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Distribution and factors affecting β-glucosidase activity in the the cotton leafworm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae)

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B-glucosidases from the cotton leafworm, *Spodoptera littoralis* (Boisd.) were studied to detect their titer during the life cycle, distribution, and response of β-glucosidases to some physiological processes such as moulting and starvation. In addition, some inorganic inhibitors were evaluated to explore any chemical inhibitors may be involved later in integrated pest management programs. The enzyme reached its maximum activity in larval and adult stages and dramatically declined during pupation. Both haemolumph and larval gut comprised about 87.22 % of the entire larval β-glucosidases. The detected elevation of β-glucosidases during and after moulting may point out its involvement in this process. B-glucosidases are not highly affected by different food host plants, but affected severely by starvation. Incubation of β-glucosidases with different molar concentrations of inorganic salts showed inhibitory effects upon 4th larval instar. Salts could be arranged ascendingly according to their inhibitory effect as follows: EDTA>NaCl>NaF>$CaCl_2$>$CuSO_4$. The paper represents a preliminary comprehensive study of the insect β-glucosidases in the cotton leafworm; *S. littoralis*.

Keywords: β- glucosidases, *Spodoptera littoralis*, titer, moulting, starvation, host plant, inhibitors

INTRODUCTION

The cotton leafworm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) is considered as one of the most destructive polyphagous pests in the tropical and subtropical regions all over the world causing great damage of variable economic important crops as cotton, orchard and ornamentals trees (Amin and Salam, 2003; Hatem et al. 2009).

The current strategy of controlling *S. littoralis* is still relying to a great extent on applying pesticides and eventually the evolution pest resistance as well as environmental contamination (Abo-El Ghar et al. 2005; Costa et al. 2008; Relyea, 2009; Abd El-Mageed and Shalaby, 2011). The need of developing more environmentally acceptable alternatives became necessary for the management of such a serious pest.

One the other hand, the insect digestive enzyme system is the most important interphase between an insect and its environment (Lwalaba et al. 2010). A better understanding of this system will helpfully enable us to develop better methods of controlling such pests.

Glycosidases are a type of digestive enzymes that are widespread almost in all living organisms including bacteria and fungi as well as both invertebrates and vertebrates with different functions among these organisms (Mostafa et al. 2014). In insects, glycosidases have a crucial role in carbohydrate digestion. β-glucosidases in

particular is responsible for the terminal digestion of hemicellulose and cellulose (Esen, 1993). β-glucosidase was detected in many insect orders and in particular those phytophagous ones related to order Lepidoptera (Santos and terra, 1985; Pratviel-Sosa et al. 1987; Franzl et al. 1989; Ferreira et al. 1997; Zibaee et al. 2009; Ghadamyaria et al. 2010; Aghaali et al. 2012; Riseh et al. 2012).

Insects are able to convert carbohydrates into di- and oligo-β-saccharides by some carbohydrases in their digestive system. β-glucosidases hydrolyse β1–4 linkages between these di-and oligosaccharides converting them into glucose which is later absorbed and metabolized by the insect (Terra and Ferreira, 1994; Terra et al. 1996). In some insects, β-glucosidases are able also to hydrolyse the toxic β-glucosides found in plants as a defensive weapon against insect attack (Ferreira et al. 1998).

Most of the previous studies were focusing mainly upon the characterization and biochemical kinetics of β-glucosidases rather than investigating its *in vivo* activity (Zibaee et al. 2009; Ghadamyari et al. 2010; Ramzi and Hosseininaveh, 2010; Sharifi et al. 2011; Aghaali et al. 2012; Dikshit and Tallapragada, 2015). However, very little available knowledge of the digestive physiology of this enzyme. Accordingly, the present work aimed to study (1) activity titre of β-glucosidases in the cotton leafworm *S. Littoralis* throughout its life cycle, (2) distribution of enzyme in different larval tissues, (3) response of β-glucosidases to some physiological processes as moulting and starvation and (4) *in vitro* effects of some inorganic salts on β-glucosidases catalysis.

