BIOCHEMICAL CHARACTERIZATION OF GLUCOSIDASES AND GALACTOSIDASES FROM ROSACEAE BRANCH BORER, *OSPHRANTERIA COERULESCENS* REDT. (COL.: CERAMBYCIDAE)

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α-/β-glucosidase and α-/β-galactosidase are important enzymes in the carbohydrate digestion of *Osphranteria coerulescens* Redt. They reached the highest activity at acidic pH. The optimal temperatures for α-/β-glucosidase and α-/β-galactosidase activities correspond to 50°C, 50–60°C, 60°C and 40°C, respectively. Zymogram analysis of α-/β-glucosidase and α-/β-galactosidase activities in the gut of *O. coerulescens* showed that these activities correspond to 3, 4, 1 and 6 major proteins from this insect digestive system, respectively. Among the tested glucosidase and galactosidase activities, β-galactosidase had the highest specific activity in the *O. coerulescens* digestive system. Therefore, β-galactosidase could play an important role in the carbohydrate digestion of this organism, representing an excellent candidate for designing transgenic plants containing β-galactosidase inhibitors as a new control method.

Key words: *Osphranteria coerulescens;* α-/β-glucosidases; α-/β-galactosidases; digestive system.

INTRODUCTION

Xylophagous insects feed on cellulose, this polysaccharide representing their major food source. These insects obtain energy/nutriments for growth, development and reproduction *via* cellulolytic enzyme, which provide advantages to the insects by increasing available absorbable material. Many kinds of enzymes, such as α-glucosidase (EC 3.2.1.20), β-glucosidase (EC 3.2.1.21), α-galactosidase (EC 3.2.1.22) and β-galactosidase (EC 3.2.1.23), are responsible for cellulose digestion. Glucosidases and galactosidases can be classified as exo- or endo-acting polysaccharides. α-glucosidases, known as α-D-glucoside glucohydrolase, comprise exo-acting glycoside hydrolases with broad specificity on substrates in

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insects, releasing α-D-glucose from the non-reducing end of α-linked polysaccharides. These enzymes are reported in bacteria, fungi, plants, animals and insects (1-3). So far, biochemical characteristics of α-glucosidase have been studied in the digestive system and salivary glands of some insect species belonging to different orders, such as *Glyphodes pyloalis* Walker (Lep.: Pyralidae), *Apis mellifera* L. (Hymenoptera: Apidae), *Drosophila melanogaster* Meigen *(*Diptera: Drosophilidae), *Xanthogaleruca luteola* Mull. (Col.: Chrysomelidae) and *Rhynchophorus ferrugineus* Olivieri (Col.: Curculionide) (2-6). β-glucosidase can degrade cellobiose and cello-oligosaccharides to glucose in insects. Hemicelluloses and cellulose present in insect food are converted to di- and oligo-β-saccharides by some carbohydrases and digestive β-glucosidases that play an important role in their hydrolysis (7). Also, several investigations indicate the main role of β-glucosidases in insect-host plant interaction (8-11). Considering this interaction, β-glucosidases extracted and characterized from the digestive system and salivary glands of many insect species belong to different orders (12, 2, 13, 3, 14, 5, 15).

α-galactosidase, also known as α-D-galactoside galactohydrolase, plays an important role in the carbohydrate hydrolysis in some insects. So far, galactosidases from plant, humans and bacteria have been extensively characterized (16-18). However, little attention has been paid to galactosidases from insects. α-D-galactosidases hydrolyze the α- linked galactose from several carbohydrates including melibiose, raffinose, stachyose, and gluco- or galactomannans (19). α-D-galactosidases are classified as exo-acting glycoside hydrolases, while ß-D-galactosidases catalyze the hydrolysis of β-galactosides to various monosaccharides (20). Compared to glucosidases, little is known on the biochemistry of galactosidases in the digestive system of insect. So far, α- and ß-galactosidases from some insects have been studied, and biochemical properties of these enzymes have been described (3, 5).

