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Carbohydrase activities in the larval digestive system of the cigarette beetle, *Lasioderma serricorne* (Col.: Anobiidae)

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Abstract

The cigarette beetle, *Lasioderma serricorne*, is a serious infesting pest of warehouses and retail stores thorough the world. Partial biochemical characterization of carbohydrases was performed in the larval digestive system of the pest. Midgut extracts from larvae showed an optimum activity for α-glucosidase, β-glucosidase and α-amylase activity against, *p*-nitrophenyl-α-D-glucopyranoside, *p*-nitrophenyl-β-D-glucopyranoside and starch at pH 5, pH 6 and pH 7, respectively. Larval midgut α-glucosidase was more stable in acidic conditions (pH 4 to pH 6.0) than highly acidic and alkaline conditions. However, the enzyme showed to be more stable in slightly acidic condition (pH 5) when incubation time was increased. β-Glucosidase was also stable in acidic conditions (pH 5 to pH 7) and its maximum stability occurred at pH 6. The range of stability for α-amylase was determined in slightly acidic to weakly alkaline conditions (pH 6 to pH 8). Maximum activity for α-glucosidase, β-glucosidase and α-amylase incubated at different temperatures was observed at 35, 40 and 50°C, respectively. The K_M and V_{max} values for α-glucosidase and β-glucosidase were 2.77 mM and 0.017 mmol min^{-1} mg^{-1} protein and 0.6961 mM and 0.0004 mmol min^{-1} mg^{-1} proteins, respectively. The K_M and V_{max} values for α-amylase were 3.2 mg ml^{-1} and 0.0170 mmol min^{-1} mg^{-1} protein. Zymogram analysis revealed the presence of one band of α-amylase activity, one band for each α-glucosidase and β-glucosidase activity in the larval midgut extract.
Key words: *Lasioderma serricorne*, digestive, carbohydrase, α-amylase, α-glucosidase, β-glucosidase.

فعالیت کربوهیدرازها در سامانه گوارشی لارو سوسک توتون، *Lasioderma serricorne* (Col.: Anobiidae)

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چکیده

سوسک توتون، *Lasioderma serricorne*، یکی از حشرات زیان‌آور فرآورده‌های انباری در سراسر دنیا می‌باشد. تعیین نسبی ویژگی‌های بیوشیمیایی کربوهیدرازها در سامانه گوارشی لارو آفت انجام گرفت. بهینه فعالیت آلفا-گلوکوزیداز، بتا-گلوکوزیداز و آلفا-آمیلاز عصاره روده میانی لارو به ترتیب در pHهای ۵، ۶ و ۷ بدست آمد. آلفا-گلوکوزیداز در شرایط اسیدی (pH ۴ تا ۶) نسبت به شرایط خیلی اسیدی و بازی پایداری بیشتری داشت. با این حال، وقتی که زمان انکوباسیون افزایش یافت، آنزیم در شرایط کمی اسیدی (pH ۵) پایدارتر بود. بتا-گلوکوزیداز نیز در شرایط اسیدی پایدار بود و بیشترین پایداری در pH ۶ مشاهده گردید. دامنه پایداری آمیلاز در شرایط کمی اسیدی تا کمی بازی (pH ۶ تا ۸) بدست آمد. بیشترین فعالیت آلفا-گلوکوزیداز، بتا-گلوکوزیداز و آلفا- آمیلاز به ترتیب در دمای ۳۵، ۴۰ و ۵۰ درجه سلسیوس تعیین گردید. مقدار K_M و V_{max} برای آلفا-گلوکوزیداز و بتا-گلوکوزیداز به ترتیب ۲/۷۷ میلی مولار و ۰/۰۱۷ میلی مول بر دقیقه بر میلی‌گرم پروتئین، و ۰/۶۹۶۱ میلی‌مولار و ۰/۰۰۰٤ میلی‌مول بر دقیقه بر میلی‌گرم پروتئین برآورد گردید. مقدار K_M و V_{max} برای آلفا-آمیلاز ۳/۲ میلی‌گرم بر میلی‌لیتر و ۰/۰۱۷ میلی‌مول بر دقیقه بر میلی‌گرم پروتئین بدست آمد. آنالیز زایموگرام وجود یک باند با فعالیت آلفا-آمیلازی، یک باند با فعالیت آلفا-گلوکوزیدازی و بتا-گلوکوزیدازی در روده میانی لارو را مشخص نمود.
واژه‌های کلیدی: *Lasioderma serricorne*، گوارشی، کربوهیدراز، آلفا-آمیلاز، آلفا-گلوکوزیداز، بتا-گلوکوزیداز.

