Article

Biochemical properties of digestive carbohydrases from the sugar beet weevil, *Lixus incanescens* (Coleoptera: Curculionidae)

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Abstract

The sugar beet weevil, *Lixus incanescens* B., is one of the most important pests of sugar beet plant in Iran. The petioles and leaves of sugar beet are attacked by larvae and adults of the sugar beet weevil. Chemical application is currently used for controlling the pest. Digestion in the alimentary canal of the sugar beet weevil is facilitated by some carbohydrases. Results of the *in vitro* studies indicated the presence of α-amylase, β-glucosidase and β-galactosidase in the digestive tract of the pest. Highest activities of α-amylase, β-glucosidase and β-galactosidase were at pH 5, pH 5 and pH 4, respectively. No significant α-glucosidase and α-galactosidase activity was detected in the pest's digestive system. Optimum temperatures for α-amylase, β-glucosidase and β-galactosidase activity were determined at 45, 50 and 40 °C, respectively. α-amylase was more stable under acidic condition (pH 4 to pH 6) than under highly acidic and alkaline condition. Na^+ and K^+ increased α-amylase activity, but sodium dodecyl sulfate significantly decreased amylase activity. Also, the activity of α-amylase was inhibited by the other compounds such as $MgCl_2$, $CaCl_2$ and EDTA. Zymogram analysis using native-PAGE revealed one band of α-amylase activity in *Lixus incanescens*. High activity of carbohydrases in the digestive system of adults was determined and further researches are needed to be applied to design new strategies for controlling the sugar beet weevil based on natural carbohydrase inhibitors.

Keywords α-amylases; sugar beet weevil; carbohydrases; *Lixus incanescens*.

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1 Introduction

The sugar beet weevil, *Lixus incanescens* B. (Coleoptera: Curculionidae) is a key pest of sugar beet plants causing significant damage to living plants in Iran. It has been reported from many parts of Iran and some other countries (Davatchi et al., 1960; Aleeva, 1953). The sugar beet weevil has three generations per year. The petioles and leaves of sugar beet plants are attacked by larvae and adults of *L. incanescens*. The larvae can cause up to 75% root weight loss (Ocete et al., 1994). The most damage happens in the second and third generations. Loss in early-planted sugar beet is less than late-planted ones (Parvizi et al., 1988). Currently,

application of chemical pesticides has been a fundamental tool for pest control. However, it has had serious consequences such as intoxication of animals and people, contamination of air, water, and soil, high persistence in the environment, resistance in pests, and has impacted beneficial insects (Rodriguez et al., 1960; Huignard et al., 2005). The use of such pesticides in urban areas holds special risks, as most pesticides are not very selective, which has led to the search for safe and environmentally friendly alternatives (Breuer et al., 2000). It seems that an integrated pest management (IPM) program, including application of selective pesticides and some resistant cultivars bearing carbohydrase inhibitors as well as the use of field sanitation and crop rotation would provide the best management option for control of this pest (Meiners et al., 1978). Understanding the biochemistry of the enzymes present in the gut of target pests is the first step to design inhibitor-transgenic crops (Oppert, 2000; Oppert et al., 2000; Wilhite et al., 2000). α-amylases (α-1,4-glucan-4-glucanohydrolases; EC3.2.1.1) are the hydrolytic enzymes that there are in microorganisms, plants and animals such as insects. α-amylases are hydrolytic enzymes that catalyze the hydrolysis of α-D-(1, 4) glucan linkages in starch and related carbohydrates (Strobl et al., 1998; Ferreira et al., 1989). A-amylases have been detected in Coleoptera, Hymenoptera, Heteroptera, Orthoptera, Lepidoptera and Diptera (Hori, 1970; Kanekatso, 1978; Baker, 1987; Baker, 1991; Terra et al., 1988; Schumaker et al., 1993; Ferreira et al., 1999). Most insects depend on their amylases and glucosidases for efficient digestion of their diet. For both the maintenance of adult longevity and optimal larval growth, carbohydrates are essential for initial hydrolyzing of energy-producing nutrients (Dadd, 1985). α-glucosidases hydrolysis non-reducing 1,4-linked alpha-D-glucose residues and releases α-D-glucose. α-glucosidase hydrolyzes several substrates, including maltose, sucrose , maltodextrin and pNP-α-Dglucopyranoside (Terra et al., 1996). β-glucosidases catalyzes hydrolysis of β-1,4 linkages between two glucoses or glucose-substituted molecules (Terra et al., 1996). α-galactosidases are exo-acting glycoside hydrolases that cleave α-linked galactose residues from carbohydrates such as melibiose, raffinose and gluco- or galactomannans (Meier et al., 1982). β-galactosidases are the enzyme that catalyses the hydrolysis of β-galactosides into monosaccharides (Sezginturk et al., 2008). Our knowledge of β-galactosidase in insects is still rudimentary.

