Gut PH, and Isolation and Characterization of Digestive *α-D*-Glucosidase of Sunn Pest

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ABSTRACT

A study of insect digestive enzymes makes sense given that the gut is the major interface between the insect and its environment. An understanding of gut and digestive enzyme function is essential when advanced methods of insect management such as application of enzyme inhibitors and transgenic plants are developed to control insect pests. The aim of the current research project was to study midgut anatomy, midgut pH and *α*-glucosidase activity in Sunn pest, *Eurygaster integriceps* Puton (Hemip: Scutelleridae). Sunn pest midgut is comprised of four distinct regions including first ventriculus (V1), second ventriculus (V2), third ventriculus (V3) and finally fourth ventriculus (V4). The study showed that the first three regions of the Sunn pest midgut are more acidic (pH= 5–5.2), the fourth region moderately acidic (pH= 6.2–6.4), and hindgut slightly acidic (pH= 6.5–6.8). Enzyme assay showed that *α*-glucosidase activity is present in midgut and salivary glands of adult *E. intergriceps*. The specific activity of midgut enzyme was 0.17 U mg protein^{-1} while the specific activity of the salivary glands enzyme was 0.033 U mg protein^{-1}. Optimum temperature and pH values for *α*-glucosidase were determined to be 40-45°C and 5, respectively. Based on linear regression analysis of reciprocal p-nitrophenyl substrate (*p*-nitrophenyl *α-D*-glucopyranoside) concentration versus reciprocal *α*-glucosidase activity K_m and V_{max} were 17 and 0.9 mM *p*-nitrophenol min^{-1}, respectively. The effect of different ion concentrations on *α*-glucosidase activity showed that Na^+, K^+, Mg^{2+}, and Ca^{2+} ions exerted positive effects on the enzyme activity. Other compounds tested such as urea, SDS, Tween 80, Triton X-100 and EDTA had an inhibitory effect on enzyme activity.

Keywords: *α*-glucosidase, Gut, pH, Sunn pest.

INTRODUCTION

Sunn pest (*Eurygaster integriceps* Puton) is a serious pest of wheat in West and Central Asia as well as in Eastern Europe (Javahery, 1995). Adults and nymphs cause severe damage to plants by feeding on leaves, stems and grains redusing the yield and food quality. Sunn pest feeding is typical of heteropterans, piercing and cutting tissues with their stylets while injecting digestive enzymes, amylases and proteases through salivary canal to pre-digest food. Predigested food is then ingested through the food canal and passed into the alimentary canal where it is further digested by digestive enzymes and then absorbed (Cohen, 2000; Boyd *et al.*, 2002). Since the insect predigests food before ingestion, enzymes injected into the grains reduce the baking quality of the dough. If the insects feed on as little as 2 to 5% of the grain the entire lot may be rendered unacceptable for baking purposes due to the baking quality of the flour being damaged. If serious control measures not taken, even 100% yield loss can result. Annual costs of chemical control against Sunn pest are

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estimated to be 40 million $ US (Javahery, 1995). Chemical control is widespread in the region and only in Iran does the pesticide spraying area exceed one million hectares, annually. In addition to the high cost of chemical control, insecticides are hazardous to human health, to the pest natural enemies and to the environment as a whole.

Many insects including Sunn pest which constitute serious pests of grains live on a polysaccharide-rich diet and thus are dependent on the grains's carbohydrases (α-amylase and glucosidases) for their survival. α-Amylase converts starch to maltose, which is then hydrolyzed to glucose by the application of an α-glucosidase. α-Glucosidase catalyzes the hydrolysis of terminal, non-reducing α-1,4 linked glucose residues from such arylglucosides as ρ-nitrophenyl—α-D-glucoside, disaccharides or oligosaccharides (Silva and Terra, 1995) which are then absorbed into the haemolymph. For a characterization of α-Glucosidases, they have been isolated from many insects including *Dysdercus peruvianus* (Hemip: Pyrrhocoridase), *Sitophilus zeamais* (Col: Curculionidae), *Apis mellifera* (Hymen: Apidae), *Drosophila melanogaster* (Dip: Drosophillidae), *Musca domestica* (Dip: Muscidae) and *Thaumetopoea pityocampa* (Lep: Notodontidae) (Silva and Terra, 1995; Baker, 1991; Huber and Mathison, 1976; Tanimura *et al.,* 1979; Jordao and Terra, 1991; Pratviel-Sosa *et al.,* 1986). A common feature of insect α-glucosidases is their optimum pH values (in the range of 5–6.5) and their inhibition by Tris (Terra *et al.,* 1996).