Osphranteria coerulescens inaurata Holzschuh is widely distributed in Iran and has been reported from Khorasan, Kerman, Qazwin, Fars, Tehran and Yazd and Alborz provinces as a serious pest of *Rosa* spp., peach and apricot orchards. Larvae of this pest feed within the living branches of various fruit and ornamental trees belonging to the rose family (*Rosaceae*), and can cause significantly economic damage to fruit trees when too abundant (21). There is relatively little information about the control of rosaceae branch borer in orchards, and the control of this pest is generally difficult due to the location of larvae in twigs. Once the larvae enter to twigs and branches, chemical control would be difficult even with systemic and semi-systemic insecticides. The transgenic plants expressing carbohydrases inhibitors are one of the safe and practical methods for controlling this pest, and the study of the physiology and digestive biochemistry of the insect midgut is necessary for achieving this goal. Glucosidases and galactosidases play a

main role in hydrolysing plant secondary metabolites, such as glucosinolate and host plant-herbivorous interaction (22, 23).

Therefore, the purpose of the present study is to characterize α-/β-glucosidase and α-/β-galactosidase from the rosaceae branch borer *O. coerulescens*, as a model for xylophagous insects.

MATERIAL AND METHODS

INSECT

O. coerulescens was collected from *Rosa* sp. in the national botanic park, Research Institute of Forests and Rangelands, Karaj, Iran. The last larval instar was used for measuring the enzyme activity.

CHEMICALS

P-nitrophenol and bovine serum albumin were purchased from Merck (Merck, Darmstadt, Germany). P-nitrophenyl-α-D-glucopyranoside (pNαG), p-nitrophenyl-ß-D-glucopyranoside (pNßG), P-nitrophenyl-α-D-galactopyranoside (pNαGa), p-nitrophenyl-ß-D- galactopyranoside (pNßGa), 4-methylumbelliferyl-ß-D-glucopyranoside (4-MUßG), 4-methylumbelliferyl-α-D-glucopyranoside (4-MUαG), 4-methylumbelliferyl-ß-D-galactopyranoside (4-MUßGa) and 4-methylumbelliferyl-ß-D-galactopyranoside (4-MUαGa) were obtained from Sigma (Sigma, St Louis, MO, USA).

SAMPLE PREPARATION

Larvae were dissected under a stereo microscope in ice-cold saline buffer. Digestive systems were removed and content (the malpighian tube and the fat bodies) was eliminated. The digestive system was divided into three distinct divisions, M1, M2 and M3 (Fig. 1). The samples were frozen at –20°C and further homogenized in cold double-distilled water using a hand-held glass homogenizer, then centrifuged at 11,000 rpm for 10 min at 4°C.

α-/β-GLUCOSIDASE AND α-/β-GALACTOSIDASE ASSAYS

α-/β-glucosidases and α-/β-galactosidases were assayed using the colorimetric method described by Ghadamyari *et al*. (2010), in the presence of pNαG, pNβG, pNαGa and pNβGa as substrate, respectively. The reaction was performed at 35°C

using 10 µl enzyme, 45 µl substrate (25 mM) and 115 µl 40 mM glycine-phosphate-acetic-citric buffer. After 20 min incubation, the reaction was terminated by adding 600 µl of 0.25 M NaOH. The absorbance of the resulting p-nitrophenol derivate was measured at 410 nm using microplate reader (Stat Fax 3200, Awareness Technology, USA), and the specific activity was expressed as µmoles of nitrophenol *per* minute *per* mg of protein. Controls without enzyme or without substrate were used in the experiment. A standard curve monitoring the absorbance of different concentrations of p-nitrophenol was used for calculating the specific activity.

