Article

Biochemical characterization of digestive carbohydrases in Moroccan locust, *Dociostaurus maroccanus* Thunberg (Orthoptera: Acrididae)

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Abstract

The Moroccan locust, *Dociostaurus maroccanus* (Thunberg), is an agricultural pest which has caused considerable agricultural damage and economic importance. α-/β-Glucosidases and α-/β-Galactosidases are essential enzymes in the carbohydrate digestion of insect pest. Characterization of digestive enzymes is essential for gathering knowledge to production of resistant plants, in this research α-/β-Glucosidases and α-/β-Galactosidase from *D. maroccanus*. Results showed that the activities of α-/β-Glucosidases were higher than those of α-/β-Galactosidase in digestive system Moroccan locust. Maximum activity for α-Glucosidase was at pH 4. Optimum activity for β-Glucosidases and α-/β-Galactosidase were found at pH 5. The *D. maroccanus* α-/β-Glucosidases and α-/β-Galactosidases have an optimum temperature activity at 45, 55, 45, and 55°C, respectively. As calculated using Lineweaver-Burk plots, the Km were about 3.96 ± 0.005, 1.927 ± 0.04, 1.051 ± 0.07, 0.558 ± 0.02 mM for α-/β-Glucosidases and α-/β-Galactosidase, respectively. Fe^{2+}, Hg^{+}, Zn^{2+}, and EDTA inhibited carbohydrases activity, whereas the addition of Mg^{2+} and K^{+} increased enzyme activity. The zymogram pattern in the native gel revealed that α-/β-Glucosidases and α-/β-Galactosidases in the digestive system showed 3, 2, 1 and 1 major bands, respectively. Biochemical characterization of digestive carbohydrases is essential for achieving new and safe methods for pest control.

Keywords *Dociostaurus maroccanus*; carbohydrases; α/β-Glucosidases; α/β-Galactosidases; digestive system.

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1 Introduction

The Moroccan locust, *Dociostaurus maroccanus* (Thunberg) (Orthoptera: Acrididae) is a polyphagous pest with migratory capability. This pest is distributed throughout Europe, Africa, Central Asia, the Middle East, and north-eastern Iran. Crop damage by *D. maroccanus* has been reported in more than 25 countries. This insect is a serious pest in wheat, barley, millet, rice, and maize grain crops and attack the leaves, stalks and grain of legumes (peas, beans, lentil, alfalfa, clover) (Rafiei et al., 2016).

Although the use of chemical pesticides is the most decisive way of reducing damages of many agricultural pests, their side effects on humans, the environment, increase of pest' resistance and residual effects of pesticides led to considering alternative to control pests (Isman, 2006; Zhang and Zhang, 2019, 2020).

The use of transgenic plants and genetic modification of important crops by insecticidal proteins are considered as one of modern methods of pest control that genes expressing insect digestive enzymes' inhibitors will be transferred to the plant and the mentioned genes' expression makes the plant resistant to pests. As a result, during insect feeding the inhibitors enter the lumen of the gut and cause blocked digestive enzymes and disordered food digestion. The first step in the production of transgenic plants with protein inhibiting digestive enzymes is biochemical study of these enzymes in the gut of pest insect (Sharma et al., 2000; Oppert, 2000).

Carbohydrates are one of the key molecules in insects to produce energy from food sources available. These molecules should be digested in the digestive system to be absorbed through the intestinal epithelial cells. Enzymes that are involved in digestion of carbohydrates are called carbohydras. The main role of these enzymes is breaking glycoside bonds that would cause monosaccharaides' connection to each other. Glucosidases and Galactosidases are a group of carbohydrases in insects' digest that are involved in the final step of digestion of carbohydrates (Hemming et al., 2000; Ghadamyari et al., 2010).

Glucosidases and Galactosidases are classified as exo or endo acting polysaccharides. Hemicelluloses and cellulose are hydrolyzed by insects' digestive glucosidases to di and oligosaccharides. α-/β-Glucosidases are not important in digestion; they play a fundamental role in deactivating plant secondary metabolites. Glucosidases are involved in insect-host plant interactions (Ferreira et al., 1998; Moller and Svensson, 2016).