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Introduction

The cigarette beetle, *Lasioderma serricorne* Fabricius (Coleoptera.: Anobiidae), is a worldwide cosmopolitan pest of many raw and finished stored products especially spices, seeds, grains, dried potatoes, raisins and tobacco(Oppert *et al.*, 2002). Alpha-amylases are a group of hydrolases that are widely dispersed in microorganisms, plants, and animal tissues. The enzymes catalyze the hydrolysis of α-1,4-glucan linkages in both starch and glycogen (Janecek, 1997; MacGregor *et al.*, 2001). α-Amylase activity has been detected in larval midguts of members of the orders Orthoptera, Hymenoptera, Diptera, Lepidoptera and Coleoptera (Terra and Ferreira, 1994). Digestive α-amylases from crude larval midguts and purified samples in Coleoptera have been characterized in details at the biochemical and structural level (Applebaum *et al.*, 1965; Lemos *et al.*, 1990; Terra and Ferreira, 1994; Grossi de Sa and Chrispeels, 1997; Strobl *et al.*, 1998). Properties of α-amylase inhibitors and their effects on α-amylase have also been also examined in some insects (Mendiola-Olaya *et al*., 2000; Valencia-Jimenez *et al.*, 2000; Silva *et al.*, 2001). Biochemical characterization of digestive α-amylase is studied in *Eurygaster maura* (Hem.: Scutelleridae) (Ravan *et al.*, 2009). Some properties of α-amylase in Indian meal moth, *Plodia interpunctella* (Lep.: Pyralidae), was studied (Farshbaf *et al.*, 2010). α-Amylase activity in digestive glands of the snail *Caucasotachea lencoranea* Mousson (Stylommatophora: Helicidae) was also studied (Bigham *et al.*, 2011).

Glucosidases are the enzymes widely spread in living organisms involved in final phases of carbohydrase digestion. Glucosidases have been found in several lepidopteran insects, such as *Chilo suppressalis* Walker (Pyralidae) *Thaumetopoea pityocampa* Schiffermuller (Notodontidae) and *Parnassius apollo* L. (Papilionidae), and some coleopteran insects such as *Rhynchophorus palmarum* L. (Curculionidae) (Yapi *et al.*, 2009) and *Rhagium inquisitor* (Cerambycidae) (Chipoulet and Chararas, 1985) and larvae of *Zabrotes subfasciatus* (Col.: Bruchidae) (Silva *et al.*, 2001) and *Tenebrio molitor* (Col.: Tenebrionidae) (Ferreira *et al.*, 2001). Alpha-glucosidase catalyzes the hydrolysis of 1,4-alpha-glucosidic linkages and releases alpha-glucose. The enzyme effectively hydrolyzes sucrose, maltose, maltodextrin and pNP-a-D-glucopyranoside (Ghadamyari *et al.*, 2010) .

This paper reports some characteristics of three main carbohydrate degrading enzymes including α-amylase, α-glucosidase and β-glucosidase, in the alimentary canal of the one of the most important stored product pest, *Lasioderma serricorne*. The resulted information hopefully will lead to new strategies for the management of this pest. To our knowledge, this is the first research on digestive carbohydrases in *L. serricorne*.

