Article

Enzymatic activity of α-amylase in alimentary tract *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae): Characterization and Compartmentalization

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Abstract

The Egyptian cotton leafworm, *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) damages a wide variety of crops in Middle East. Their hosts include cotton, alfalfa, eggplant, tomato, lettuce, bean and some ornamental crops. The intensive use of broad-spectrum insecticides against *S. littoralis* has led to the development of resistance to many registered pesticides use for its control. The purpose of the present study is biochemical characterization of digestive enzymes of this pest to gain a better understanding of the digestive physiology. The physiology and biochemistry of the insect digestive enzyme had an important role in the study of novel insecticidal strategies. The Egyptian cotton leafworm alimentary canal consists of a short foregut, a long midgut and a short hindgut. Application of pH indicators showed that alimentary canal was alkaline. Our results showed that activities of gut α- amylase were different in three parts of the insect gut. Also shown the greatest activity of α-amylase observed in the midgut followed by hindgut and foregut, respectively. However, there were not significant differences in activity of the enzyme in the midgut and hindgut. The optimal pH α-amylase in foregut, midgut and hindgut were 10.0. Zymogram analysis of different part of gut showed four bands in midgut, hind gut and two bands in foregut. Therefore, in midgut of *S. littoralis*, four isoenzymes were present. These results explain why more amylase activity was seen in these regions in the spectrophotometric assay.

Keywords Egyptian cotton leafworm; *Spodoptera littoralis*; α-amylase; Zymogram.

1 Introduction

The Egyptian cotton leafworm, *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) damages a wide variety of crops in countries around the Middle East and Southeast Asia (Sneh et al., 1981). Main damage is

recorded on cotton, alfalfa, eggplant, tomato, lettuce, bean and some ornamental crops. In addition to the direct damage caused by reducing photosynthetic area, the occurrence of larvae, feeding damage and excrement reduces marketability of vegetables and ornamentals (Pluschkell et al., 1998). The intensive use of broad-spectrum insecticides against *S. littoralis* has led to the development of resistance to many registered pesticides use for its control (Aydin and Gurkan, 2006; Smagghe et al., 1999). The environmental hazards of insecticides require introduce of new effective and safer ways and negligible effects on ecosystem (Korrat et al., 2012).

The physiology and biochemistry of the insect midgut has an important role in the study of novel insecticidal strategies. Digestive enzymes could be aimed by plant inhibitors to interfere in food digestion and its absorption in pests (Jongsma and Bolter, 1997; Gatehouse and Gatehouse, 1999). Therefore, understanding the biochemical characteristics of different enzymes in insect's gut is essential (Wilhite et al., 2000). Many caterpillars live on a polysaccharide-rich diet and require digestive α-amylase to breakdown starch in their food. These amylases play an important role in starch digestion and insect survival (Valencia-Jiménez et al., 2000).

The α-amylase (α-1,4-glucan-4-glucanohydrolases; EC3.2.1.1) is a hydrolytic enzyme, found in microorganisms, plants and animals. This enzyme catalysis and hydrolysis the α-D-(1,4)-glucan linkage in starch and related carbohydrates (Strobl et al., 1998). Starch digestion by insect amylases has been demonstrated and described in several insect species, including, *Rhynchophorus ferrugineus* Olivier (Coleoptera: Curculionidae) (Darvishzadeh et al., 2012), *Ceratitis capitata* (Wiedemann)(Diptera: Tephritidae) (Darvishzadeh et al., 2013) *Sitophilus oryzae* Hustache (Coleoptera: Curculionidae) (Baker and Woo, 1985), and *Zabrotes subfasciatus* (Bohemann) (Coleoptera: Bruchidae) (Lemos et al., 1990), *Macrosiphum rosae* (Hemiptera: Aphididae) (Darvishzadeh and Bandani, 2012)and *Tecia solanivora* Povolny larvae (Lepidoptera:Gelechiidae) (Valencia-Jimé-nez et al., 2000).

Inhibitors of insect digestive enzymes have important role in the control of insect pests. Dias et al. (2010) demonstrated that rye α-amylase inhibitor expressed in transgenic tobacco seeds (*Nicotiana tabacum*) caused 74% mortality in *Anthonomus grandis* first larval instar when transgenic seed flour mixture used in artificial diet. Understanding the biochemistry and physiology of nutrition and feeding adaptation is important in inhibitors application in insect pest control programs. Hence, the aim of present study is biochemically characterization of digestive enzymes in *S. littoralis*, to gain a better understanding of the digestive physiology of this insect.

