Stein, 1960; Takagi et al., 1971; Baker, 1983, 1987, 1991). α-Amylase converts starch to maltose, which is then hydrolyzed to glucose by an α-glucosidase. In insects, only α -amylases have been found that hydrolyse long α -1,4 glucan chains, such as native starch or glycogen (Terra et al., 1996).

The Green Semi Looper, *Naranga aenescens* Moore (Lepidoptera: Noctuiudae) is an important pest in rice fields and causes severe decrease in rice production (Alinia, 1993). The Green Semi Looper has three larval instar that all of them consume of rice leaves (Reissig et al., 1986). Pupation takes place on the top of the leaves and then fall on the rice field, where provides a suitable environment for pupal overwintering (Alinia, 1993). The *N. aenescens* has three generation that can be found from April to September (Alinia, 1993). First instar larvae feed on the margins of the young leaves and second as well as third instar larvae feed all of the leaves and causes severe damages (Alinia, 1993). Control strategies for *N. aenescens* rely on widely spraying by chemical insecticides especially organophosphorous ones (Alinia, 1993).

Because of living on a starch-rich diet, many insects depend on the effectiveness of their amylases for survival. In insects α-amylases are synthesized and secreted by midgut epithelial cells, along with other digestive enzymes (Baker, 1983; Terra & Ferreira, 2005). Amylases have been investigated in

Coleoptera, Hymenoptera, Diptera, Lepidoptera and Heteroptera by several researchers (Hori, 1970, 1971, 1972, 1975; Buonocore et al., 1976; Kanekatsu, 1978; Terra & Ferreira, 1983; Baker & Woo, 1985; Colepicolo-Neto et al., 1986; Santos & Terra, 1986; Baker, 1987, 1989, 1991; Terra et al., 1988; Ferreira & Terra, 1989; Schumaker et al., 1993; Ferreira et al., 1994). Amylases are one of the most important enzymes in digestive biochemistry of Lepidoptera because this order includes destructive herbivores. Also, high basic condition in alimentary tract of caterpillars cause the enzyme very specific and favorable in case of biochemistry of digestion. Hence, The aim of this study was extraction and characterization of a α-amylases from the alimentary canal of *N. aenescens* to reach a better understanding of the digestive physiology and to find a winning procedure to use amylase specific inhibitors for control of this pest on rice.

MATERIALS AND METHODS

Insect

Larvae in 3th instar were collected from the paddy fields around Amoul (Mazandaran province) and transferred to the laboratory of entomology at the University of Tehran.

Sample preparation and enzyme assays

Whole guts of third instar larvae were removed by dissection and homogenized in distilled water; the samples were put in the 1.5 ml centrifuge tubes and centrifuged at 15000 rpm for 15 min at 4 °C. Supernatant was separated from plate and kept at -20 °C for subsequent analysis.

α-amylase assay

α-Amylase activity was assayed using the dinitrosalicylic acid (DNS) procedure (Bernfeld, 1955), using 1 % soluble starch (Merck, Darmstadt, Germany) as the substrate.Ten microliter of the enzyme in addition to 50 microliter substrate (soluble starch) and 150 microliter universal buffer (0.02 M) containing succinate, glycine and 2-morpholinoethanesulfonic acid (pH 6.5) were incubated in 30 min in 35 °C (in Ben Mari). After addition of 100 microliter DNS and heating in the boiling water during 10 min, the reaction was stopped. Then, absorbance was read at 540 nm using a spectrophotometer. As a blank, instead of enzyme, distilled water was used. All assays were repeated at least three times.

Effect of PH and temperature on enzyme activity

The effects of temperature and pH on α-amylase activity were examined using enzymes from the larval whole gut. Optimal pH was determined using universal buffer with pH set at 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12. The effect of temperature on α-amylase activity was determined by incubating the reaction mixture at 10, 20, 25, 30, 35, 40, 45, 50 and 60 °C for 30 min followed by measurement of activity.

Effect of activators and inhibitors on enzyme activity

To test the effect of different ions on the enzyme activity, assays were performed in the presence of different concentrations of chloride salts of NaCl (5,10, 20 and 40 mmol/l), KCl (5,10, 20 and 40 mmol/l), $CaCl_2$ (5, 10, 20 and 40 mmol/l), $MgCl_2$(5, 10, 20 and 40 mmol/l), and ethylenediaminetetraacetic acid (EDTA; 0.5, 1, 2 and 4 mmol/l), sodium dodecylsulfate (SDS; 2, 4, 8 and 16 mmol/l) and urea (2, 4 and 8 mol/l). These compounds were added to the assay mixture, and activity was measured after 30 min. A control was also measured.