α-Glucosidase (EC 3.2.1.3), known as α-D-glucoside glucohydrolase, comprise exo-acting glycoside that can catalyze the releases of α-D-glucose from terminal 1, 4-linked alpha-D-glucose residues. β-Glucosidases (3.2.1.21) catalyzes the hydrolyzes β 1–4 linkages between two glucoses or glucose-substituted molecules. β-Glucosidase (EC 3.2.1.21), can degrade cellobiose and cello-oligosaccharides to glucose in insects.Considering that about 33% of the plant matter is cellulose, which represents the most prevalent substrate on the Earth, it is obvious why a study of β_glucosidasein herbivorous species is important (Ferreira et al., 2010; Vlahović et al., 2020).

α-Galactosidases (EC 3.2.1.22) are exo-acting glycoside hydrolases that cleave α-linked galactose residues from carbohydrates such as melibiose, raffinose, stachyose, and gluco- or galactomannans β-Galactosidase (EC 3.2.1.23) is a hydrolase enzyme that catalyses the hydrolysis of β-galactosides into monosaccharides (Riseh et al., 2012; Sezgintürk and Dinçkaya, 2008).

α-/β-Glucosidasess and α-/β-Galactosidases can be found in the alimentary canal, salivary secretions of insects, haemolymph and hypopharyngeal glands of some insects (Ramzi and Hossininaveh, 2010; Ghadamyari et al., 2010; Asadi et al., 2010; Sharifi et al., 2011; Ricks and Bradling, 2011; Riseh et al., 2012). But no study has been conducted on *D. maroccanus* physiology and digestive enzymes yet; hence knowledge of its digestive enzymes is essential for using new techniques of pest control, especially molecular techniques of genetic engineering (transgenic plants). For this reason, in this study biochemical properties of α-/β-Glucosidasess and α-/β-Galactosidases in Moroccan locust digestive system were investigated. It is hoped that the findings of this study are useful in order to achieve new strategies to control pest.

2 Materials and Methods

2.1 Location and insects

The insects were collected from the cultivated farmlands in Golestan province of Iran (Coordinates: 37°00′50″N 54°27′18″E) and transferred to the laboratory of entomology at the University of Giulan. The last instar nymphs of were used for measuring the enzyme activity.

2.2 Chemicals

p-Nitrophenol and bovine serum albumin were purchased from Merck (Darmstadt, Germany). p-Nitrophenyl-α-d-glucopyranoside (pNαG), p-nitrophenyl-β-d-glucopyranoside (pNβG), p-nitrophenyl-α-d-galactopyranoside (pNαGa), p-nitrophenyl-β-d-galactopyranoside (pNβGa), 4-methylumbelliferyl-β-d-glucopyranoside (4-MUβG), 4-methylumbelliferyl-α-d-glucopyranoside (4-MUαG), 4-methylumbelliferyl-β-d-galactopyranoside (4-MUβGa) and 4-methyl-umbelliferyl-β-d-galactopyranoside (4-MUαGa) were obtained from Sigma (St. Louis, USA).

2.3 Sample preparation

The synchronized adults were randomly selected and immobilized on ice and dissected under light microscope in ice-cold saline buffer. The Digestive systems removed and cleaned from adhering unwanted tissues. Then, the guts were transferred to a freezer (-20°C). For measuring enzyme activity, the samples were homogenized in cold double-distilled water and/or buffer using a hand-held glass homogenizer and centrifuged at 16000×g for 15 min at 4 °C.

2.4 Determination of α-/β-glucosidases and α-/β-galactosidase activity

α-/β-Glucosidasess and α-/β-Galactosidases activities were measured using pNaG, pNbG, pNαGa and pNβGa as the substrates, respectively. The reaction was performed at 35°C using 10 µl homogenates, 45 µl substrate (25 mM) and 115 µl of 40 mM glycinephosphate- acetic-citric buffer. After 20 min incubation, the reaction was stopped by addition of 600 µl of 0.25 M NaOH. Optical density was measured at 405 nm using a microplate reader (Stat Fax 3200, Awareness Technology, USA). In the control tubes, the enzyme added to the reaction mixture after addition of NaOH for 30 min. The reaction was stopped by addition of 50 µL of NaOH (1 M). 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. The specific activity was expressed as µmoles of nitrophenol per minute per mg of protein. A standard curve of absorbance against the amount of p-nitrophenol released was constructed to enable the calculation of the amount of p-nitrophenol released during the -/β-Glucosidasess and α-/β-Galactosidases assays.