Materials and methods

Insects: Last larval instars of *Lasioderma serricorne* were reared on pea at 30°C and were used as the source of enzymes in the subsequent experiments.

Sample preparation: Last larval instars were cold-immobilized, dissected under a stereoscopic microscope, and the midguts removed and cleaned from adhering unwanted tissues. The midguts were collected into a known volume of distilled water and homogenized with a hand-held glass grinder on ice. The homogenates were centrifuged at 16000×g at 4°C for 15 min. The resulting supernatants were transferred to new tubes and frozen at -20 °C for further use.

α-Amylase assay: Amylase activity was assayed according to Bernfeld (1955) using dinitrosalisylic acid (DNS) and 1% soluble starch as the substrate. Briefly, 10 µL enzyme extract was incubated with 85 µL buffer and 5 µL soluble starch for 30min at 37°C. The experiment was stopped with the addition of 50 µl DNS and heating in boiling water for 10 min. The absorbance of the reaction mixture was recorded at 540 nm using a microplate reader (ELX 808). In the blanks, the enzyme added to the reaction mixture after DNS addition. Effect of some chemicals was assayed on α-amylase activity. To experiment the effect of ions on enzyme activity, assays were performed in the presence of different concentrations of chloride salts of Na^+ (15, 25, 35 and 45 mM), K^+ (15, 25, 35 and 45 mM), Ca^{2+} (15, 25, 35 and 45 mM), Mg^{2+} (15, 25, 35 and 45 mM), and sodium dodecylsulfate (SDS; 1.5, 2.5, 3.5 and 4.5 mM). All assays were done in triplicate.

Glucosidase assay: Activities of α-glucosidase and β-glucosidase were detected using *p*-nitrophenyl-α-D-glucopyranoside (pNaG), *p*-nitrophenyl-β-D-glucopyranoside (pNbG) as the substrates, respectively. The enzyme extract

was incubated with 5 µL of pNaG (5 mM) or pNbG (5 mM) and 85 µL of 40mM citrate-phosphate-borate buffer at 30ºC for 30 min. The reaction was stopped by addition of 50 µL of NaOH (1M). Optical density was measured at 405 nm using a microplate reader (ELX 808,). In the control tubes, the enzyme added to the reaction mixture after addition of NaOH.

pH profile of α-amylase, α-glucosidase and β-glucosidase activity: The effects of pH on the activity of midgut α-amylase, α-glucosidase and β-glucosidase were assayed. The optimum pH for α-amylase, α- and β-glucosidase activity was determined using sodium citrate-phosphate-borate buffer at a pH range of 3 to 10 and 4 to 10, respectively. The assays were performed according to the sections "α-Amylase assay" and "Glucosidase assay".

Effect of temperature on α-amylase, α- and β-glucosidase activity: The effect of temperature on the activity of midgut α-amylase, α- and β-glucosidase was assayed. Incubation of the reaction mixture was done at a temperature set of 20, 30, 35, 40, 45, 50, 55, 60, 70 and 80°C for 30 min for the enzymes. The assay was performed according to the sections "α-Amylase assay" and "Glucosidase assay".

pH stability of amylase and glucosidase enzymes: Stability of α-amylase and the glucosidases was determined at a pH set of 3 to 9 and 4 to 10, respectively and two incubation time periods. Enzyme extract was mixed with the buffer and incubated for 1 and 10 hours at 37°C. The substrate was then added to the buffered enzyme extract and α-amylase and α- and β-glucosidase activity was determined as before.

Kinetic parameters: The Michaelis–Menten constants (K_M) and the maximal reaction velocities (V_{max}) of α-glucosidase, β-glucosidase and α-amylase were determined. For α-glucosidase and β-glucosidase, the homogenate was incubated at 30°C in an appropriate buffer toward the substrates pNaG (pH 5) and pNbG (pH 6), respectively, in concentrations ranging from 0.159 mM to 2.544mM. In the case of α-amylase, the homogenate was incubated at 50°C in an appropriate buffer (pH 7) toward starch in concentrations ranging from 0.94 mg ml^{-1} to 7.52 mg ml^{-1}. The experiments were performed in triplicate. The K_M and V_{max} were evaluated by non-linear regression analysis using the software Sigmaplot.