Considering the importance of carbohydrate digestion as a target for Sunn pest control, it becomes clear that carbohydrases need more attention. As a strategy of control, inhibitors of insect digestive enzymes have already been demonstrated to be an important biotechnological approach to the control of insect pests. Pea and azuki transgenic

plants expressing α-amylase inhibitors from common beans (α-AI) were completely resistant to the weevils *Bruchus pisorum* and *Callosobruchus chinensis* (Morton *et al.,* 2000). So, this study was carried out to determine gut pH, isolate and characterize α-glucosidase activity in *E. integriceps* to gain a better understanding of the digestive physiology of the insect. The gained knowledge will lead to new management strategies for this economically important pest.

MATERIALS AND METHODS

Insects

The insects studied in this work were collected from wheat farms during spring when feeding started in Karaj, Tehran Province of Iran. They were fed and maintained on wheat grains in the laboratory conditions at $25 \pm 2°C$ and a photoperiodism of 14: 10(L:D).

pH Determination of Gut Lumen

To have a clear understanding of the process of digestion in *E. integriceps* and to determine pH in the alimentary canal, adult insects were dissected under light microscope and their alimentary canal removed. Gut pH was determined according to the methods of Bignell and Anderson (1980), and Silva *et al.* (1999).

Each section of gut was cut and mounted on a microscope slide, then five micro-liters of pH indicator solutions added to each. Indicators used were; 0.1% bromophenol blue (pH= 3.0–4.6), 0.1% methyl red (pH= 4.4–6.2), 0.1 % bromcresol purple (pH= 5.2–6.8), 0.1 % bromophenol blue (pH 6.2 – 7.6), 0.1 % natural red (pH 6.8 – 8.0), 0.1 % cresol red (pH= 7.2–8.8), 0.1% thymol blue (pH= 8.0–9.6) and 0.1 % Alizarin yellow (pH= 10–12).

Enzyme Preparation

Enzyme samples from midguts and salivary glands of adults were prepared by the method of Cohen (1993) with slight modifications. Briefly, adults were randomly selected with midgut and salivary gland complexes (SGC) from these individuals removed by dissection under a light microscope in ice-cold 0.04M Citric acid – Phosphate (Na_2HPO_4) buffer at pH 5.0 containing 10 mM NaCl and 10 mM $CaCl_2$.

Midgut was also separated from the insect body, rinsed in ice-cold buffer, placed in a pre-cooled homogenizer and ground in one ml of phosphate buffer.

The homogenates from both preparations (midgut and SGC) were separately transferred to 1.5 ml centrifuge tubes, centrifuged at 15,000×g for 20 minnutes at 4˚C.

The supernatants were pooled and stored at -20˚C for subsequent analysis.

Enzyme Assay

α-Glucosidase activity was assayed spectrophotometrically by assessing the hydrolysis of the *p*-nitrophenyl substrate (*p*-nitrophenyl *α*-D-glucopyranoside) according to the method of Silva *et al.* (1999). Assays were performed in duplicate, at 40˚C, in 0.04M citric acid–phosphate (Na_2HPO_4) buffer at pH 5.0 containing 10 mM NaCl and 10 mM $CaCl_2$. Reaction mixture (170 μl) consisted of buffer (115 μl), enzyme extract (10 μl) and substrate (45 μl). The reactions were terminated after 10 minutes by addition of 1 ml 0.25M NaOH, and the production of p-nitrophenol was measured at wavelength of 405 nm. All Experiments were repeated at least three times.

Production of Standard Curve

A standard curve of absorbance against amount of *p*-nitrohenol released was constructed to enable calculation of the amount of *p*-nitrophenol released during assays. Serial dilution of *p*-nitrophenol in citrate–phosphate buffer at pH 5.0 were made to give final concentration of 7.5, 15, 30, 61, and 122 nM. Duplicates were incubated for 3 minnutes at 40^oC before addition of of 1 ml 0.25M NaOH whereupon the absorbance measurements were read.