2.5 Determination of ph optimum and effect of temperature on α-/β-glucosidases and α-/β-galactosidase activity

The activity of α-/β-Glucosidasess and α-/β-Galactosidases towards pNαG, pNsG, pNαGa and pNsGa was determined at several pH (2–12) values using 40mM glycine-phosphate-acetic-citric buffer.

The effect of temperature on enzymes activities were measured using the homogenate by incubating the reaction mixture at 20, 30, 40, 50, 60 and 70^{o}C for 30 min, followed by measurement of activity.

2.6 Polyacrylamide gel electrophoresis and zymogram analysis

Non-denaturing polyacrylamide gel electrophoresis (PAGE) was performed according to the method presented by Davis (1964) modified by Riseh et al. (2012) for zymogram of α-/β-Glucosidasess and α-/β-Galactosidases. The samples were mixed with sample buffer and loaded to non-denaturing polyacrylamide gel (10%). Electrophoresis was carried out in 100 V at 4°C. After the electrophoresis, the gel was submerged in 3 mM 4-MUβG, 4-MUαG, 4-MUβGa and 4-MUαGa in 0.1M sodium acetate (pH 5.0) for 15 min at room temperature to develop fluorescents bands corresponding to α-/β-Glucosidasess and α-/β-Galactosidases activities, respectively. The blue-fluorescent bands were appeared under UV and photographed with gel documentation

apparatus.

2.7 Kinetic parameters

The Michaelis-Menten constant (Km) and maximal velocity (Vmax) of α-/β-Glucosidasess and α-/β-Galactosidases were estimated by Lineweaver-Burk plots. Homogenates were incubated in 40 mM phosphate, glycine and acetate buffer (optimum pH for each enzyme) at 35°C with different concentrations of pNαG, pNβG, pNαGa and pNβGa over the range of from 5 to 80 mM. The kinetic values are the averages of three experiments.

2.8 Effect of various metal ions and chemicals on enzymes activities

Enzyme assays were determined in the presence of chloride salts of Zn^{2+}, Mg^{2+}, Ca^{2+}, K^{+}, Co^{2+}, Ba^{2+}, Fe^{2+}, Mn^{2+}, Hg^{2+}, Hg^{+} and Ethylenediaminetetraacetic acid (EDTA) (10 mM) for α-/β-Glucosidasess and α-/β-Galactosidases. The activities of enzymes were investigated by adding ions in glycinephosphate-acetate mixed buffer (optimum pH), and activity was measured after 20 min pre-incubation. All experiments were performed in three replicates, and for all of them, appropriate blanks were run.

2.9 Protein concentration

Protein concentrations were estimated by the Bradford Method (1976), using bovine serum albumin as standard.

2.10 Data analysis

The data were compared by one-way analysis of variance (ANOVA) followed by Tukey's test when significant differences were found at $p = 0.05$ using the SAS program, Version 8.0.

3 Results

3.1 α-/β -glucosidase and α-/β -galactosidase activities

The activities of α-/β-Glucosidases and α-/β-Galactosidases measured in the adult digestive system of *D. maroccanus* and the results are shown in Table 1. The α-galactosidase activity in gut of Moroccan locust was higher than other enzymes assayed.

Table 1 The specific activities (μmol min^{-1} mg^{-1} protein) in the digestive system of *D. maroccanus*.

Enzyme	Activity (μmol/min/mg protein)
α-Glucosidase	15.68 ± 0.04
β-Glucosidase	10.68 ± 0.06
α-Galactosidase	3.53 ± 0.03
β-Galactosidase	1.98 ± 0.05

3.2 Effects of pH and temperature on α-/β -glucosidase and α-/β -galactosidase activities

The optimal pH for α-Glucosidase activity was pH 4.0. Maximum activities of on β-Glucosidasess and α-/β-Galactosidases in the digestive system of *D. maroccanus* were observed at pH 5.0 (Fig. 1). Moroccan locust α-Glucosidase and α-Galactosidase have an optimum temperature activity at 45°C. Also, the optimal temperature for β-Glucosidase and β-Galactosidase were 55°C (Fig. 2).