Visualization of α-amylase activity: In-gel α-amylase assay was performed using native-PAGE for visualizing the enzyme activity. Enzyme extract was diluted in electrophoresis sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 0.01% (w/v) bromophenol blue) and loaded in gel of 5% stacking and 10% separating polyacrylamide gels**.** To observe the bands with α-amylase activity after native-PAGE, the resolving gel was incubated in a 1% (w/v) starch solution for 60 min with gentle shaking. Finally, it was stained with a solution of 14 mM KI and 10 mM I_2. The bands of α-amylase activity were appeared as clear areas in the field of black background of the gel.

Visualization of glucosidase activity: Visualizing of the α-glucosidase and β-glucosidase activities was performed as the section "Visualization of α-amylase activity" with after gel modification steps. When the enzyme gets to the end of the gel, it was immersed in the substrates 4-methyl umbelliferyl-α-D-glucopyranoside and 4-methylumbelliferyl-β-D-glucopyranoside (3mM) and the gel was checked under UV lamp in 366nm after 20 min. The bands of the enzyme activities were appeared as distinct light bands in the dark background of the gel.

Protein concentration: Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Results and Discussion

Alpha-amylase activity: Alpha-amylase activity was determined in the larval midgut of *L. serricorne.* One band of α-amylase activity was revealed in zymogram analyses (Figure 1) suggesting the presence of one form of amylase enzyme in the midgut of *L. serricorne.* The K_M and V_{max} values for α-amylase was 3.2 mg ml^{-1} and 0.0170 mmol min^{-1} mg^{-1} protein.

The enzyme retained more than 60% of its activity at a broad slightly acidic pH range (5 to 7) with maximum activity at pH 7.0 (Figure 2). No α-amylase activity was observed at pH 2 and pH 11. Lower α-amylase activity was observed at pH 3 to pH 4 and pH 8 to pH 10. The enzyme was remarkably stable in slightly alkaline conditions (pH 7 to pH 8) (Figure 3). However, α-amylase was more stable in slightly alkaline conditions than slightly acidic conditions (pH 6 to pH 7) in short term incubation (1 hr incubation time).

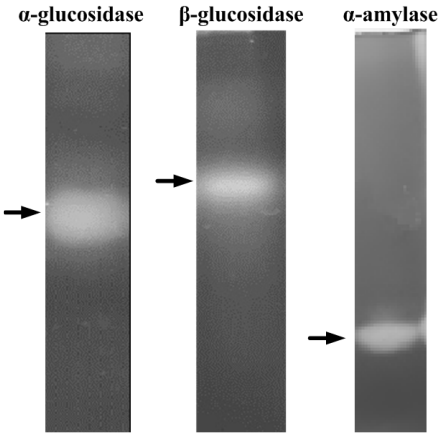

Figure 1. Gel assays of α-amylase, α-glucosidase and β-glucosidase activities in larval midgut of *Lasioderma serricorne*.

However, there was remarkable decrease in amylase activity after 10 hr incubation time compared with 1 hr incubation time. Digestive α-amylase was remarkably active at a broad temperature range from 30 to 60°C (Figure 4). Optimum temperature for α-amylase activity was obtained at 50°C. Alpha-amylase activity was significantly decreased at temperatures above 60°C.