Effect of Temperature on Enzyme Activity

The effect of temperature on *α*-glucosidase activity was determined by incubating the reaction mixture at different temperatures including 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, and 70˚C for 3 minutes. Also the thermo-stability of enzyme over 10 days at specified temperature was determined. Samples were maintained at 4, 24, 34, and 44˚C for 10 days followed by determination of residual activity by enzyme assay as described above.

Effects of pH on Enzyme Activity

The pH optima of α-glucosidase were determined using universal buffer (Hosseinkhani and Nemat-Gorgani, 2003). The pH values tested were 2, 3, 4, 5, 5.5, 6, 6.5, 7, 7.5, 8, 9, and 10. Also, the effect of pH on α-glucosidase stability was determined by pre-incubating the enzyme at the above pH values for 1.0 and 10 hours, followed by measurement of enzyme activity.

Effect of Activator and Inhibitos on the Enzyme Activity

To test the effect of different ions on the enzyme activity, midguts were first dissected in distilled water. Enzyme assays were then performed in the presence of 10 and 20 mM concentrations of NaCl, $CaCl_2$, KCl, $MgCl_2$, $CuSO_4$, EDTA (ethylenediaminetetraacetic acid), SDS
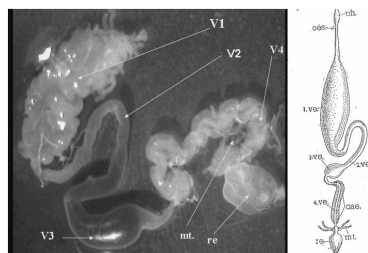
Figure 1. Alimentary canal of *Eurygaster integriceps* (Hemip: Scutelleridae) (Left) and its comparison with alimentary canal of *Leptocorisa varicornis* (Hemip: Coreidae) (Right). Cae: Caeca of the fourth ventriculus (V4), mt: Malpighian tubules, ph: Pharynx, re: Rectum, V1: First ventriculus, V2: Second ventriculus, V3: Third ventriculus, V4: Fourth ventriculus.

(sodium dodecylsulfate), Urea, Triton X-100 and Tween-80. These compounds were added to the assay mixture, and enzyme activity measured after incubation. A control (no chemical compounds added) was also included.

Protein Determination

Sample protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as standard.

Kinetic Studies

Enzyme kinetic studies were performed in duplicate, employing a range of substrate concentrations (S) (0.5, 1, 2, 3, 4, and 5 mM) with constant enzyme level in a final volume of 170 μl. All reactions were carried out at pH 5.0 and stopped through addition of 1 ml 0.25M NaOH after 10 minutes.

Controls in which distilled water replaced the enzyme for each substrate concentration were run in parallel. Double reciprocal plots (Lineweaver-Burk plots) of enzyme activity versus substrate concentration were used to establish the fact that Michaelis Menten kinetics were obeyed over this substrate concentration range and to obtain values for

the maximum velocity (V_{max}) and for Michaelis constant (K_m).

RESULTS

Gut Luminal pH

Sunn pest alimentary canal consists of a short narrow oesophagus, a large midgut and a short hindgut (rectum) (Figure 1). The midgut shows four distinct regions which according to Saxena (1954) are referred to as the 1st (V1), 2nd (V2), 3rd (V3), and 4th ventriculus (V4) (Figure 1). Application of pH indicators showed that the first three regions of the midgut (V1-V3) were acidic (pH 5.0 – 5.2), the fourth region (V4) was less acidic (pH 6.2 – 6.4) while the rectum only slightly acidic (pH= 6.5–6.8).

α-Glucosidase Activity

Our studies showed that α- glucosidase activity is present in all regions of the midgut as well as in the salivary glands of adult *E. intergriceps* (Table 1). The activity of the enzyme in the midgut was higher than that in the salivary glands. As an example cited the specific activity of midgut enzyme was 0.17 U mg protein^{-1} while the specific activity of the salivary gland enzyme 0.033 U mg protein^{-1}. So, the specific activity of

Table 1. A Comparison of the activity of α-glucosidase in salivary glands and in midgut of *Eurygaster integriceps*.