The destructive effects of the pest *L. incanescens* on the production and handling of sugar beet plants have made this insect an important target for biochemical study. There are significant variations among the properties of insect digestive enzymes. To develop the control strategy utilizing plant-carbohydraceous inhibitors, it is necessary to have more information on the gut enzymatic activities of insects (Wilhite et al., 2000). In the current study, we have carried out a detailed biochemical analysis of digestive carbohydrolytic activity in *L. incanescens* adults.

2 Materials and Methods

2.1 Test insect

Adults of the sugar beet weevil, *Lixus incanescens*, were collected from the fields of sugar beet plants at Qazvin province, Iran and were used as the enzyme source for subsequent experiments.

2.2 Sample preparation

Whole gut of the adults were removed by dissection under a stereo-microscope in ice-cold saline buffer, and homogenized in distilled water using a hand-held glass homogenizer. The homogenates were centrifuged at 16,000 × g for 15 min at 4°C. Resulted supernatants were removed and kept at -20 °C until needed.

2.3 α-amylase assay

α-amylase activity was assayed using dinitrosalicylic acid (DNSA) as the reagent (Strobl et al., 1998) and 1% soluble starch (Merck) as the substrate. Ten microliters of the enzyme source was added to 5 µl substrate and 85 µl of the universal buffer (0.02 M; sodium citrate- phosphate-borate). The reaction mixture was incubated at

35°C for 30 min. Thereafter, 100 µl DNSA was added to the reaction mixture and heated in boiling water for 10 min. After cooling the mixture, the absorbance was measured at 540 nm. All assays were performed in triplicate.

2.4 Glucosidase and galactosidase assay

Glucosidase and galactosidase assays was carried out by incubating 10 µl of the enzyme extract with 85 µl of 50 mM universal buffer (pH 4 to pH 11; sodium citrate-phosphate-borate) and 5 µl of each pnitrophenyl- α-D-glucopyranoside (pNPαGlu, 5 mM) for α-glucosidase or p-nitrophenyl-β-D-glucopyranoside (pNPβGlu, 5 mM) for β-glucosidase at 35 °C for 30 min. After the addition of NaOH (2 M) to the reaction mixture, released p-nitrophenol was determined by measuring the absorbance at 405 nm. In the blanks, enzyme extract was added to the reaction mixture after the addition of NaOH. The same procedure was used for detecting α- and β-galactosidase activity but with p-nitrophenyl-α-D-galactosidase (pNPαGal, 5 mM) and p-nitrophenyl-β-D-galactosidase (pNPβGal, 5 mM), respectively, as the substrates.

2.5 Effect of pH and temperature on enzyme activity

The effects of temperature and pH on the activity of α-amylase, β-glucosidases and β-galactosidase were examined using enzymes extracted from the whole gut. Effect of temperature on α-amylase, β-glucosidases and β-galactosidases activity was determined by incubating the reaction mixture at 20, 30, 35, 40, 45, 50, 60 and 70 °C for 30 min followed by measurement of the enzyme activity. The optimum pH for α-amylase, β-glucosidases and β-galactosidase activity was determined using the universal buffer (sodium citrate-phosphate-borate) at a pH range 3 to pH 11 with one degree interval.

2.6 pH stability of α-amylase

Stability of α-amylase was determined over a broad pH range and two incubation time periods. Enzyme extract was mixed with buffer and incubated for 1 and 10 hr at 37°C. The substrate was then added to the buffered enzyme extract, and α-amylase activity was determined as described above.