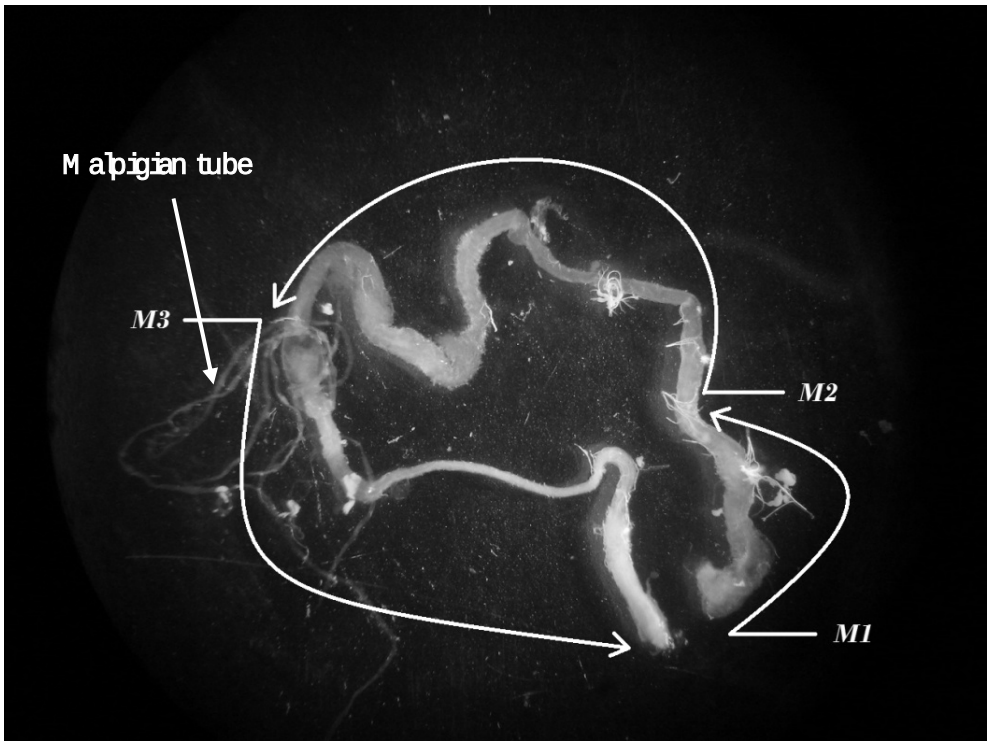


Fig. 1. – Digestive system from the larvae of *Osphranteria coerulescens.* M1, M2, M3: divisions of the digestive system.

EFFECT OF pH AND TEMPERATURE ON α-/β-GLUCOSIDASE AND α-/β-GALACTOSIDASE ACTIVITIES

The activity of α-/β-glucosidases and α-/β-galactosidases was determined at several pH values using the universal 40 mM glycine-phosphate-acetic-citric buffer. The effect of temperature on α-/β-glucosidase and α-/β-galactosidase activity was determined using the homogenate larvae. The reaction was carried on by

incubating the reaction mixture at 20, 30, 40, 50, 60 and 70°C for 30 min, and the activity was measured as previously described.

PROTEIN CONCENTRATION

Protein concentration was measured by the Bradford method (24), using bovine serum albumin as standard.

POLYACRYLAMIDE GEL ELECTROPHORESIS AND ZYMOGRAM ANALYSIS

In the zymogram analysis, non-denaturing polyacrylamide gel electrophoresis (PAGE) was carried out based on the method of Davis (1964). Briefly, the samples were mixed with sample buffer and applied onto a 10% non-denaturing polyacrylamide gel. Electrophoresis was performed at constant voltage (100 V) and 4°C. The gel was incubated in 3 mM 4-MUαG, 4-MUßG, 4-MUαGa and 4-MUßGa prepared in 0.1 M sodium acetate (pH 5.0) for 10 min at room temperature to develop colored bands indicating the α-/β-gluctosidase and α-/β-galactosidase activity, respectively. The blue-fluorescent bands developed in few minutes under UV exposure.

STATISTICAL ANALYSIS

The data were compared by one-way analysis of variance (ANOVA), followed by Tukey's test using the SAS program (25).

RESULTS

α-/β-GLUCOSIDASE AND α-/β-GALACTOSIDASE ACTIVITIES

The midgut of *O. coerulescens* was divided into three main sections, M_1, M_2, M_3 (Fig. 1) and the α-glucosidase specific activity of each of this section was measured for the last larval instar, as indicated in the Material and Methods section. Under these conditions, the corresponding specific activities were 4.55±0.06 $\mu mol.min^{-1}mg^{-1}$ for M1, 0.51±0.04 $\mu mol.min^{-1}mg^{-1}$ for M2, and 0.33±0.01 $\mu mol.min^{-1}mg^{-1}$ for M3.

The specific activity of α-/β-glucosidase and α-/β-galactosidase measured in these three sections of last larval instar (Table 1) indicate a higher activity in M1 than that determined in M2 and M3. In particular, β-galactosidase activity in M1 was 2.32-fold and 28.56-fold higher than in M2 and M3 of last larval instar, respectively (Table 1).