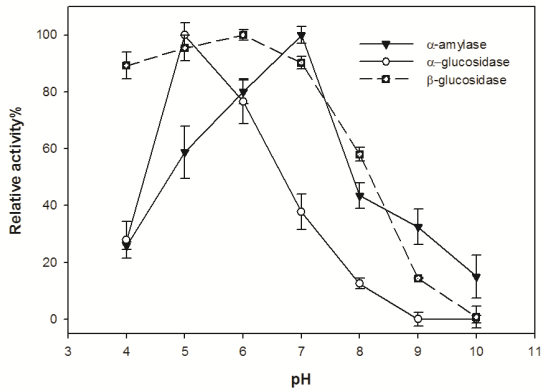

Figure 2. pH profile of α-amylase, α-glucosidase and β-glucosidase activities in larval midgut of *Lasioderma serricorne*. Each datum is the mean of three replicates and vertical bars represents standard error of the mean.

Effect of some chemicals was examined on α-amylase activity. Among the ions, Mg^{2+} (R^2=0.74, p=0.15) and SDS (R^2=0.97, p=0.004) significantly decreased the α-amylase activity (Figure 5). The effect of the ions Ca^{2+} (R^2=0.19, p=0.31), K^+ (R^2=0.2, p=0.74), Na^+ (R^2=0.18, p=0.77) and EDTA (R^2=0.1, p=0.86) on the α-amylase activity was not significant.

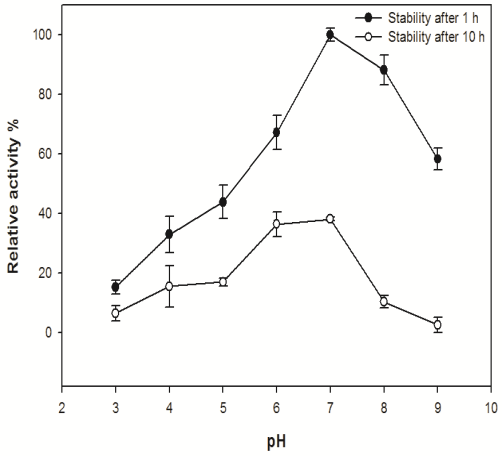

Figure 3. Stability of α-amylase from larval midgut of *Lasioderma serricorne* at different pHs under 1 and 10 hr incubation time. Each datum is the mean of three replicates and vertical bars represents standard error of the mean.

Glucosidase activities: Glucosidase activity was observed in the larval midgut of *L. serricorne*. The α-glucosidase was optimally active in acidic conditions (pH 5 to pH 6) (Figure 2). Lower activity was observed at pH 3 to pH 4 and pH 7 to 8 and no activity was revealed at pH 9 to pH 11.The β-glucosidase was active in acidic conditions (pH 4 to pH 7) (Figure 2). Lower activity was observed at pH 8 to pH 9 and no activity was revealed at pH 10 to pH 11. The results revealed that α-glucosidase was stable at pH 5 and 6 in a short incubation time (1 hr) (Figure 6). However, stability of the enzyme was significantly decreased at pH 5 and pH 6 in long incubation time (10 hr). β-Glucosidase is remarkably (above 80%) stable at a pH range of 4 to 7 in short incubation time (1hr) (Figure 7). However, stability of the enzyme was decreased after long incubation time (10 hr) so that the enzyme was most active at pH 6. α-Glucosidase retained more than 70% of its activity at a broad temperature range of 20 to 50°C with a maximum activity at temperatures 30 to 35°C (Figure 4). The enzyme activity was gradually decreased near to 20% at high temperature (70°C). β-Glucosidase remained

active (more than 60%) at a broad temperature range 20 to 70°C and its maximum activity was at 40 to 45°C (Figure 4).

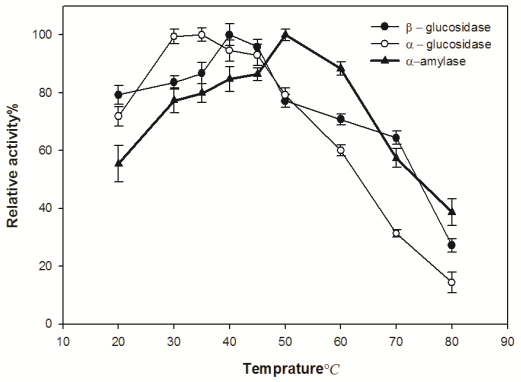

Figure 4. Activity of α-amylase, α-glucosidase and β-glucosidase from larval midgut of *Lasioderma serricorne* at different temperatures. Each datum is the mean of three replicates and vertical bars represents standard error of the mean.