MATERIALS AND METHODS

Insects

The cotton leafworm, *S. littoralis* was obtained as egg masses from an established laboratory colony maintained in plant protection research institute, Sharkia province branch, Egypt. The colony was reared at 25± 2 ^{0}C, 70% R.H., and photophase period 12:12 (L:D) hr. Every 500 individuals of newly hatched larvae, were transferred to 1-litre capacity glass jar and were fed by fresh castor bean leaves (otherwise mentioned). The number of larvae/jar was gradually decreased due to larval growth, reaching 20 larvae/jar during the sixth instar. Moths were fed by a 10% sugar solution via pieces of cotton moistened with this solution (El-Defrawi et al. 1964).

Insects (two-days old) from each stage from eggs to adults were collected to determine enzyme activity changes throughout the cotton leafworm life cycle. Fourth larval instar represented the larval stage as a median larval instar.

Fourth instar larvae were starved for 12, 24 and 36 hours for starvation experiments. Another group of the same instar was used to determine β-glucosidase activity changes during moulting period. Specimens were collected at different time intervals (before and during moulting, and just after the entrance to the fifth instar). The above experiments were replicated 3-5 times (20 insect or 3 egg patches/ replicate).

Host plants

The effect of different nutritional sources on β-glucosidase activity was tested. Castor, bean, cabbage, and grapevine leaves, besides an artificial diet, were fed by larvae. The chosen host plants are among many plant species known to be attacked by the cotton leaf worm in Egypt. They were collected as fresh leaves from Banha province cultivars and introduced to caterpillars for feeding from egg hatching till fourth instar larvae. The artificial diet prepared as described by Shorey and Hale (1965).

Apparatus:

Insect bodies and tissues were homogenized in a chilled glass Teflon tissue homogenizer (ST-2 Mechanic-Preczina, Poland). After centrifugation, supernatants were kept in a deep freezer at -20 ̊C until use. Centrifugation was done in a refrigerated centrifuge at 4 ̊C. A double beam ultraviolet/visible spectrophotometer (Spectronic 1201, Milton Roy Co., USA) was used to measure the absorbance of β-glucosidase reaction products.

Insect preparation for analysis:

B-glucosidases activity was determined in different developmental stages of the cotton leafworm total body homogenate. Besides, the distribution of activity was studied in different body tissues.

Sixth instar larvae were dissected as reported by Amin (1998). The dissection dish was filled with cold (5 ̊C) Nacl 0.9 % and the larvae were fitted by small pins. Then with the aid of a sharp small razor, the head of each larva was cut off and the ventral side of the body was longitudinally

opened. The alimentary tract was removed and integument with the adhering tissues and rinsed twice with distilled water. On the other hand, haemolymph was bled by cutting one the larval abdominal prolegs, and collected in a chilled centrifuge tube, then centrifuged at 5000 x g for 10 minutes.

Each 100 mg of insect total body or tissue was homogenized in 1 ml of 0.1 M ice-cold phosphate buffer (pH 6). The homogenate was centrifuged at 14000 g for 10 min at 4 ̊C, and the supernatants were collected, quantified, and used as enzyme source.

β-glucosidase assay

β-glucosidase activity was measured by assaying glucose liberated by enzymatic hydrolysis of salicin as described by Lindroth (1988).

One ml of the reaction mixture consisted of 200 ul enzyme solution, 0.1 M phosphate buffer (pH 6), and 50 u mole salicin. Mixtures were incubated at 35 ̊C for 30 min, then boil for 2 min to stop the reaction. Glucose that liberated by salicin hydrolysis was measured enzymatically by a glucose kit (Sigma kit, Sigma Co.). Optical densities were measured against a blank containing boiling enzyme. Enzyme activity was expressed as µg glucose liberated / min/ mg protein.