2 Materials and Methods

2.1 Insects and enzyme extraction

Last instar larval of *S. littoralis* were collected from damaged Cotton farm located at Golestan province, North of Iran. Enzyme samples from different parts of alimentary tract (Foregut, Midgut and Hindgut) were prepared based in Darvishzadeh et al (2013). Briefly, larvae were placed on ice (about 5 min) for immobilization and dissected under binocular. After dissection, different parts of alimentary tract were removed and homogenized using a hand-held glass grinder. The homogenates were centrifuged at 15,000 ×g for 15 min at 4 ℃ and the supernatants were kept at -20 ℃ as the enzyme source.

2.2 Gut pH determination

The gut pH of *S. littoralis* larvae was determined based in Bignell and Anderson (1980) method. A set of colored reagents for different alkaline pH (with an interval of 0.5), including 0.1% Cresol-red for pH 7.0-8.8, 0.004% Thymol-blue for pH 8.0-9.6, 0.1% Phenolphthalein for pH 8.2–9.8, 0.1 % Alizarin yellow for pH 10–12, and 0.25% Indigo-carmine for pH 11.5-14 were prepared. After removing the guts, 5 μl of the solutions were added to separately.

2.3 α-Amylase activity

α-Amylase activity was assayed by the dinitrosalicilic acid (DNS) procedure (Bernfeld, 1955), using 1% soluble starch (Merk, Product Number 1257, Darmstadt, Germany) solution as substrate as described by Bandani et al. (2009). One unit of α-amylase activity was defined as the amount of enzyme required to produce 1 mg maltose in 30 min at 35ºC. A blank without substrate but with α-amylase extract and a control containing no α-amylase extract with substrate were run simultaneously with reaction mixture. All assays were performed in triplicates.

2.4 Effect of pH and temperature on α-amylase activity

Enzymatic activity of α-amylase was assayed according to Bernfeld (1955) using dinitrosalicylic acid (DNS) as the reagent and 1% soluble starch as the substrate. Twenty microliters of the enzyme extract was incubated for 30 min at 35 °C with 100 μl universal buffer at pH 5.0 to pH 12 and 10 μl soluble starch. The reaction was stopped by the addition of 100 μl DNS and heating in boiling water for 10 min. The absorbance of the mixture at 540 nm was then measured. All assays were performed in triplicate. Optimum temperature for the enzyme activity was determined by incubation of the reaction mixture at a temperature set of 20, 30, 40, 50 and 60° C for 30 min and the residual activities measured. All assays were performed in three replications.

2.5 Electrophoresis of α-amylase enzyme

The amylase present in crude homogenates of the gut after Native –polyacrylamide gel electrophoresis (PAGE) was visualized using the procedure described by Laemmli (1970) and Campos et al. (1989), with minor modification. Native–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. Electrophoresis was conducted at room temperature at 100 V until the blue dye reached the bottom of the slab gel. To prepare gels for α-amylase assay, the gel was cleansed by water and washed by shaking gently with 1 % (v/v) Triton X-100 in phosphate buffer containing 2 mmol/l $CaCl_2$ and 10 mmol/l NaCl for 1.5 h.

2.6 Protein determination

Protein concentration was measured according to the method of Bradford (1976), using bovine serum albomin (Bio-rad, Munchem, Germany) as standard.

2.7 Statistical analysis

One way ANOVA data analysis performed followed by Duncan multiple range test when significant differences were found at P = 0.05.

3 Results

3.1 Alimentary tract morphology

The gut alimentary canal of *S. littoralis*, as in the other caterpillar, is morphologically divided into three distinct divisions (Fig. 1). The foregut is very short and connected to four gastric caeca. The midgut is long and comprises most of the gut. It extends from the proventriculus to the point where the malpigian tubles attached the gut. The hindgut seems to be compromised of a narrow intestine connected to rectum.

3.2 Gut luminal pH

Application of pH indicators showed that foregut were alkaline (pH 8.2 – 9.4), the midgut was more alkaline (pH 8.7 – 10.8) and the hindgut is also alkaline (pH 8.0–9.5) (Fig. 2).