Kinetic parameters of α-glucosidase and β-glucosidase were estimated at pH 5 and 6 against pNaG and pNbG at 30°C, respectively. The K_M and V_{max} values for α-glucosidase and β-glucosidase were 2.7707 mM and 0.017 mmol min^{-1} mg $protein^{-1}$ and 0.6961 mM and 0.0004 mmol min^{-1} mg^{-1} protein, respectively.

α-Glucosidase and β-glucosidase activities were detected in the gel using fluorescent substrates (Figure 1). One bands of activity was revealed for α-glucosidase and β-glucosidase activity in the midgut extract.

Our results show α-amylase in *L. serricorne* larvae have a neutral optimal pH (7) which is approximately congruent with the optimal pH for amylase activity reported for other coleopteran species (Silva *et al.*, 2001; Baker 1991; Dojnov *et al.*, 2007). The digestive amylases of several insects of the orders Hemiptera and Coleoptera are active in slightly acidic to neutral conditions. Digestive α-amylase in *Hypothenemus hampei* (Col.: Scolytidae), *Callosobruchus chinensis* (Col.: Bruchidae), *Tribolium castaneum* (Col.: Tenebrionidae) shows an optimal activity at pH 5, 5.2 and 5.4, respectively (Podoler and Applebaum, 1971; Applebaum and Konijn, 1965; Valencia-Jimenez *et al.*, 2000). However, in some coleopteran such as *Trogoderma granarium* (Col.: Dermestidae), optimum α-amylase activity mostly occurs in weakly acidic to slightly alkaline pH (6-8). In *T. granarium*, optimum pH for α-amylase activity is not completely congruent with the pH prevailing in the midgut (Hosseininaveh *et al.*, 2007). The optimal pHs were found at 5 and 6 for digestive amylases in the midgut and salivary glands of *Brachynema germari* (Hemiptera: Pentatomidae) (Ramzi and Hosseininaveh, 2010), respectively. Optimal pH for α-amylase activity in the first, second and third part of midgut of *Eurygaster maura* is found at acidic pH (5-5.5) and for the forth part obtained at pH 6.5-6.9. (Ravan *et al.*, 2009). In *Caucasotachea lencoranea* optimal pH is obtained at pH 2 (Bigham *et al.*, 2011).

Activity of digestive α-amylase in larval midgut of *L. serricorne* was optimum at 50°C which is similar to the other reported cases in some insects. α-amylase activity in midgut of *Caucasotachea lencoranea* obtained optimum at 40°C. (Bigham *et al.*, 2011). Optimal temperature for α-amylase activity in midgut and salivary glands of *Eurygaster maura* were found at 40°C and 35°C respectively. (Ravan *et al.*, 2009) Some chemicals can change activity of α-amylase in insects. Inhibitory effect of the ions K^+ and Mg^{2+} was also reported in *Brachynema germari* (Ramzi and Hosseininaveh, 2010). SDS also could decrease α-amylase activity which may be due to its denaturing effect on protein structure.