Protein determination

Total proteins were determined by the method of Bradford (1976). Sample solution (50 µl) or for preparation of standard curve 50 µl of serial concentrations containing 10 to 100 µg bovine serum albumin were pipetted into test tubes. The volume in the test tube was adjusted to 1 ml with phosphate buffer (0.1 M, pH 6.6). Five millimeters of Comassie brilliant blue G250 reagent was added to the test tube and the contents were mixed by either inversion or vortexing. The absorbance at 595 nm was measured after 2 min and before 1 hr against blank prepared from 1 ml of phosphate buffer and 5 ml protein reagent.

Incubation with inorganic salts to detect inhibitors of β-glucosidases

Some anions and cations were tested against β-glucosidases from 4th larval instar of *S. littoralis* to detect their effect on enzyme catalysis. Different molar concentrations of ethylene diamine tetraacetic acid (EDTA), Sodium fluoride (NaF), Copper sulphate ($CuSO_4$), Sodium chloride (NaCl) and Calcium chloride ($CaCl_2$), were prepared and mixed with the enzyme solution. Two hundred microliters of the enzyme were incubated with 100 µl of each compound concentration for 10 min at 25 ̊C before starting salicin hydrolysis by β-glucosidase. The residual activity was determined as described above and compared with the control reaction, containing everything except salt solution. Preliminary studies were performed to determine the used range of each compound concentrations used.

Data analysis

Using costat statistical software (Cohort software, Brekeley), means and standard deviations were obtained. Experimental results were pooled from at least triplicate determinations. Data were analyzed by completely randomized ANOVA. The means were separated using Duncan^s multiple range tests ($P< 0.01$).

RESULTS

β-glucosidase titer during *S. littoralis* life cycle

B-glucosidase activity was measured in egg, larval, perpupal, pupal and adult stages of the cotton leaf worm, *S. littoralis* as demonstrated in Fig. (1). The enzyme reached its maximum activity in larval and adult stages. The activity was rather high in the egg stage but significantly lower than those in larval and adult ones. A dramatic decrease of β-glucosidase activity was detected during prepupal and pupal stage by 69.44 and 75.64% respectively, as compared to the enzymatic level in the larval stage.

β-glucosidase activity distribution in different larval tissues of *S. littoralis*

When β-glucosidase in the 4th larval instar of *S. littoralis* was measured in haemolymph, gut, and integument, a significant difference in the enzyme activity was detected between all the tested tissues. The major activity of β-glucosidase was detected surprisingly, in haemolymph and not in the gut. The activity in the former tissue increased by 44% than in the latter. Both haemolymph and larval gut comprised about 87.22% of the entire larval β-glucosidase. The lowest enzymatic activity was detected in the larval integument, which represented only 12.77% of the larval enzyme activity as observed in Fig. (2).