2.7 Effect of activators and inhibitors on α-amylase

To investigate the effect of several salts on α-amylase activity, assays were performed in the presence of different concentrations of K^+, Na^+, Ca^{2+}, and Mg^{2+} chloride salts as well as sodium dodecyl sulfate (SDS) and Ethylenediaminetetraacetic acid (EDTA). The enzyme sample was pre-incubated with the compounds for 15 min.

2.8 Zymogram analysis

Activity of α-amylase present in the crude homogenates of the whole gut of the adults was visualized using native polyacrylamide gel electrophoresis (native-PAGE). Native-PAGE was performed in a 10% (w/v) resolving gel and a 4% stacking gel. The sample buffer contained 25% stacking buffer (0.5 M Tris–HCl; pH 6.8), 20% glycerol, 0.005% (w/v) bromophenol blue, but no mercaptoethanol and no boiling. After electrophoresis, the gel was rinsed in distilled water and incubated for 1 h in phosphate buffer containing 1% starch and 20 mM CaCl2 at pH 5. The gel was then rinsed with distilled water and incubated with a solution of 10 mM I_2 and 14 mM KI to stop the reaction and to stain the unreacted starch background. Areas bearing α-amylase activity appeared as light bands against a dark background.

2.9 Protein determination

Protein content was determined using the method of Lowry et al. (1951). Bovine serum albumin was used as the standard.

3 Results

3.1 α-amylase activity

The effect of pH on amylolytic activity from the whole gut extracts is shown in Fig. 1. Relatively higher

activities were detected over a broad range of acidic condition (pH 4 to pH 6) with maximum activity at pH 5.

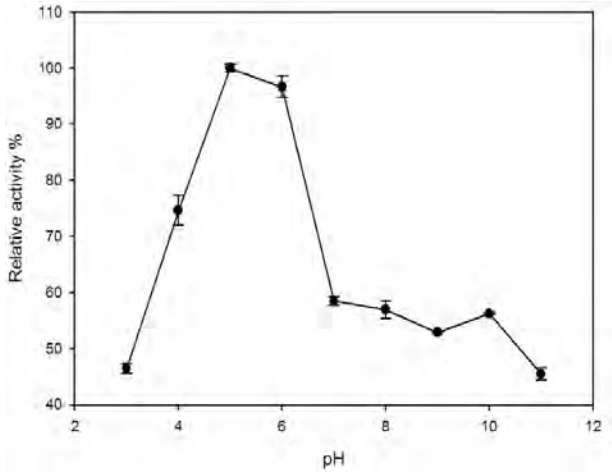


Fig. 1 The effect of pH on the activity of α-amylase extracted from the digestive system of *L. incanescens* adults.

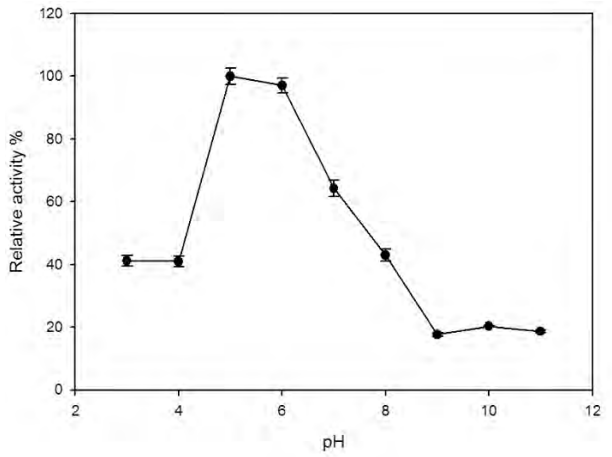


Fig. 2 The effect of pH on the activity of β-glucosidases extracted from the digestive system of *L. incanescens* adults.