Tissues	α-Glucosidase activity	
	Unit activity (U ml^{-1})	Specific activity (U mg protein^{-1})
Midgut	0.42	0.17
Salivary glands	0.015	0.033

the enzyme in the midgut was five times higher as compared to that in the salivary glands.

Effect of pH and Temperature on *α-Glucosidase Activity*

Similar to most insect *α*-glucosidase, which have their optimal activities at acidic pH values, *α*-glucosidase in Sunn pest also showed an optimal pH of 5 (Figure 2). These studies showed that the enzyme activity increased steadily in pH media from 3 to 6 and then the activity decreased with increasing pH values. As can be seen from Figure 2, Sunn pest *α*-glucosidase has a broad range of activity from pH 4 to 6.

Residual activity of the enzyme following pre-incubation at different pHs and at different times is shown in Figure 3. Our results showed that stability of the enzyme at different pHs was not significantly affected because pre-incubation of the enzyme in different acidic pH value media for one and ten hours did not greatly affect enzyme activity very much. However, when the enzyme was pre-incubated at alkaline pHs for 10 hours, enzyme activity was reduced

more as compared with pre-incubation of the enzyme in acidic pH media.

α-Glucosidase was considerably active over a broad range of temperatures. The optimum activity was found to be in temperature between 40 to 45°C (Figure 4). A rapid decrease in the enzyme activity was at temperature observed above 45°C and the activity reached about zero at 65 to 70°C temperature. *α*-Glucosidase thermal stability was monitored by measuring residual activity following incubation of the enzyme at 4, 24, 34, and 44°C for 10 days. The activity decreased at higher temperatures. For instance at 4 and 44°C, losses of enzyme activity over a 10 day period were 2 and 30%, respectively (Figure 5).

Effect of Activators and Inhibitors on *α-Glucosidase Activity*

The effect of different ion concentrations on *α*-glucosidase activity showed that Na$^+$, K$^+$, Mg^{2+}, and Ca^{2+} ions had positive effects on the enzyme activity (2). In all cases except for NaCl, the effect of 20 mM ion concentrations was greater than those
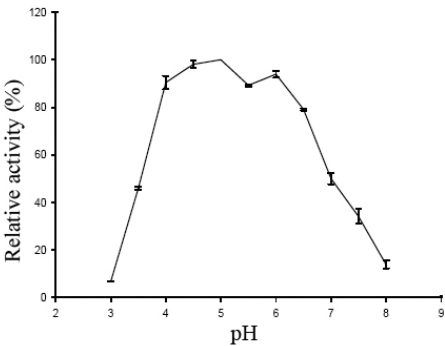
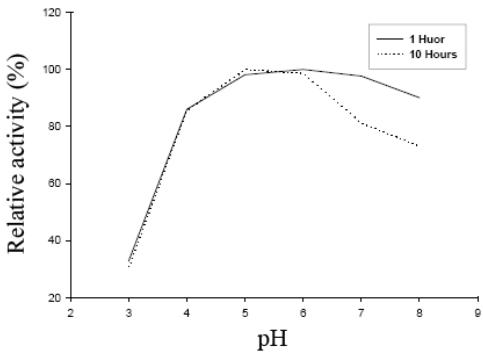**Figure 2**. The effect of pH on α-glucosidase activity in Sunn pest (*Eurygaster integriceps*).

Figure 3. The effect of pH on α-glucosidase stability. The enzyme was pre-incubated for 1 and 10 hours at different pHs prior to enzyme activity assessment.
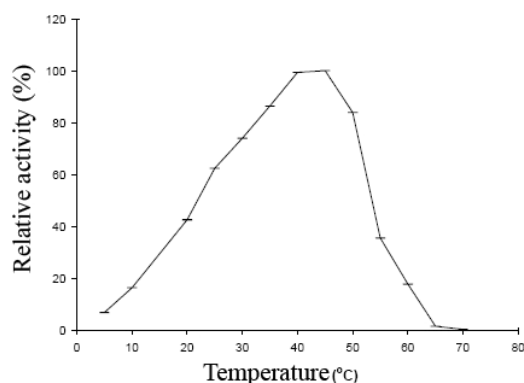
Figure 4. The effect of Temperature on α-glucosidase activity in Sunn pest (*Eurygaster integriceps*).