3.3 α-Amylase activity

Results showed that activities of gut α-amylase were distinct in different parts of the insect gut (Table 1). Specific activity of α- amylases in foregut, midgut and hindgut were 0.54 ± 0.08, 1.14 ± 0.11and 1.07 ± 0.09

(μmol/min/mg protein), respectively. As shown the greatest activity of α- amylases observed in the midgut followed by hindgut and foregut, respectively. However, there were not significant differences in activity of the enzyme in the midgut and hindgut. The least activity of the enzyme was seen in the foregut (Table 1).

Table 1 α-Amylase activities of different parts of the gut of *Spodoptera littoralis*.

Enzyme	Organ	Total activity (μmol/min)	Total protein (mg)	Specific activity (μmol/min/mg protein)
	Foregut	0.68 ± 0.11	1.25	0.54 ± 0.08[a*]
α-amylase	Midgut	2.42 ± 0.25	2.11	1.14 ± 0.11[b]
	Hindgut	1.97 ± 0.18	1.83	1.07 ± 0.09[b]

*Different letters (a and b) indicate significant differences according to Tukey test at α= 0.05. Values with the same letter are not significantly different.
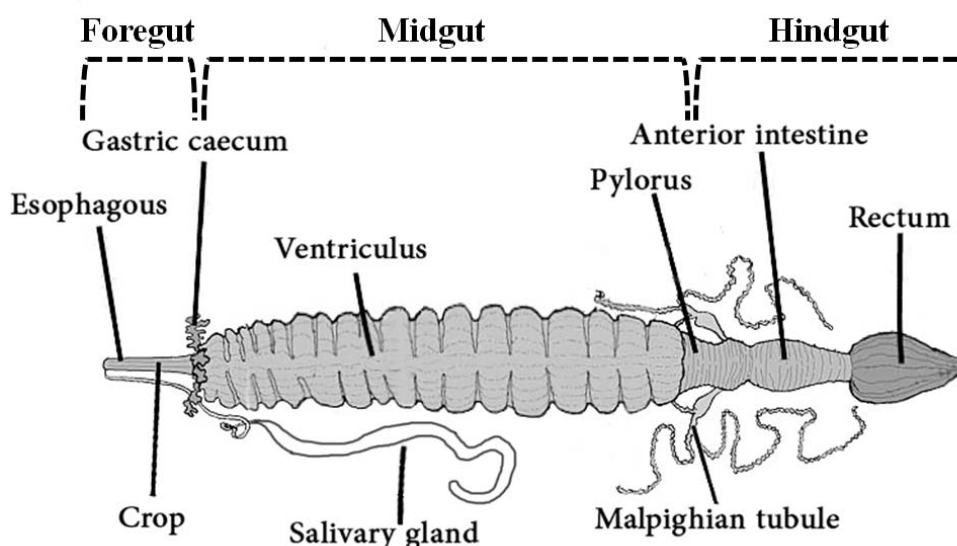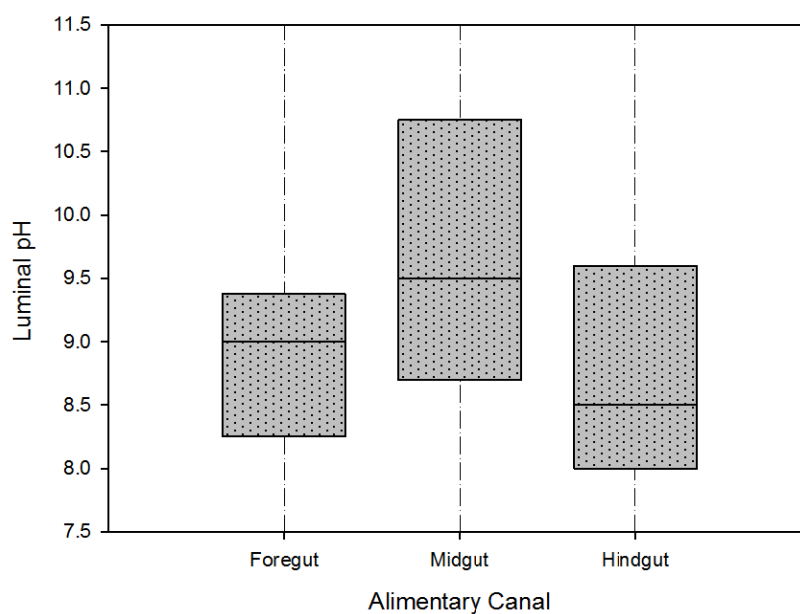

Fig. 1 Gut luminal pH of Egyptian cotton leafworm alimentary canal (Foregut, Midgut and Hindgut).