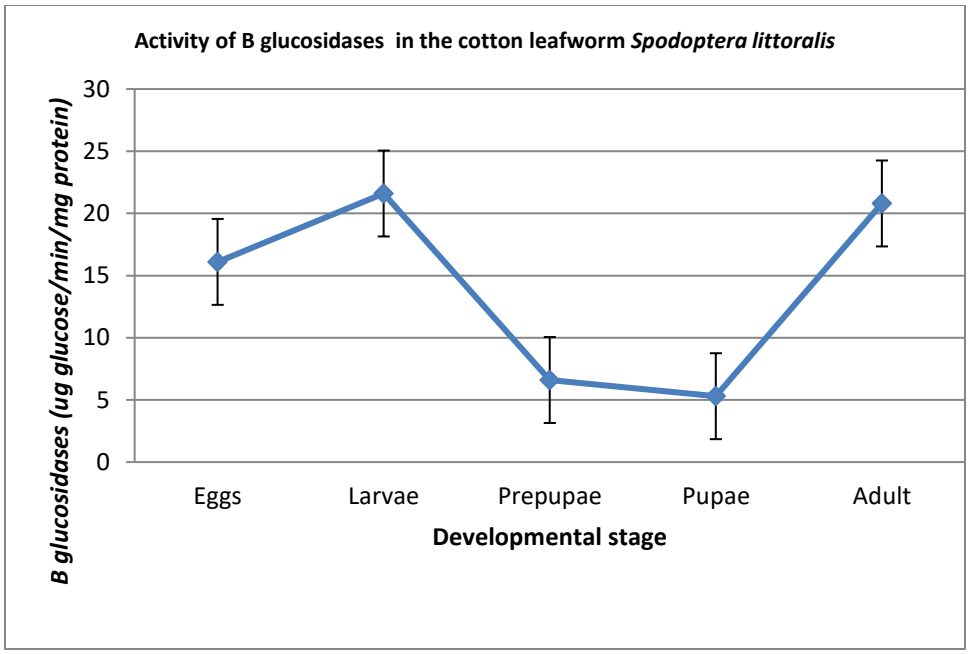

Figure1: β-glucosidase activity of different developmental stages of *S. littoralis*

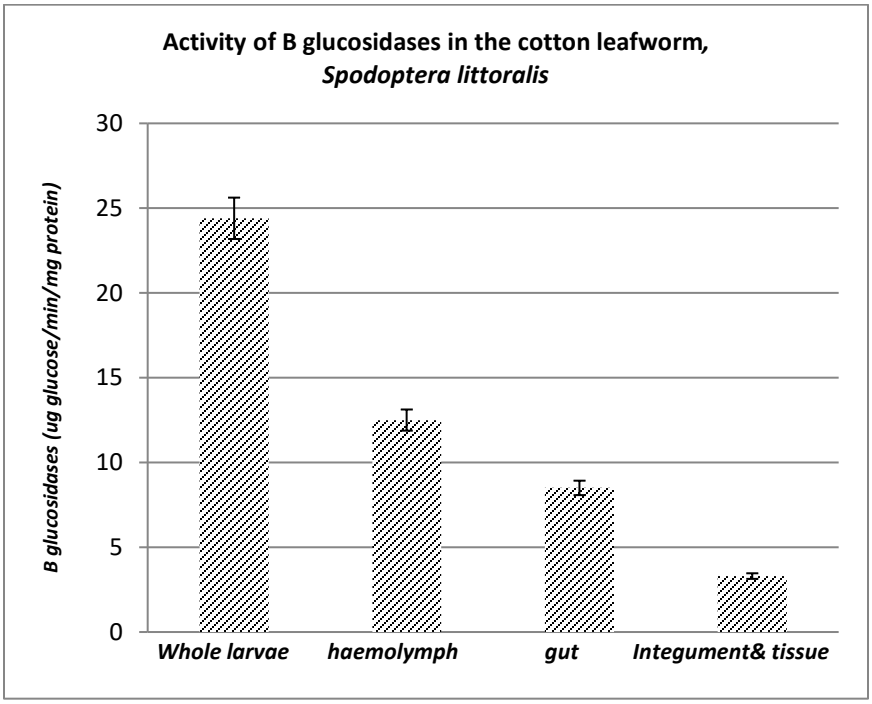

Figure2: β-glucosidase activity distribution in different larval tissues of fourth larval instar of *S. littoralis* fourth instar larvae