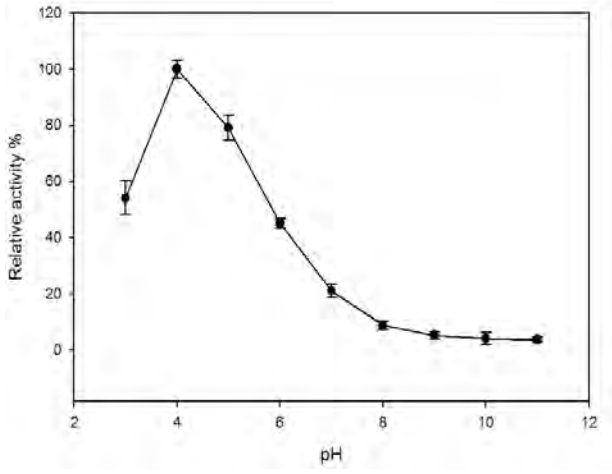


Fig. 3 The effect of pH on the activity of β-galactosidases extracted from the digestive system of *L. incanescens* adults.

3.2 Glucosidase activity

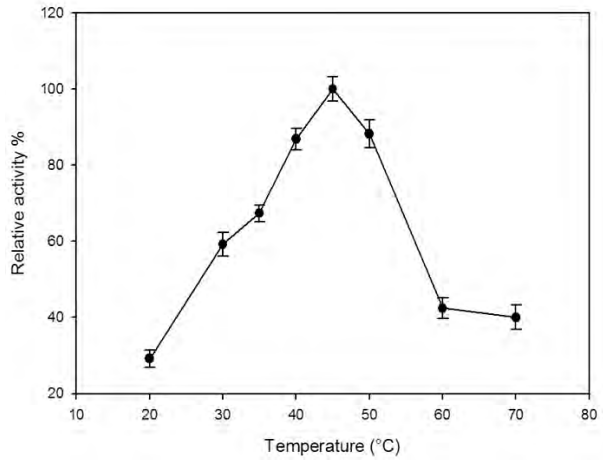
β-glucosidase activity in the gut increased steadily from pH 3 to pH 6, and then decreased with increased pH above 6 (Fig. 2). The optimal pH for β-glucosidase activity occurred at 5.0. Activity for α–glucosidase was not significantly detected in digestive system of *L. incanescens* adults.

3.3 Galactosidase activity

β-Galactosidase activity increased steadily from pH 3 to pH 4, then decreased with increased pH above 4. The optimal pH for β-galactosidase activity occurred at pH 4.0 (Fig. 3). Activity for α–galactosidase was not detected in digestive system of *Lixus incanescens* adults.

3.4 Effect of temperature on α-amylase activity

The optimum temperature for α-amylase activity was 45 °C (Fig. 4). Digestive β-glucosidase was optimally active at 50 °C (Fig. 5). Optimum temperature for β-galactosidase activity was determined at 40 °C (Fig. 6).



F**ig. 4** The effect of temperature on the activity of α-amylase extracted from the digestive system of *L. incanescens* adults.

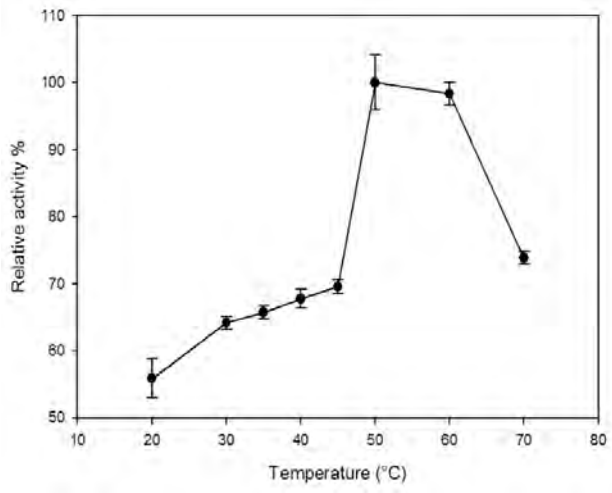


Fig. 5 The effect of temperature on the activity of β-glucosidases extracted from the digestive system of *L. incanescens* adults.