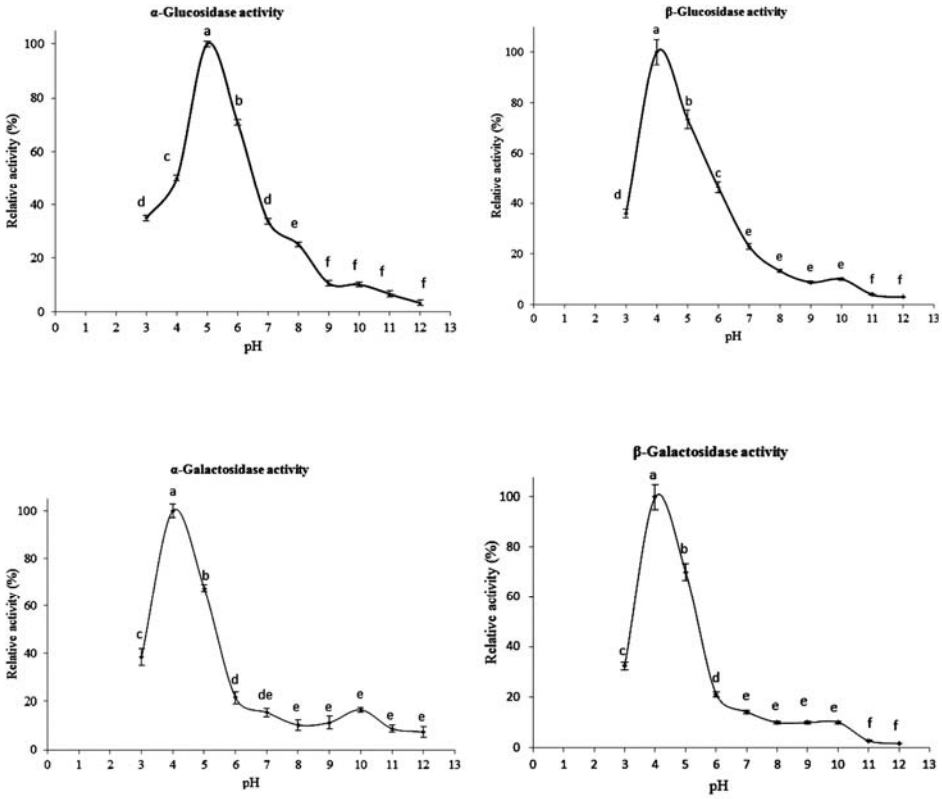

Fig. 1 The effect of pH on α-/β-Glucosidases activities from the digestive system of *D. maroccanus*. Different letters indicate that the activity of enzymes is significantly different from each other by Tukey's test ($p < 0.05$).