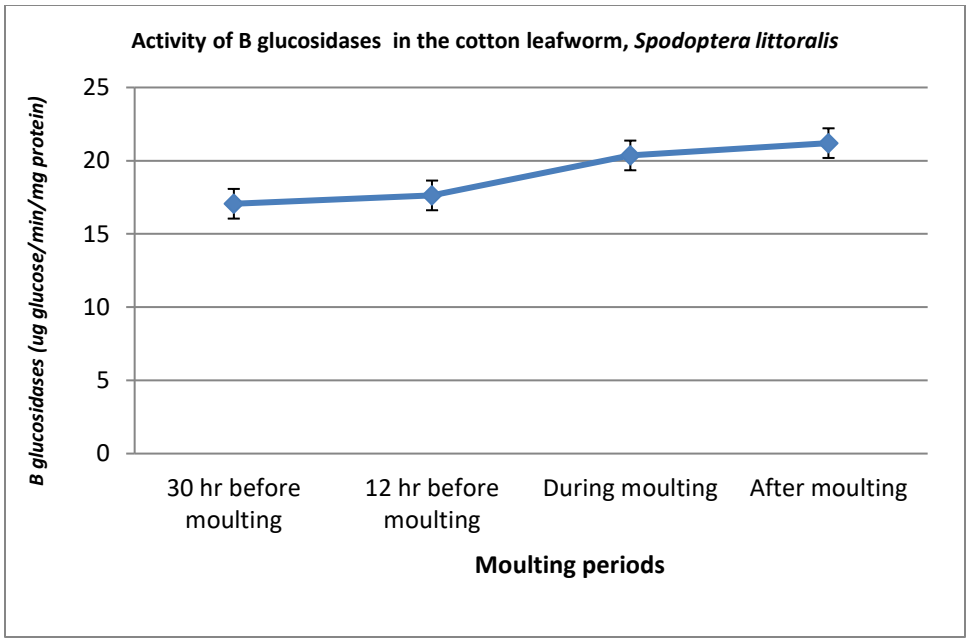

Figure3: β-glucosidase activity in *S. littoralis* during moulting period of fourth instar larvae

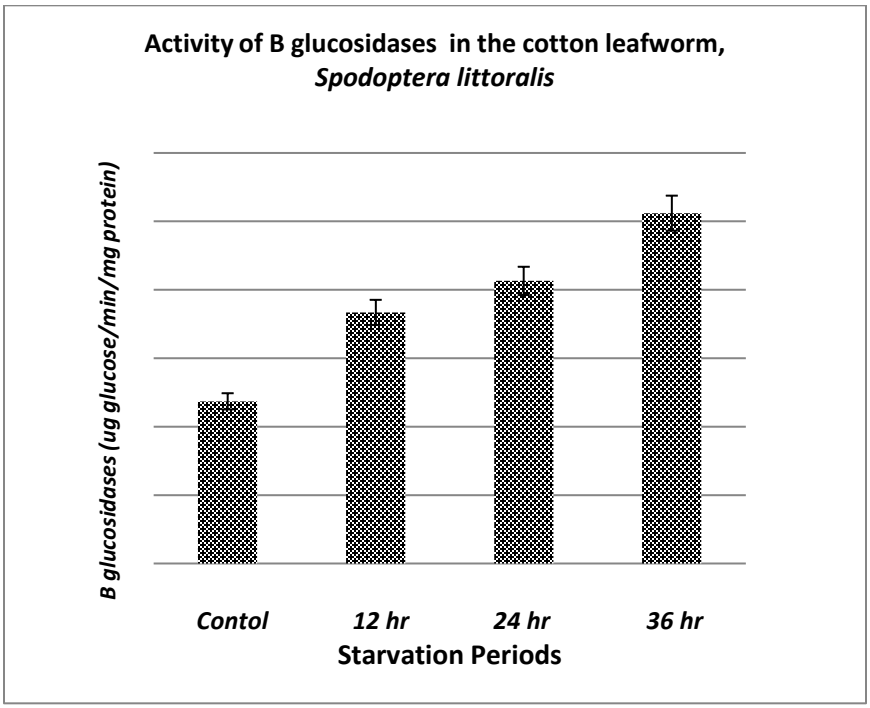

Figure4: β-glucosidase activity in 4th instar larvae of *S. littoralis* during starvation periods.