Electrophoresis

The amylase present in crude homogenates of Whole gut after SDS–polyacrylamide gel electro-phoresis (PAGE) was visualized using the procedure described by Laemmli (1970) and Campos et al. (1989), with minor modification. SDS–PAGE was performed in a 10 % (w/v) separating gel and a 5 % stacking gel, both with 0.05 % SDS. The electrode buffer was prepared based on the method of Laemmli (1970), but SDS was not used. The sample buffer contained 25 % stacking buffer (0.5 mol/ L Tris–HCl [pH 6.8]), 20 % glycerol, 2 % SDS, 0.005 % (w/v) bromophenol blue, but no mercaptoethanol, and it was not heated. Electro-phoresis was conducted at room temperature at 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 buffercontaining 2 mmol/l$CaCl_2$ and 10 mmol/l NaCl for 1.5 h. The gel Fig. 1.

Protein determination

According to the method of Bradford (1976), bovine serum albumin (Bio-Rad, München, Germany), used as a standard for measuring the concentration of protein.

Statistical analysis

A completely randomed design was used to set up the experiments. To comparison of means and show the variance, Tukey's studentisized test was considered at probability less than 5 % (p<0.05).

RESULTS

α-amylase activity

α-amylase activity was present in the gut of larvae. The activity of enzyme in the guts were 0.41 mOD/min.

Effect of PH and temperature on enzyme activity

The α-amylases extracted from guts of *N. aenescens* showed an optimal pH of 10 (Fig. 1). The enzyme activity from whole gut increased steadily from pH 6 to 10, and then decreased with increasing pH 11 (Fig. 1). Amylase was active over a broad range of temperatures. The optimal temperatures for α-amylase activity in gut was 30 °C (Fig. 2).

Effect of activators and inhibitors on enzyme activity

Tested Chemicals in this study had different results on the activity level of α-amylase in gut of larvae (Fig. 3). Activity level of α-amylase in whole gut of *N. aenescens* larvae decreased with increasing concentrations of NaCl and $MgCl_2$ (Fig. 3). However, enzyme activities in gut increased in the presence of remaining compounds.

Electrophoresis

Analysis of gut homogenates by electro-phoresis indicated a band in polyacrylamide gel (Fig. 4).

DISCUSSION

Amylases from different origins have been characterized (Fisher & Stein, 1960; Takagi et al., 1971; Baker, 1983, 1987, 1991). In the silkworm, *Bombyx mori*, Yokoyama (1959) reported the presence of two types of amylase activities in digestive fluid and haemolymph. In this study we measured α-amylase of *Naranga aenescens* larvae in whole gut and our results showed that this enzyme exists in a high level. There is a significant difference between activity of α-amylase in the midgut and the large amounts of the enzyme presents in the midgut (Zibaee et al., 2008). In fact complete breakdown of starch has observed in the midgut (Somadder & Shrivastava, 1980; Kfir et al., 2002).

Optimal pH for activity of α-amylase in this insect was 10. This result was similar to other Lepidopterans that their gut amylase acts in alkaline environment. In fact, high gut pH has been shown in such insects that feed on plant materials rich in tannins (Chapman,1998). In this order, optimal pH for midgut lumen of *Acherontia atropos* (Sphingidae) was 12, for midgut lumen of *Lasiocampa quercus* (Lasiocampidae) 10.8, for midgut lumen of *Manduca sext* (Sphingidae) 11.3 and for midgut lumen of *Lichnoptera felina* (Noctuidae) 10.8 (Dow, 1984). Zibaee et al. (2008) showed that optimal pH in *Chilo suppressalis* was 9 in both midgut and salivary glands. In other orders the results are different and show a correlation between enzyme pH and luminal pH in insect midgut (Terra et al., 1996a,b). Kazzazi et al. (2005) explained that the optimal pH for α-amylase activity of *Eurygaster integriceps* (Hemiptera: Scutelleridae) was 6.5 and Mehrabadi and Bandani (2009) agreed this result. Baker (1983) illustrated that amylase from midguts in larvae of *Sitophilus zeamais* and *Sitophilus granaries* are generally most active in the neutral to slightly acid pH condition.