Figure 5. Thermal stability of Sunn pest α-glucosidase at different temperatures over a 10 day period.

observed for 10 mM ion concentrations.

Other compounds tested such as urea, SDS, Tween 80, Triton X-100 and EDTA exhibited an inhibitory effect on enzyme activity (Table 2). Inhibitory effects of urea, SDS, Tween 80, Triton X-100 and EDTA at concentrations of 20 mM were 41, 92, 25, 66 and 25%, respectively.

Kinetic Studies

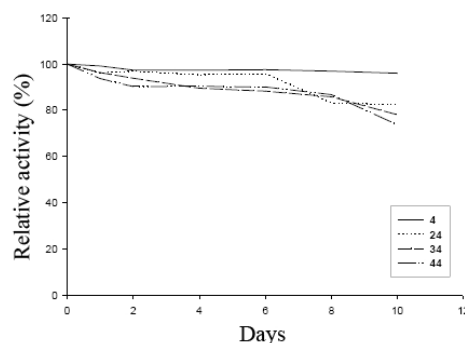Hydrolytic activity of *α*-glucosidase was assessed over the concentration range of 0.5 to 5 mM *p*-nitrophenyl *α-D*-glucopyranoside as a substrate (Figure 6). Saturation occurred at 5mM concentration of substrate. Based upon linear regression analysis of reciprocal substrate concentrations versus reciprocal α-glucosidase activity, K_m and V_{max} values were 17 and 0.9 mM *p*-nitrophenol min^{-1}, respectively.

DISCUSSION

In this study Sunn pest midgut anatomy as well as midgut pH were determined. As is the case in some other heteropterans

Table 2. Relative activity of *E. integriceps* α-glucosidase toward different compounds. Values are Means±S.E (Standard error), n = 6.

Compound	Relative activity (%)	
	(10 mM)	(20 mM)
Control	100 ± 4.2	100 ± 5.6
NaCl	117.63 ± 6.1	115.36 ± 5.4
CaCl$_2$	117.69 ± 4.7	119.17 ± 4.5
KCl	117.58 ± 3.8	118.88 ± 5.2
MgCl$_2$	115.74 ± 5.1	117.71 ± 4.3
CuSO$_4$	58.42 ± 6.2	57.78 ± 5.3
EDTA	81.77 ± 4.8	74.01 ± 4.2
SDS	9.7 ± 2.5	7.67 ± 2.3
Urea	66.79 ± 3.6	59.73 ± 4.7
Tween 80	58.33 ± 4.3	44.39 ± 4.5
Triton X-100	58.33 ± 5.1	44.39 ± 6.3

The enzyme was pre-incubated for 10 min at 35ºC with the listed compounds at the final concentration indicated prior to substrate addition. Activity in absence of compounds was taken as 100%. Each value represents the average of three independent experiments, each of two replicates.
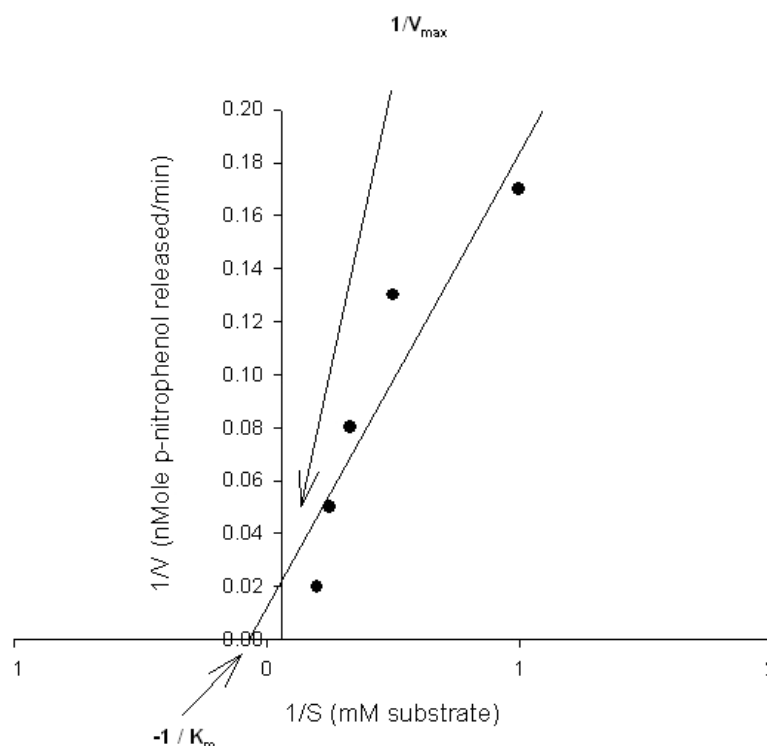
Figure 6. Double reciprocal plot (Lineweaver-Burk plot) of enzyme activity (velocity) versus p-nitrophenyl α-D-glucopyranoside concentration (nM) to obtain values for the maximum velocity (V_{max}) and for Michaelis constant (K_m).