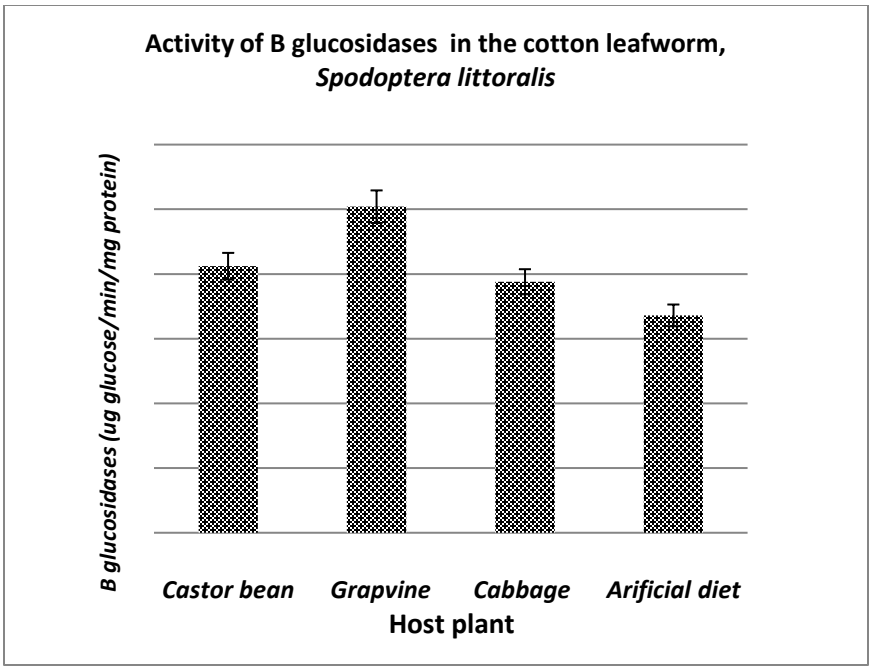

Figure5: β-glucosidase activity in fourth larval instar of *S. littoralis* fed upon different host plants.

Table1: β-glucosidase activity in the fourth larval instar of *S. littoralis* treated with different chemical compounds.

Chemical compounds	Concentration (mM)	B-glucosidase activity (Mean ± SD)	Control ratio %
Control		16.6 ±1.25 a	
EDTA	500 mM	10 ±0.2 c	-39.76
	250 mM	13.6 ±1.3 b	-18.07
	50 mM	15 ±1.05 ab	-9.63
	10 mM	15.9 ±0.5 a	-4.22
$CuSo_4$	50 mM	0.00 9± 0.002 d	-100
	25 mM	2.78 ±0.24 c	-83.25
	10 mM	9.00 ±0.5 b	-63.86
	2 mM	15.7 ±1.07 a	-5.42
NaF	50 mM	12.3 ±0.37 b	-25.90
	10 mM	13.3 ±0.49 b	-19.88
	2 mM	15.4 ±0.21 a	-7.23
$CaCl_2$	250 mM	4.9 ±0.36b	-70.48
	50 mM	6.76 ±0.49b	-59.28
	10 mM	15.1 ±0.62a	-9.040
NaCl	250 mM	11.1 ±0.65c	-33.13
	50 mM	13.5 ±0.61c	-18.67
	10 mM	20.9 ±1.37a	+25.90

β-glucosidase activity during moulting period

β-glucosidases activity was measured at 30 and 12 h before moulting, during moulting of 4th larval instar, as well as after entrance to the fifth instar as observed in Fig. (3). Generally, moulting process had no effect on β-glucosidases activity, or in other words, β-glucosidases showed no special activity changes during this critical period. Generally, β-glucosidase activity had a slightly higher increase during and just after moulting than the hours preceding it.

Effect of starvation on β-glucosidase activity

When 4th instar larvae were starved for 12, 24, and 36 hours, β-glucosidase activity had a significantly gradual increase which was proportional with time as compared to normally fed larvae (control). After 36 hours of starvation, β-glucosidase activity increased by two-folds relative to the normally fed ones as appears in Fig. (4).

Effect of host plant on β-glucosidase activity

When 4th instar larvae of *S. littoralis* were allowed to feed upon different host plants namely, castor bean, grapevine, cabbage as well as artificial diet, β-glucosidase activity showed some significant changes according to the source of nutrition as shown in Fig. (5). The activity was 20.6, 25.6, 19.4, and 16.8 µg glucose/ min/ mg protein for larvae fed castor bean, grapevine, cabbage, and artificial diet, respectively.