Our study shows that there is one form of α-amylase in the larval midgut extract while the existence of multiple amylases has been reported in *C. maculatus* (F.) (Col.: Bruchidae) (Campos *et al*., 1989). Baker (1991) detected two major isoamylases in *Rhyzopertha dominica* (Col.: Bostrichidae) and several minor protein bands with α-amylase activity in *Acanthoscelides obtectus* (Col.: Bruchidae) (Franco *et al.*, 2005). The influence of geographical origin on electrophoretic amylase isozymes also have been observed in *Sitophilus zeamais* (Col.: Curculionidae) (Baker and Halliday, 1989; Baker, 1991), but no electrophoretic variation was detected within or among strains of *T. confusum* (Col.: Tenebrionidae) (Wool and Noiman, 1980). Two major isoforms of α-amylase have been reported in *Prostephanus truncates* (Col.: Bostrichidae) (Mendiola-Olaya *et al.*, 2000) and *Zabrotes subfasciatus* (Col.: Bruchidae) (Lemos *et al.*, 1990) and *Morimus funereus* (Col.: Cerambycidae) (Dojnov *et al.*, 2007). Glucosidase activities were determined in larvae of *L. serricorne*. Optimum glucosidase activity

occurred at slightly more acidic pHs than α-amylase activity which is not completely consistent with the pH current in the larval midgut which is weakly acidic (Oppert *et al.*, 2002) (Hosseininaveh *et al.*, 2007). Weakly acidic optimum pH for β-glucosidase activity has been reported in some insects such as *Rhynchophorus palmarum* (Col.: Curculionidae), the adult moth *Heliothis zea* (Lep.: Noctuidae) and B. germari (Burton, 1975; Yapi *et al.*, 2009; Ramzi and Hosseininaveh, 2010).