Although the optimal temperature for α-amylase activity was 30 °C, the enzyme was active over a broad temperature range from 10 to 40 °C. The optimal α-amylase activity in *Chilo supprressalis*, was 35 °C and 40 °C in midgut and salivary gland, respectively (Zibaee et al., 2008). In coleopteran order, enzyme activity had shown 35 °C in *Cerambyx cerdo* L. (Cerambycidae) (Applebaum, 1985) and 25 °C in *Tenebrio molitor* L. (Tenebrionidae) (25 °C; Barbosa Pereira *et al.*,1999) while in Heteroptera, *Dolycoris baccarum* L. (Pentatomidae) it was 40 °C (Hori, 1969). In the current study, Mg^{2+} declined effect of α-amylase of *Naranga aenescens* larvae but K^{+} acted as activator. Hori (1969, 1970, 1972) studied different activators on the salivary amylase activity of *Lygus disponsi* and found NaCl was one of the activators. Similarly, Agblor et al. (1994) reported that α-amylases from both *L. hesperus* and *L. lineolaris* were activated by NaCl. However, amylases in some insect species (e.g., *Callosobruchus chinensis* (Linaeus) (Coleoptera: Bruchidae) and *B. mori* (Linnaeus) (Lepidoptera: Bombycidae)) are inhibited by Cl^{-} (Terra et al., 1996a,b). Dojnov et al. (2008) reported concentrations of Ca^{2+} from 0.005 to 5 mM activated α-amylases crude extract of *Morimus funereus* larvae. In contrast, concentrations above 10 mM were inhibitory. This phenomenon has not been previously reported for other insect α-amylases, but it bears resemblance to that reported for pea leaf α-amylase (Ziegler, 1988). In this experiment, we got the result obtained by Dojnov et al. (2008) but results showed a slight increase in 20 mM. Chelating agent (EDTA), urea and SDS in present study, acted as activator and it was against the results have come from other authors' measurements that showed these three chemicals as inhibitor (Zeng & Cohen, 2000; Zibaee et al., 2008; Mehrabadi & Bandani, 2009). Also, EDTA and SDS showed inhibitory effect in extracellular α-

amylase from *Thermus* sp. But urea had no influence effect on amylase activity (Shaw et al., 1995).

Existence of a band in whole gut homogenates after Native-polyacrylamide gel electro-phoresis (PAGE) indicated that there is a single isozyme. Zibaee et al. (2008) clarified three bands in the midgut and two in the salivary glands. Doani (1967) reported different α-amylases isozymes in fruit fly *Drosophila melanogaster* Meigen (Diptera: Drosophilidae). In other cases, different researchers showed several isoforms of this enzyme in coleopteran order (Terra et al., 1977; Baker, 1991; Chen et al., 1992; Oliveira-Neto et al., 2003).

Study on digestive enzymes of insects especially pest ones is one of the new and winning area to reach a safe and effective way to decrease the damages of the pest to the agricultural products so that determination of different specific inhibitors are necessary to reach the real results by reliance.

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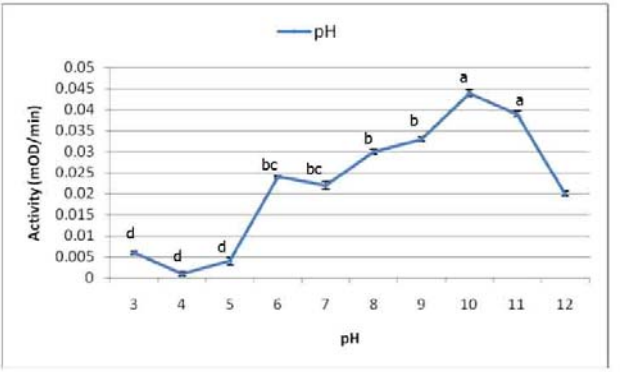


Figure 1. Effect of pH on the α-amylase activity of *Naranga aenescens* larvae.

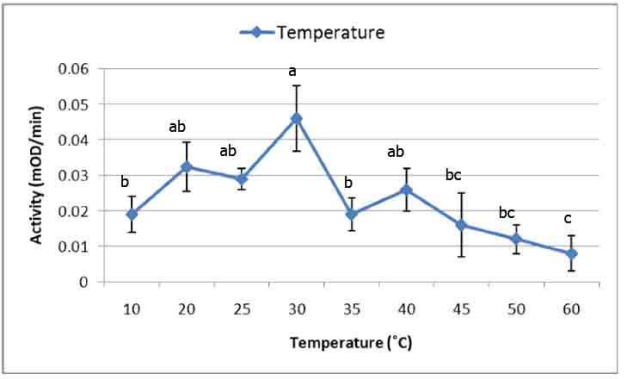
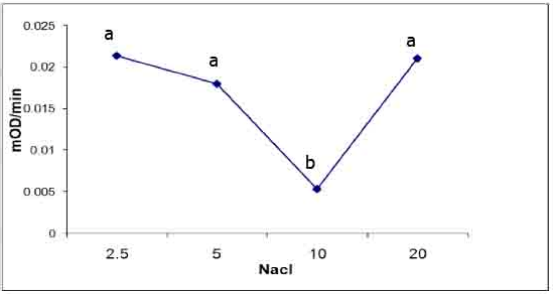