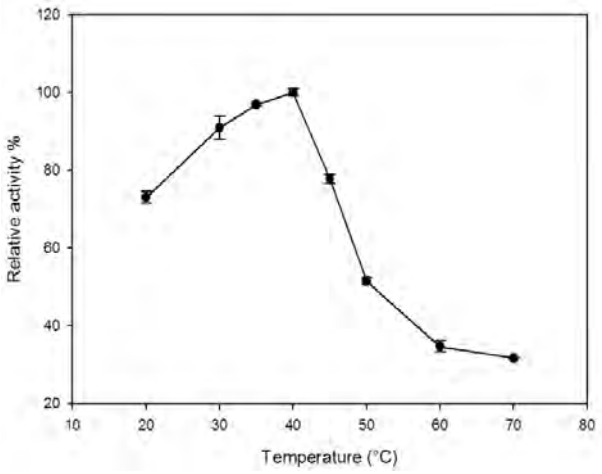


Fig. 6 The effect of temperature on the activity of β-galactosidases extracted from the digestive system of *L. incanescens* adults.

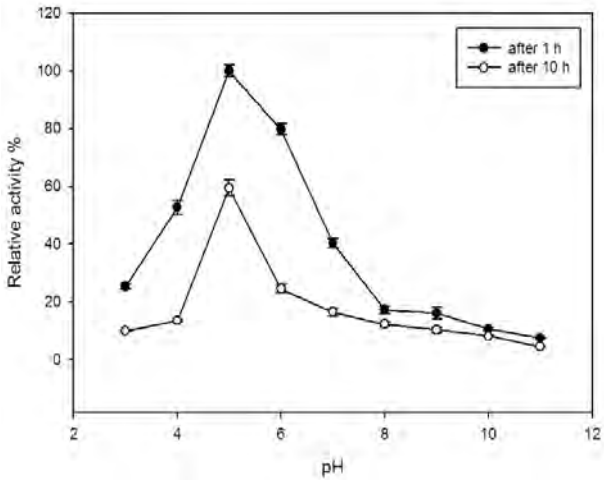


Fig. 7 Stability of digestive α-amylase from *L. incanescens* at different pHs after 1 and 10 hrs incubation period.

3.5 pH stability of α-amylase

The results revealed that α-amylase was more stable in acidic to slightly acidic condition (pH 4 to pH 6) with a short incubation period (1 hr; Fig. 7). However, α-amylase stability decreased under alkaline condition and a longer time incubation period (10 hr; Fig. 7).

3.6 Effect of activators and inhibitors on α-amylase activity

Na^+ and K^+ enhanced α-amylase activity, whereas sodium dodecyl sulfate (SDS) significantly decreased α-amylase activity. Activity of α-amylase was inhibited by other compounds such as $MgCl_2$, $CaCl_2$ and ethylenediaminetetraacetic acid (EDTA) (Fig. 8).

3.7 Zymogram analysis

Further characterizations of the α-amylase activity of the gut extract from the adults of *L. incanescens* using starch as the substrate under native-PAGE are shown in Fig. 9. At least one band of α-amylase activity was revealed from the gut extract.