***In vitro* effects of inorganic compounds on β-glucosidase activity**

As shown in table (1), incubation of β-glucosidases for 10 minutes with different molar concentrations of inorganic salts showed inhibitory effects upon 4th larval instar β-glucosidase ability to hydrolyze salicin. Inhibition depended on concentration and compound type. Residual β-glucosidases activity was 15, 12.3, 0.009, 6.76 and 13.5 µg glucose /min/mg protein when enzyme incubated with 100 µl of 50 mM EDTA, NaF, $CuSO_4$, $CaCl_2$ and NaCl, respectively. Control (without inhibitor) activity was 16.6 µg glucose/min/mg protein. Salts could be arranged in ascending order according to their inhibitory effect as follows: EDTA < NaCl < NaF < $CaCl_2$ < $CuSO_4$. NaCl (10 mM) was the only salt that caused activation to β-glucosidases activity, however when used at higher concentrations, it showed also an inhibitory effect.

DISCUSSION

The present study showed a logic increase of β-glucosidases activity in both larval and adult stages rather than non-feeding ones i.e. eggs and pupae. Similar findings were recorded in many insects such as the coleopteran, *Tenebrio motitor* (Dastranj et al. 2013), the hemipteran pentatomid, *Acrosternum arabicum* (Mohammadzadeh and Izadi, 2016), the elm leaf beetle; *Xanthogaleruca luteola* (Sharifi et al. 2011), the red palm weevile, *Rhynchophorus ferrugineus* (Riseh et al. 2012) as well as lesser mulberry pyralid; *Glyphodes pyloalis* (Ghadamyari et al. 2010). This may be attributed to the need of polysaccharides degradation into monosaccharide for energy supply (Murray et al. 1994). Sharifi et al. (2011) concluded that feeding is intensified at the larval instars especially the last one due to the need for energy saving for further growth and development. He also demonstrated that oviposition needs a high-energy demand, which eventually leads to high metabolic rates in adults.

In the present work, a considerable activity of β-glucosidase was also detected in the egg stage. This may point to another undiscovered non-nutritional role of this enzyme. On the other hand, De Moraes and Bowen (2000) demonstrated that the activity of this enzyme in *Apis mellifera* may depend upon several physiological and nutritional factors such as age and environmental conditions.

β-glucosidases, in the present work, showed also a variable activity in different tissues of the 4th larval instar of *S. littoralis*. The maximum activity was detected surprisingly in haemolymph, and not in the gut as expected. The detection of high such activity may direct our thinking again to another non-digestive role of this enzyme. Pentzold et al. (2013) concluded that some insects use β-glucosidase haemolymph as a defensive weapon against predators and pathogens via the β-glucosidase-mediated release of toxic aglycones from glucoside precursors as cyanogenic, and phenolic glucosides. The same finding was achieved in Juvenile Chrysomelina leaf beetles (Rahfeld et al. 2015), and in *Lutzomyia longipalpis* larvae (Moraes et al. 2012; Vale et al. 2012).

Prior to and during moulting, larvae stop feeding (Waterhohouse, 1957). Accordingly, the detected activity of β-glucosidase found prior, during, and after the larval moulting of 4th larval instar into the 5th one, in the present work, points out strongly that this enzyme may somehow be involved in the moulting process. This seems logical because, during moulting, many enzymes are working in a harmony, for instance, trehalase is activated to degrade trehalose to glucose for chitin build-up (Candy and Kilby, 1962; Abd El-aziz and Fahmy, 2008). No study has yet demonstrated a relation between β-glucosidase and moulting to our knowledge but the present data shed the light on this expected relation.

On the other hand, starvation experiment was carried out to discover whether β-glucosidases are secreted only in response to feeding or not. The 4th larval instar of *S. littoralis* recorded a significant gradual increase in β-glucosidase activity as the starvation hours lasts. The enzyme level was nearly twice in larvae starved for 36

hours relative to the normally fed ones. i.e. enzyme not secreted as a response of food intake, and may point out that β-glucosidases might play another non-digestive role. Ghadamyari et al. (2010) showed that there is no significant difference in β-glucosidase activity at 5th instar of *G. pyloalis* midgut when containing food and without food. In addition, insect severe dehydration during starvation, in the present experiments, might be responsible for such a dramatic increase in β-glucosidases activity.