3.4 Effect of pH and temperature on α-amylase activity

The optimal pH α-amylase in foregut, midgut and hindgut were 10.0. (Fig. 3). The enzyme activity increased stably from pH 4 to 10 ,and then decreased until pH 12 (Fig. 3). α-Amylase was active over a broad range of temperatures. The optimal temperature for α-amylase activity in different part of gut was 45 °C (Fig. 4).

3.5 Zymogram analysis of α-amylase activity

Zymogram analysis of different part of gut electrophoresis showed four bands in midgut and hind gut (a, b, c and d), two bands in foregut (a and b) (Fig. 5). Therefore, in midgut of *S. littoralis*, four isoenzymes were present. These results explain why more amylase activity was seen in these regions in the spectrophotometric assay.

Fig. 2 Gut luminal pH of Egyptian cotton leafworm alimentary canal (Foregut, Midgut and Hindgut).
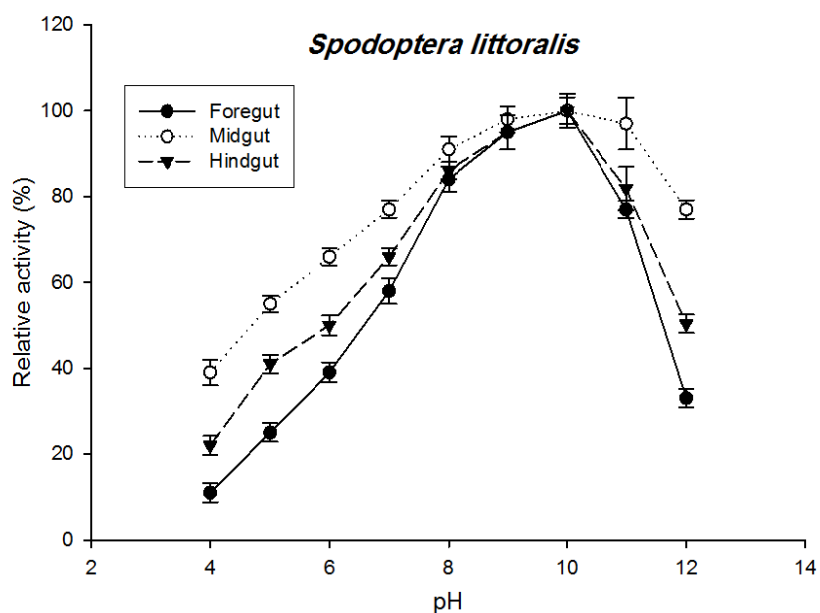
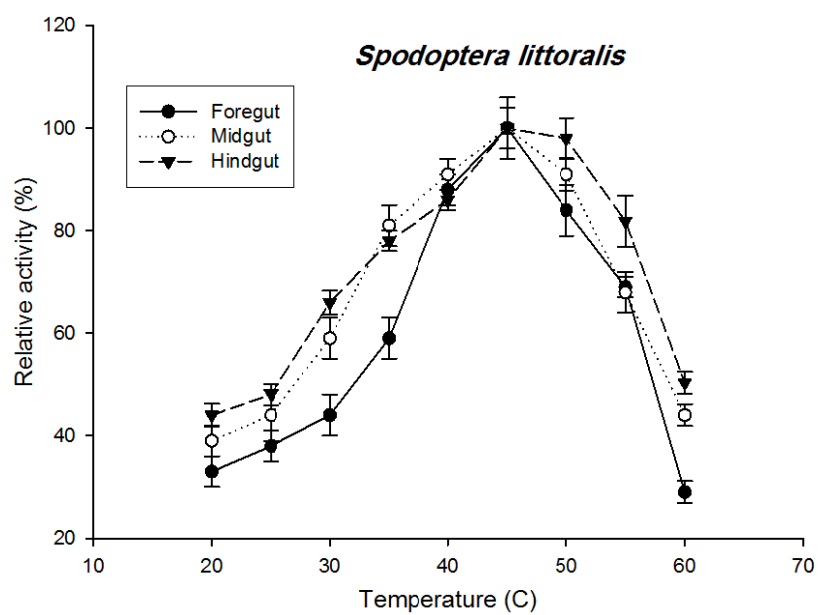**Fig. 3** The optimal pH α-amylase in foregut, midgut and hindgut of Egyptian cotton leafworm.