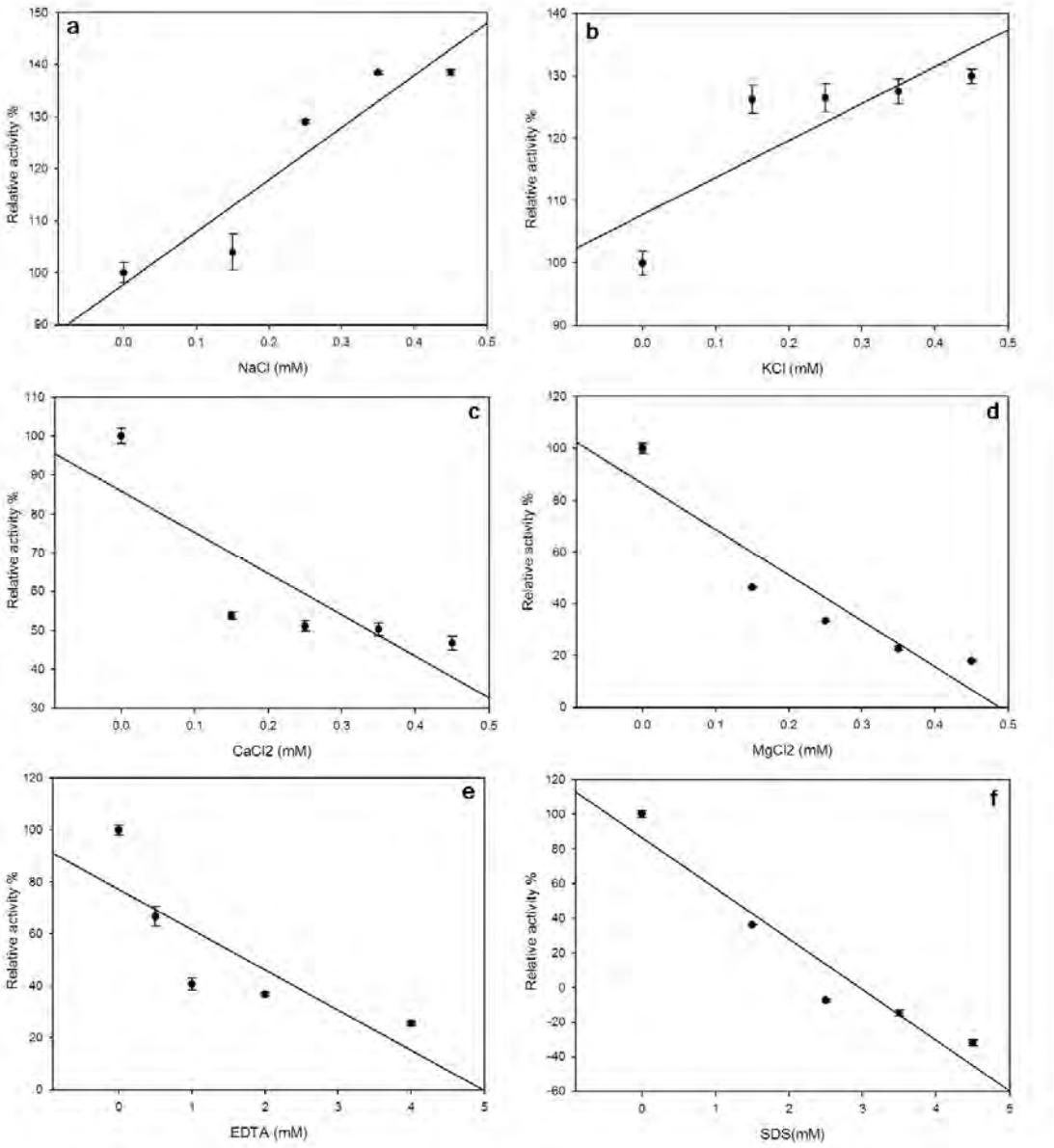


Fig. 8 Effects of different compounds on the midgut digestive amylase activity in *L. incanescens* adults.

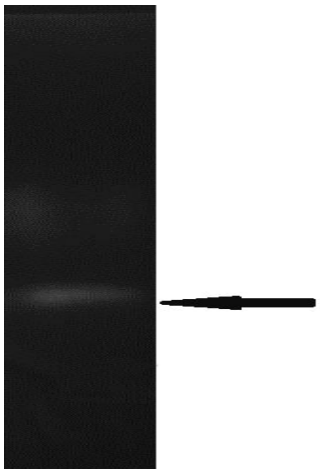


Fig. 9 Zymogram of α- amylase extracted from the digestive system of *L. incanescens*.