When 4th instar larvae were allowed to feed upon different food types, β-glucosidases activity was significantly higher in larvae fed on grapevine than those fed upon rather cabbage or artificial diet. The enzymatic activity also showed an insignificant difference between castor bean and cabbage fed larvae compared to those fed on an artificial diet.

Plants are able to form different allelochemicals such as glycosides, phenols, and alkaloids as defensive barriers against phytophagous pests (Biere et al. 2004). β-glucosidases in insects besides being involved in carbohydrates metabolism play an important role in insect-plant interactions (Ghadamyari et al. 2010). They are able to cleave plant toxic glycosides, which may produce cyanide after their hydrolysis or other toxic inhibitors to vitally important enzymes such as glucose 6-phosphate dehydrogenase (Desroches et al. 1997).

Some insects can overcome such toxicity via lowering β-glucosidases as observed in lepidopteran insects such *Chilo suppressalis* (Zibaee et al. 2009) and *G. Pyloalis* (Ghadamyari et al. 2010). On the other hand, Nakonieczny et al. (2006) observed increased β-glucosidase activity in the lepidopteran larvae, *Parnassius apollo*. Such variable response among different insects can be attributed to the differences in performance of insect detoxification mechanism after the hydrolysis of glycoside or may depend on differential β-glycosidase specificity (Ferreira et al. 1997). Zibaee el al. (2009) concluded that the enhanced activity of β-glucosidase can be detrimental to plant-feeder insects since they ingest plant glycoside.

The present work showed a trial to detect some compounds, which may cause an inhibitory effect to β-glucosidases and thus could be utilized later in integrated pest management programs. When β-glucosidase was incubated with different chemical compounds, all tested compounds at low concentrations (*i.e.* 10 m M or less) showed no effect upon the enzymatic activity except with NaCl salt which caused a significant activation to β-glucosidases activity. At higher concentrations, all compounds inhibited β-glucosidase activity and $CuSO_4$ was the strongest inhibitor among all tested compounds.

β-glucosidases response was tested to different chemical substances varies in different insect species. The enhanced effect was detected in the presence of NaCl and $CaCl_2$ while the enzyme was inhibited with different concentrations of EDTA in the rice striped stem borer, *C. suppressalis* (Zibaee et al. 2009). On the other hand, $CaCl_2$ didn't show any effect on β-glucosidase of the palm weevil, *R. palmarum* (Yapi et al. 2009). Inhibition of digestive enzymes is an important practice for controlling insect pests using enzyme inhibitors (Zibaee et al. 2009).

The present study shed the light for the first time on a preliminary correlation between β-glucosidases in the cotton leafworm, *S. littoralis* at different physiological states such as moulting and starvation, its distribution among larval tissues, its titer throughout its life cycle as well as *in vitro* effects of some inorganic salts.

Detection and understanding of insect digestive enzymes is a fundamental step to develop a successful pest control strategy. β-glucosidase in insects seems also to play a multifunctional role rather than being only a digestive one. These roles are still obscured and undiscovered. Further studies are needed to trace the enzyme in detail especially during important physiological phases such as oviposition and we need also to discover the relation (if present) between β-glucosidase and moulting.

CONCLUSION

The present study showed that B-glucosidases in the cotton leafworm; Spodoptera littoralis (Boisd.) seemed to have a variable activity during different developmental stages. The maximum activity was detected during larval and adult stages. Both larval gut and haemolymph comprised the major content of B-glucosidases compared to the other larval tissues. The noticeable elevation of β-glucosidases during and after moulting may point to its involvement in this process. Both starvation and incubation with inorganic salts inhibit B-glucosidases. Accordingly, B-glucosidases in *S. littoralis* seems to play a multifunctional role rather than being only a digestive one.

CONFLICT OF INTEREST

The authors declared that present study was

performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

NMF and TRA designed the experiments of the entire work. TRA performed the biochemical analysis and data statistics. NMF wrote the manuscript and designed figures and tables. Both authors read and approved the final version.

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