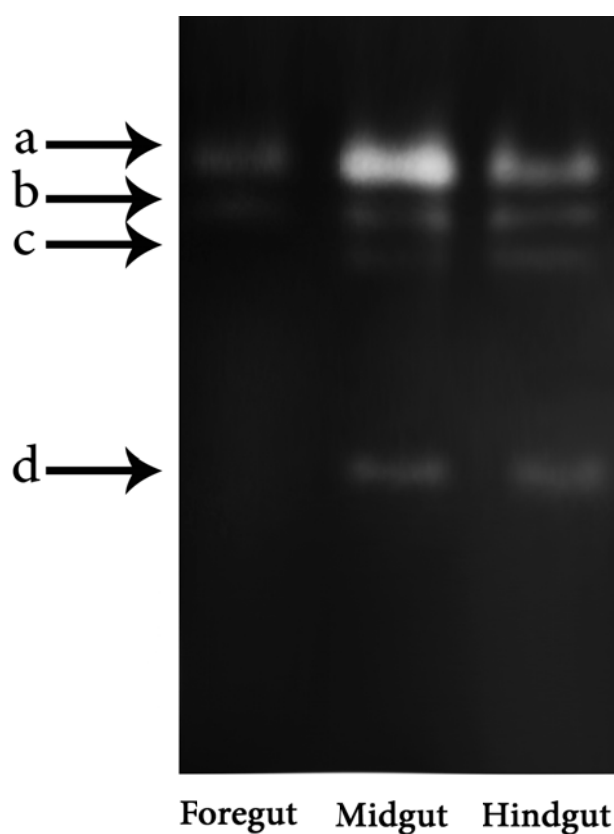**Fig. 4** The optimal temperature of α-amylase in foregut, midgut and hindgut Egyptian cotton leaf worm.

Fig. 5 Native PAGE of α-amylase in Egyptian cotton leafworm larvae. Different arrows show four amylase isoforms against the starch substrate.

4 Discussion

Our data present evidence that α-amylase is present in different part of digestive system of the last larval instar of *S. littoralis*. In this study we measured α-amylase of *S. littoralis* larvae in different part of gut and our results showed that this enzyme exists in a high level. There is a significant difference between activity of α-amylase in foregut and midgut. Optimal pH for activity of α-amylase in this insect was 10. Generally, optimal pH is corresponding to the pH prevailing in the midgut from which the enzyme has been extracted so these discrepancies seen in midgut pH are related to the different feeding habits and feeding sources. These data support the previous finding that there is a correlation between enzymes pH optima and luminal pH in insect midguts (Terra and Ferreira, 1994). The optimal temperature for *S. littoralis* α-amylase activity was 45°C, the enzyme was active over a broad temperature range from 20 to 60°C.

In insects, α-amylase has been reported from various orders and families, with the majority isolated from the intestinal tract (Terra and Ferreira, 1994). Our zymogram analysis revealed the presence four bands of activity for α-amylase activity. A mixture of different α-amylase isoenzymes has been reported for other insects such as *Choreutis nemorana* and *Hypera positica* (Bigham et al., 2013; Vatanparast and Hosseininave, 2010). Presence of different α-amylase isoenzymes could be related to importance of this enzyme in the insect food digestion.

The continuous application of chemical pesticides for control of agricultural pests threats the health of human and environment as well as non-target organisms including natural enemies and pollinators. Using pest-resistant transgenic plants help us to reduce the application of chemical pesticides. The first step in designing a controlling strategy based on inhibition of digestion is to understand and characterize digestive enzymes of the target insect biochemically (Strobl et al., 1998). The biochemical characterization of insect digestive enzymes will facilitate the understanding of the mechanisms responsible for the inhibitory potential of the plant inhibitors and will help to design new and more specific strategies for insect control.

Understanding biochemical features of the digestive physiology of this insect and especially biochemical characterization of the α-amylase of *S. littoralis* is important when new management strategies for this economically important pest are devised.

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