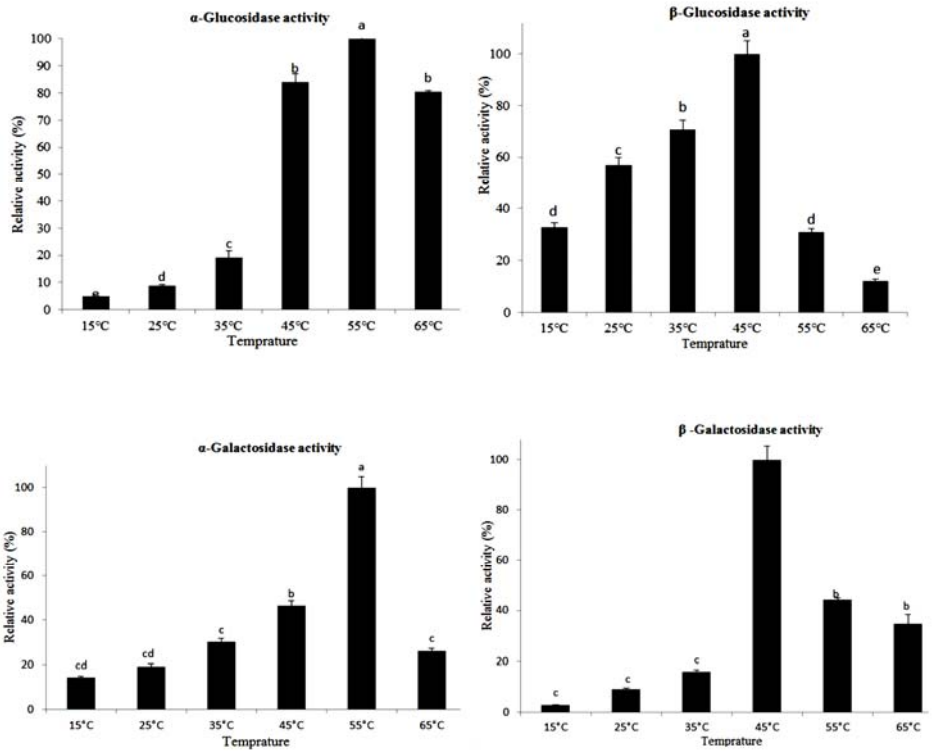

Fig. 2 The effect of temperatures on the mean relative activities of -/β-Glucosidases and α-/β-Galactosidases from the digestive system of *D. maroccanus*. Different letters indicate that the activity of enzymes is significantly different from each other by Tukey's test ($p < 0.05$).

3.3 Effects of metal ions and EDTA on the α-/β-glucosidases and α-/β-galactosidases activities

Effect of metal ions on the activities of *D. maroccanus* α-/β-Glucosidases and α-/β-Galactosidases observed that Mg^{2+} and K^{+} increased activities and Ca^{2+} increased β-Glucosidase activities. In the presence of Fe^{2+}, strongly inhibited the α-Glucosidase and Hg^{+} more than 96% inactivation has been observed for β-Glucosidas. Cu^{2+} significantly decreased β-Galactosidase activity. Activities of α-/β-Glucosidases and α-/β-Galactosidases were inhibited by other compounds (Table 2).

Table 2 Effect of various metal ions and EDTA (10 mM) on relative *D. maroccanus* α-/β-Glucosidases and α-/β-Galactosidases activities。

	Relative activity (%) (mean ± SE)			
Compound	α-Glucosidase	β-Glucosidases	α-Galactosidase	β-Galactosidase
Control	100 c	100 b	100 d	100 b
EDTA	56.50 ± 0.4 e	64.50 ± 0.6 e	38.66 ± 0.2 h	87.65 ± 0.8 d
Hg^{2+}	67.91 ± 0.2 e	93.25 ± 0.7 c	99.73 ± 0.6 d	75.32±0.1 e
Mn^{2+}	39.20 ± 0.3 f	62.04 ± 0.1 d	42.81 ± 0.7 gh	79.09 ± 0.3 e
K^{+}	131.80 ± 0.2 b	108.75 ± 0.2 b	122.56 ± 0.2 b	103.45 ± 0.3 b
Fe^{2+}	4.80 ± 0.7 h	37.89 ± 0.3 e	62.09 ± 0.5 g	65.06 ± 0.8 e
Hg^{+}	11.25 ± 0.9 g	3.95 ± 0.8 g	19.54 ± 0.3 i	15.67 ± 0.1 g
Co^{2+}	30.54 ± 0.6 f	77.40 ± 0.3 cd	93.62 ± 0.5 de	88.00 ± 0.5 d
Ba^{+}	59.50 ± 0.5 e	75.19 ± 0.9 c	110.67 ± 0.2 c	105.06 ± 0.2 b
Zn^{2+}	53.90 ± 0.8 e	79.81 ± 0.5 c	64.05 ± 0.4 g	76.04 ± 0.1 e
Mg^{2+}	142.8 ± 0.1 a	128.93 ± 0.7 a	198.02 ±0.2 a	113.02 ± 0.6 a
Ca^{2+}	91.50 ± 0.5 d	130.97 ± 0.2 a	81.21 ± 0.4 f	94.45 ± 0.7 c
Cu^{2+}	69.70 ± 0.2 e	13.11 ± 0.5 f	59.20 ± 0.2 g	46.03 ± 0.8 f

3.4 Kinetic parameters

The Km and Vmax values were estimated for the α-/β-Glucosidases and α-/β-Galactosidases as 3.96 ± 0.005, 1.927 ± 0.04, 1.051 ± 0.07, 0.558 ± 0.02 mM and 0.786 ± 0.02, 0.143 ± 0.03, 2.045 ± 0.06, 0.99 ± 0.01 mmol/min/mg protein, respectively (Table 3).