4 Discussion

Carbohydrases play a vital role in food digestion in insects. The present study clearly showed that α-amylase, β-glucosidase and β-galactosidase are active in the gut of *L. incanescens* adults. The enzymes are optimally active in acidic condition which is congruent with the pH prevailing in the gut of Coleoptera. Stability of amylase was also consistent with the pH value of the midgut and showed its maximum stability at acidic to slightly acidic condition in *L. incanescens*. The optimal pHs for hemolymph and gut α-amylases of the red palm weevil, *Rhynchophorus ferrugineus* Olivier (Col.: Curculionidae) were 5 to 6 and 4 to 5, respectively (Saberi et al., 2012). The optimum pH for digestive α- amylase activity in the salivary glands and midgut of *Brachynema germari* Kol. was determined at 6 and 5, respectively (Ramzi et al., 2010). In insects, amylases are usually most active in neutral to slightly acidic condition (Baker, 1983; Terra et al, 1996). The optimal pH values in larvae of various coleopterans were from 4 to 5.8 for amylases (Baker, 1983). The optimum activity for α-amylase from the midgut of *Hypera postica* larvae was determined at pH 5.0 (Vatanparast et al., 2010). *Hypera postica* α-amylase was more stable at pH 5 to pH 6 than highly alkaline and acidic pH in larval midgut of the pest (Vatanparast et al., 2010). The pH in the tissues from which the enzymes are isolated usually corresponds to the optimum pH for digestive enzyme activity. Biochemical properties of many partially purified or purified insect gut α- glucosidases are known, and regardless of the corresponding midgut pH value, their optimal pH range from 5 to 6.5 (Terra et al., 1994). Activity for α–glucosidase and α–galactosidase were not detected in digestive system of *Lixus incanescens* adults. Insect β-glucosidases have pH optima of 4.5 to 6.5 (Terra et al, 1994; Azevedo et al, 2003). β-glucosidase activity has been reported in *Diatraea saccharalis* Fabricius (Lepidoptera: Pyralidae) (Azevedo et al., 2003), *Parnassius apollo* ssp. *frankenbergeri* (Lepidoptera: Papilionidae) (Nakonieczny et al, 2006). Also, digestive β-glucosidase activity has been reported in *Rhynchophorus palmarum* L. (Coleoptera: Curculionidae) (Yapi et al., 2009). In *Dysdercus peruvianus* Guerin (Hemiptera: Pyrrochoridae), galactosidases have an optimum pH of 5.0 (Silva et al., 1997). In *Abracris flavolineata* De Geer (Orthoptera: Acrididae) midguts, there are two α-galactosidases and these enzyme have maximum activity at pH 5.4 (Ferreira et al., 2001). β-galactosidase activity has been detected in *Spodoptera frugiperda* (Marana et al., 2000) and *Diatraea saccharalis*, *Tenebrio molitor* (Franco et al., 2000). The highest activities of α- and β-glucosidases of *Rhynchophorus ferrugineus* were at pH 5 and of α- and β-galactosidases at pH 4 (Saberi et al., 2012). Data on the biochemical characterization of β-galactosidase in insects are not complete.

Maximum carbohyrolytic activity was obtained at 40 to 50 °C congruent with the other insects. Higher gut α-amylase activity of the red palm weevil was obtained at 40 to 50 °C (Saberi et al., 2012). Digestive α-amylase from *H. postica* showed its maximal activity at 35 °C (Vatanparast et al., 2010). For *α-amylase* activity, optimum temperature was 60 °C in *Bombyx mori* L. (Lepidoptera: Bombycidae) (Kanekatso, 1978). In midgut of *Brachynema germari* Kol. (Hemiptera: Pentatomidae) optimum temperature for α- and β-galactosidase was 30 °C (Ramzi et al., 2010). The sugar beet weevil β-glucosidase has optimum temperature activity at 50 °C (Fig. 5). Also, the optimal temperature for β-galactosidase in the digestive system this pest was 40 °C (Fig. 6). Alpha and β-glucosidases and α- and β-galactosidases from the red palm weevil had an optimum temperature activity at 50, 50–60, 40–60, and 40 °C, respectively (Saberi et al., 2012). Activity of digestive α- and β-glucosidases، α- and β-galactosidases in the digestive system of *Xanthogaleruca luteola* were optimum at 60, 50, 40, 60 °C , respectively (Sharifi et al., 2011).

In the presence of Ca^{2+}, Mg^{2+} and SDS, digestive α-amylase activity of *Hypera postica* significantly decreased, while Na^{+} and K^{+} did not significantly affect the enzyme activity (Vatanparast et al., 2010). Zymogram analysis showed the presence of one band of α-amylase activity in the gut of *L. incanescens* similar to other coleopteran pests (Vatanparast et al., 2010). However, zymogram analysis revealed that red palm

weevil hemolymph and gut α-amylases had two isoforms (Saberi et al., 2012).

Producing pest-resistant transgenic plants help us to decrease the use of chemical pesticides and the entering of these chemicals in the environment. Understand digestive enzymes of the target insect is first step in designing a controlling program based on inhibition of digestion (Strobl et al., 1998). The biochemical characterization of insect digestive enzymes will greatly help to design new strategies for insect control and will facilitate the understanding of the mechanisms responsible for the inhibitory potential of the carbohydraceous plant inhibitors.

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