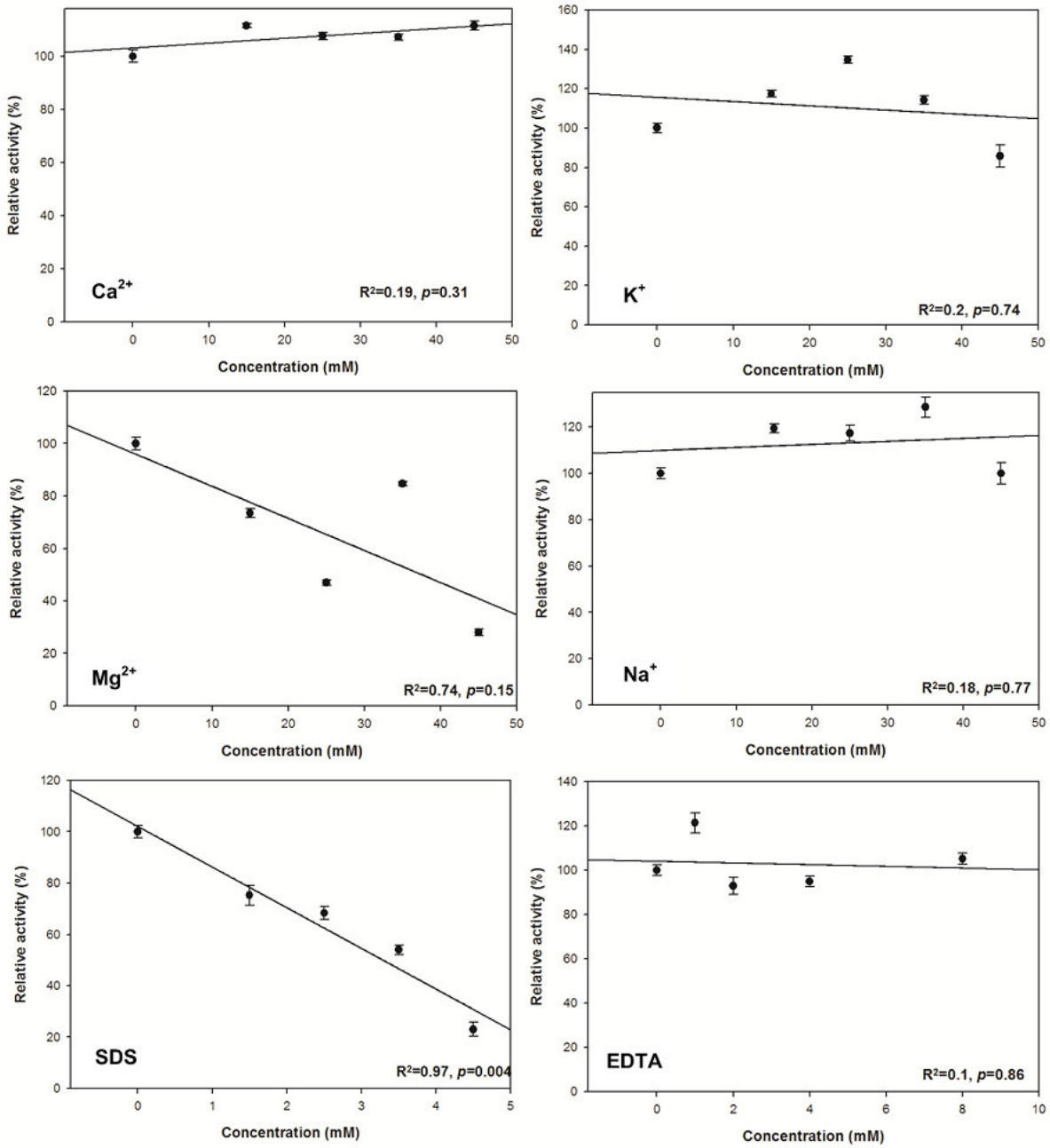

Figure 5. Effect of some ions and compounds on α-amylase activity from larval midgut of *Lasioderma serricorne*. Each datum is the mean of three replicates and vertical bars represents standard error of the mean

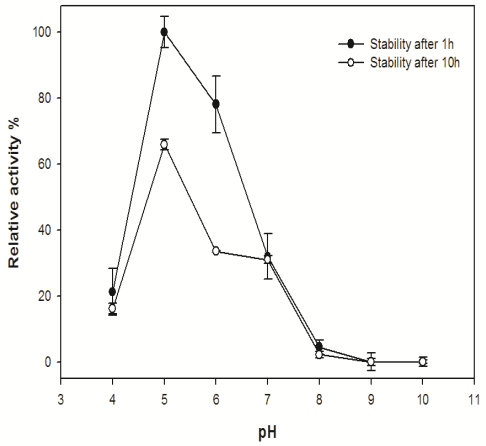

Figure 6. Stability of α-glucosidase from larval midgut of *Lasioderma serricorne* at different pHs under 1 and 10 hr incubation time. Each datum is the mean of three replicates and vertical bars represents standard error of the mean.

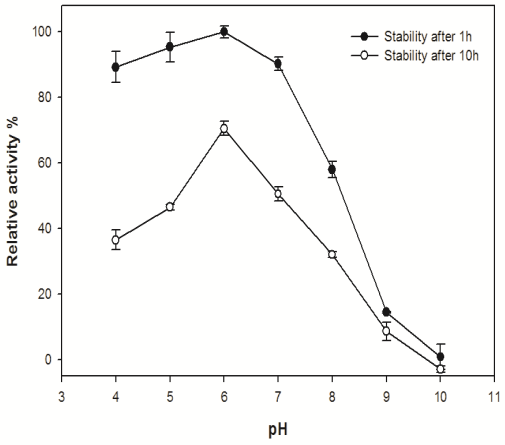

Figure 7. Stability of β-glucosidase from larval midgut of *Lasioderma serricorne* at different pHs under 1 and 10 hr incubation time. Each datum is the mean of three replicates and vertical bars represents standard error of the mean.

Conclusion

The current study demonstrated the presence of α-amylase, α-glucosidase and β-glucosidase in the midgut of *L. serricorne* larvae. Optimum activity of the enzymes was observed at weakly acidic to neutral pH. α-Amylase was more stable at slightly acidic to weakly alkaline pH. Glucosidases were more stable in weakly acidic condition. one forms of α-amylase activity and one form of α-glucosidase and β-glucosidase activity were detected in the midgut of the pest larvae.

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