Figure 2. Effect of Temperature (˚C) on the α-amylase activity of *Naranga aenescens* larvae.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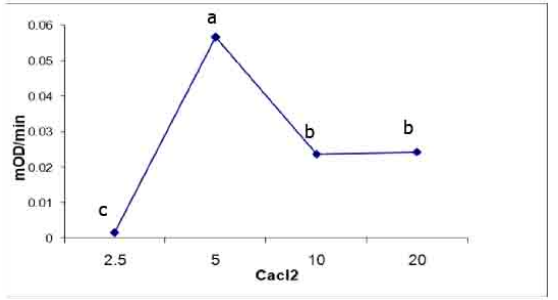
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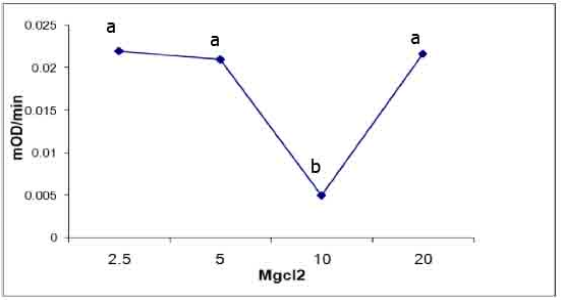
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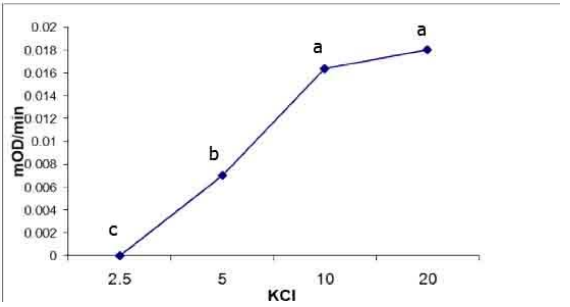
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20
KCl

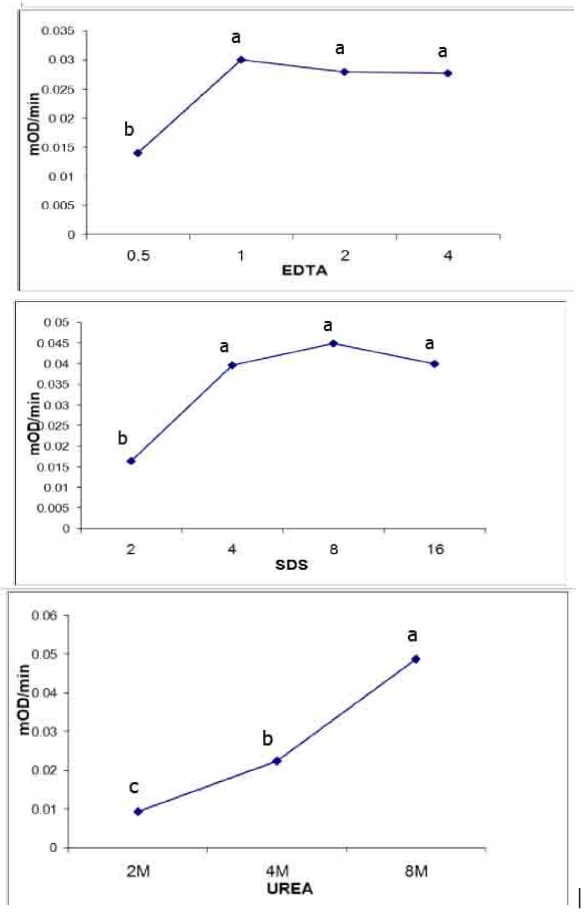

Figure 3. Effect of different compounds on the activity of α-amylase in *Naranga aenescens* larvae. The activity of enzyme in absence of any component was 0.038 ,OD/min.

Figure 4. Native-PAGE gel electrophoresis of α-amylase in *Naranga aenescens* larvae.