(Chapman, 1998), Sunn pest midgut is composed of four distinct regions including first ventriculus (V1), second ventriculus (V2), third ventriculus (V3) and a fourth one (V4). The study showed that the pH in the different gut regions decreased from the anterior to posterior. So, regions V1-V3 of the Sunn pest midgut are more acidic (5–5.2), the fourth region (V4) being moderately acidic (6.2–6.4) and the hindgut pH slightly acidic (6.5 – 6.8). Saxena (1954) reported that in *Leptocorisa varicornis* (Hemip: Coreidae) the midgut is composed of four different regions (V1, V2, V3, and V4) with the first two regions having a pH ranging from 5.2 to 6.8, the third and fourth regions, pHs were from 4.6 to 5.2 while the hingut pH varying 6.8 from 8.3. Hirose (2005) found that in *Nezara viridula* (Hemip: Pentatomidae) the midgut also is composed of four different regions (V1, V2, V3, and V4) with different midgut regions having pH values of 6.6 (V1), 6.1 (V2), 5.2 (V3) and 6.6 (V4).

The first two regions of the midgut (V1 and V2) have been reported to be involved in the storage and digestion of food (Goodchild, 1966). During the process of feeding the ingested food becomes mixed with salivary secretions (*α*-amylase and *α*-glucosidase) and is accumulated in the first ventriculus. In this region the food becomes also exposed to additional α-amylase and glucosidases as well as to midgut proteases, which contribute to digestion of the food in this region. Silva and Terra (1995) found major *α*-glucosidae activity going on in the V1 region in *Dysdercus peruvianus* (Hem: Pyrrhocoridae).

The third region of the midgut (V3) is where the absorption of food takes place (Goodchild, 1966; Saxena, 1954) and the fourth region (V4) is where symbionts are located in *N. viridula* and possibly in other hemipterans (Hirose, 2005; Chapman, 1998). The fourth region in Sunn pest midgut appears to also accommodate symbionts.

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In Sunn pest, α-glucosidase activity was detected in the salivary glands, however, this activity was less than that measured in the gut. This makes sense in terms of food digestion because endoase enzymes must act first to hydrolyze the substrates so that fragments of small size can be further hydrolyzed by exoases. Glycosidases are exoenzymes which hydrolyse the *o*-glycosyl bonds of carbohydrates. Insects such as Sunn pest which feed on wheat grains live on a polysaccharide-rich diet and are dependent on the grain's carbohydrases (α-amylase and glucosidases) for survival. It has been reported that Sunn pest carries at least two major α-amylases in its midgut (Kazzazi *et al.,* 2005). α-Amylase converts grain starch to maltose, which is then hydrolyzed to glucose by the action of α-glucosidase. The physiological role of α-glucosidase is to release glucose from small dietary oligosaccharides that are the products of α-amylase activity. α-Glucosidase catalyzes the hydrolysis of terminal, non-reducing α-1,4 linked glucose residues from such arylglucosides as ρ-nitrophenyl—α-D-glucoside, disaccharides or oligosaccharides (Silva and Terra, 1995) which are then absorbed into the haemolymph. α-Glucosidase activity has been shown to be optimal at acidic pH in the other hemipteran species such as *Dysdercus peruvianus* (Hemip: Pyrrhocoridae) (Silva and Terra, 1995) and *Rhodnius prolixus* (Riberio and Pereira, 1984). Optimal pH values for Sunn pest α-glucosidase were determined to be in the acidic range (pH 4 – 6) and this is comparable with the pH of the gut lumen.