Table 3 Kinetic parameters of alimentary canal α-/β-Glucosidases and α-/β-Galactosidases from *D. maroccanus*.

	Kinetic parameters	
Enzyme	Km (mg/ml)	Vmax (mmol/min/mg protein)
α-Glucosidase	0.786 ± 0.02	3.96 ± 0.005
β-Glucosidases	0.143 ± 0.03	1.927 ± 0.04
α-Galactosidase	2.045 ± 0.06	1.051 ± 0.07
β-Galactosidase	0.786 ± 0.02	0.558 ± 0.02

3.5 Zymogram

The crude extracts of *D. maroccanus* were analyzed by native PAGE. Enzymes activity staining, 3, 2, 1, and 1 isoforms of α-/β-Glucosidases and α-/β Galactosidase in the digestive system of *D. maroccanus* were clearly detected, respectively (Fig. 3).

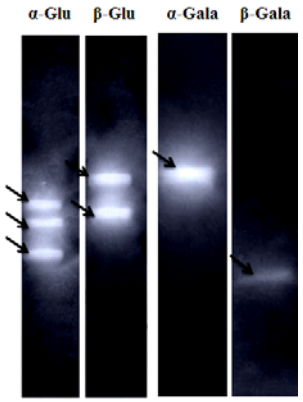

Fig. 3 Zymogram α-/β-Glucosidases (α-Glu, β-Glu) and α-/β-Galactosidases (α-Gala, β-Gala) extracted from the digestive system of *D. maroccanus*.

4 Discussion

Insect pests cause economical losses to the world's commercially important agricultural crops. Recently, various attempts have been made to control the losses caused by pest through the development of chemical pesticides. The intensive use of pesticides has led to problems like bioaccumulation in food chains, environmental degradation and development of resistance among insects against various insecticides (Kaur et al., 2018). The digestive system of insects is an excellent target for control pest that in general are not toxic to other organisms. Therefore, understand the function and physiology of digestive enzymes of insects is essential for developing alternative methods of insect control (Mohammadzadeh and Izadi, 2016; Rafiei et al., 2018). Vlahović et al. (2020) showed that understanding the activity of glucosidase explain the survival strategy of highly adapted organisms, as well as insect pest management strategies.

α-/β- Glucosidases and α-/β- Galactosidases activities were detected in the alimentary canal adult of Moroccan locust using pNαG, pNβG, pNαGa and pNβGa as substrates (Table 1). The results showed that α-Glucosidase activity was the highest among the carbohydrases tested in digestive system of *D. maroccanus*. Ferreira et al. (1999) showed that high s-glucosidase activity is found in the foliage feeder, *Abracris flavolineata* (Orth.: Acrididae). Same results were obtained on *Rhynchophorus ferrugineus* (Col.: Curculionide) (Riseh et al., 2012), *Choreutis nemorana* (Lep.: Choreutidae) (Chitgar et al., 2014), *Acrosternum arabicum* (Hem.: Pentatomidae) (Mohammadzadeh and Izadi, 2016), *Tuta absoluta* (Lep.: Gelechiidae) (Sadeghinasab et al., 2017), *Bombyx mori* (Lep.: Bombycidae) (Miyazaki and Park, 2020). In contrast to our results, β-galactosidase activity in digestive system of *Xanthogaleruca luteola* (Col.: Chrysomelidae) (Sharifi et al., 2011), *Osphranteria coerulescens* (Col.: Cerambycidae) (Aghaali et al., 2012) was higher than that of other Glucosidases and Galactosidases.

Maximum activity of α-/β-Glucosidases and α-/β-Galactosidases in the digestive system of *D. maroccanus* obtained at pH 5.0, 4.0, 4.0, and 4.0, respectively (Fig. 1). In *A. flavolineata* (Orth.: Acrididae) midguts, α-Galactosidases have maximum activity at pH 5.4 (Ferreira et al., 1999). Sharifi et al. (2011) reported that the optimal pH for α-Glucosidases and β-Galactosidase activities extracted from midgut of *X. luteola* were 5 and 4, respectively. The pH 6.0 and 5.0 were observed to be the optimum activity for α-glucosidase and α-

galactosidase in midgut of *T. absoluta*, the β-glucosidase and β-galactosidase activity were shown to have the maximum activity at pH 7.0 and 5.0, respectively (Sadeghinasab et al., 2017).

The optimum pH for purified β-Glucosidases from the digestive tract of cockroach, *Periplaneta americana* was 3.6. This variation in optimal pH between insect species may refer to their phylogenetic relations or may be in response to different diets (Nakonieczny et al., 2006; Asadi et al., 2012).

The highest activity of α-Glucosidase and α-Galactosidase at various temperatures were determined at 45°C. Maximum β-Glucosidase and β-Galactosidase activities were found at temperature 55°C, abruptly declines with further increase of temperature due to denaturation of α-/β-Glucosidases and α-/β-Galactosidases at temperatures above 45°C and 55°C. The obtained results correspond to the optimal temperature of β-Glucosidase activities in *G. pyloalis* (Ghadamyari et al., 2010). α-/β-Glucosidases of B. germari has an optimal activity in 30°C (Ramzi and Hosseininaveh, 2010).

Each enzyme has specific optimum activity temperatures and pH conditions; changes in these values can cause denaturation and loss of tertiary structure and produce a decrease in activity.

Insect α-/β-Glucosidases and α-/β-Galactosidases are activated or inhibited by chemicals and ions. Also, some endophytic fungi can be inhibited alpha glucosidase activity of insects (Kaur et al., 2018). The results of the current study showed that Fe^{2+} and Hg^{+} significantly decreased enzymes activity from the digestive system of *D. maroccanus* (Table 2). In the presence of EDTA, enzymes activities significantly decreased, confirming presence of a metal ion in active site of the enzyme. EDTA is a general chelating agent that removes all metal ions from active sites of the enzyme.

The activity of α-/β-Glucosidases *Stromatium Fulvum* (Col.: Cerambycidae) (Zibaee et al., 2013), *N. aenescens* (Lep.: Noctuidae) (Asadi et al., 2010), and *Arge rosae* (Hym.: Argidae) (Chitgar et al., 2013) all inhibited with the addition of EDTA to the assay mixture.

Kinetic parameters of the α-/β-Glucosidases and α-/β-Galactosidases in digestive system of *D. maroccanus* were estimated (Table 3). Most insect α-/β-Glucosidases show Km values in the range 0.24–3 mM (Ferreira and Terra, 1993). Research on substrate specificities of β- glycosidases in digestive system of insect orders observed the evolutionary trend from multiple enzymes with different substrate specificities to a single enzyme that is able to hydrolyse all the β-glycosides within the same site (Ferreira et al., 1998; Pothula et al., 2019).

The activity of α-/β-Glucosidases and α-/β-Galactosidases of Moroccan locust was also characterized by activity staining after native PAGE which allowed visualization of the blue fluorescent bands under UV. The zymogram pattern showed that α-/β-Glucosidases and α-/β-Galactosidases had 3, 2, 1 and 1 isoforms, respectively (Fig. 3). *A. flavolineata* (Orthoptera: Acrididae) midguts, there are two α-galactosidases (Ferreira et al., 1999). Riseh et al. 2012 showed the presence of at least 2, 3, 1 and 1 major isoforms for α-/β-Glucosidases and α-/β-Galactosidases in the digestive system of *R. ferrugineus*, respectively.

Glucosidases and Galactosidases play an important role in digestion of carbohydrates used by insects. In addition, Glucosidases play a vital role in insect gut by detoxification or deactivating of plant secondary metabolites, which are produced to cope with pest. Biochemical detection of these enzymes digestive system of *D. maroccanus* enhances the knowledge to design control methods and plant pest management using proteins inhibiting these enzymes and producing GM crops resistant to this pest potentially and provides new ways in order to control pest.

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