Temperature optima for Sunn pest α-glucosidase were determined to be between 40 to 45°C, which is in match with previous findings. For example, Huber and Mathison (1976) reported that honey bee α-glucosidase activity has an optimal temperature demand of 20 to 45°C. Whereas Cu^{2+} had a negative effect (inhibited enzyme activity) on Sunn pest α-glucosidase activity, such metal ions as Na^+, K^+, Mg^{2+}, and Ca^{2+} showed a positive effect on enzyme activity.

These ions also have positive effects on the activity of α-glucosidase and α-amylase enzymes in other insects (Kazzazi *et al.,* 2005; Hori, 1972). Some other compounds such as SDS, Triton X-100, EDTA, and urea have inhibitory effects on Sunn pest α-glucosidase activity. Kinetic parameters of α-glucosidase include a K_m value of 17 mM which is relatively high in comparison with that in the other insects. Silva and Terra (1995) reported a K_m value of 1.4 for α-glucosidase in *D. peruvianus*. They also reported that α-glucosidase in *D. peruvianus* hydrolyse maltose more than sucrose and more than any other substrate tested (Silva and Terra, 1995). This may also be true for Sunn pest because it feeds on wheat grains in which α-amylases hydrolyse starch to maltose which is then hydrolyzed to glucose by α-glucosidase. So, a study of carbohydrase enzymes makes sense since carbohydrates play a major role in Sunn pest development. Knowing the mechanism(s) of carbohydrate digestion and enzymes involved in the digestion process could be employed in new strategies for Sunn pest control.

ACKNOWLEDGEMENTS

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PH دستگاه گوارش و جداسازی و تعیین خصوصیات آلفا دی گلوکوزیداز گوارشی سن گندم

ع. ر. بندانی ، م. کزازی و م. الهیاری

چکیده

مطالعه دستگاه گوارش و آنزیم های گوارشی حشرات ازاین جهت مهم میباشد که دستگاه گوارش حشرات حد فاصل بین حشره و محیط می باشد. مخصوصا اطلاع از کارکرد دستگاه گوارش و آنزیم های گوارشی وقتی که هدف توسعه روشهای کنترل حشرات با استفاده از مهارکننده ها ی آنزیم ها و گیاهان تراریخته باشد ضروری می باشد. بنابراین هدف مطالعه حاضر بررسی آناتومی معده میانی سن گندم ، pH قسمت های مختلف معده میانی و تعیین مشخصات آنزیم آلفا گلوکوزیداز میباشد. معده میانی سن گندم دارای چهار قسمت مشخص میباشد که عبارتند از وینتریکولوس اول (V1)، وینتریکولوس دوم (V2)، وینتریکولوس سوم (V3) و وینتریکولوس چهارم (V4). مطالعات نشان داد که سه قسمت اول معده میانی سن گندم اسید یته بیشتر(۵-۵,۲ pH)از قسمت چهارم (۶,۴-۶,۲ pH) می باشد. سنجش آنزیمی نشان داد که آلفا گلوکوزیداز در غدد بزاقی و معده میانی حشره وجود دارد. فعالیت ویژه آنزیم در معده میانی برابر با ۰,۱۷ در غدد بزاقی برابر با ۰,۰۳ واحد در میلی گرم پروتئین می باشد. دما و pH بهینه فعالیت آنزیم به ترتیب برابر با ۴۰- ۴۵ درجه سانتی گراد و ۵ تعیین شد. برای محاسبه K_m و V_{max} فعالیت آنزیم روی غلظت های مختلف سوبسترا اندازه گیری شد که K_mمعادل ۱۷ و V_{max} معادل ۰,۹ میلی مولار پی-نیتروفنل در دقیقه تعیین شد. اثر غلظت های مختلف یونها روی فعالیت آنزیم نشان داد که یونهای سدیم، منیزیوم، کلسیم و پتاسیم دارای اثرات مثبت روی فعالیت آنزیم هستند درحالیکه SDS ، توین — ۸۰ ، EDTA ، اوره و تریتون دارا اثرات بازدارندگی می باشند.