Table 1

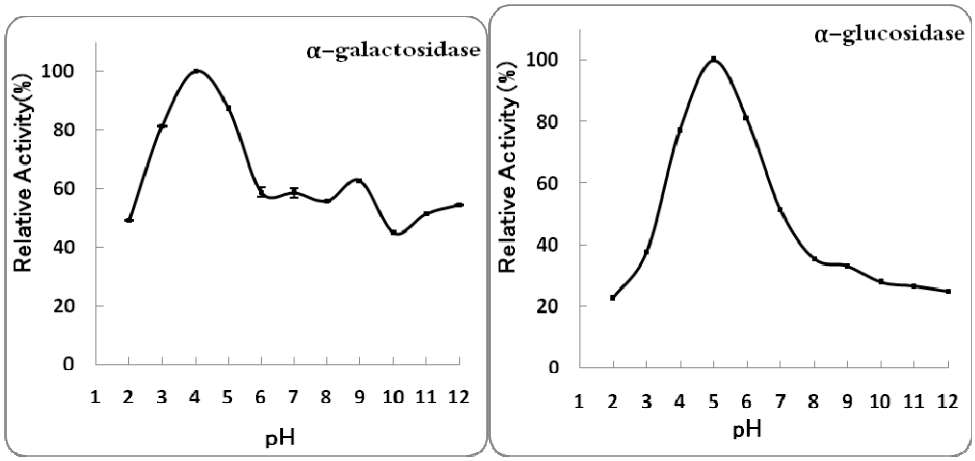
The specific activities of α-/β-glucosidases and α-/β-galactosidases in the digestive system of last larval instar of *Osphranteria coerulescens*

Enzyme	Specific activity (µmol/min/mg protein)			
	Digestive system	**M1**	**M2**	**M3**
α-gluctosidase	0.853±0.01[c*]	4.55±0.06	0.51±0.04	0.33±0.01
β-gluctosidase	9.185±0.08[b]	4.04±0.08	2.31±0.022	0.68±0.01
α-galactosidase	0.285±0.007[d]	0.338±0.003	0.234±0.014	0.119±0.027
β-galactosidase	35.39±0.2[a]	36.13±0.1	15.55±0.69	1.26±0.14

* Different letters (a-d) indicate that the specific activity of enzymes is significantly different from each other by Tukey's test ($p < 0.05$).

EFFECT OF pH AND TEMPERATURE

The effect of pH on α-/β-glucosidase and α-/β-galactosidase hydrolytic activity was tested using 40 mM phosphate-acetic-citric buffer (pH 2–12). The maximum activity in *O. coerulescens* digestive system was observed at pH 4 in the cases of β-glucosidase and α-/β-galactosidase. Also, the optimal pH for α-glucosidase activity was obtained as pH 5 (Fig. 2). The *O. coerulescens* α- and ß-glucosidase activities reached their optimum at 50°C and 60°C, respectively. Also, the optimal temperature for α-/ß-galactosidase in this organism was 60°C and 40°C, respectively (Fig. 3).



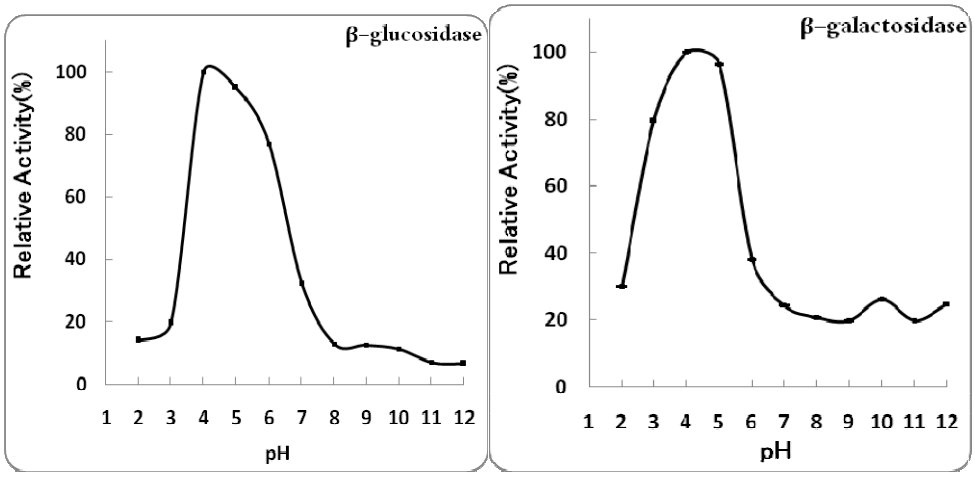


Fig. 2. – Effect of pH on α-/β-glucosidase and α-/β-galactosidase activities from the digestive system of *Osphranteria coerulescens.* All data were expressed as relative activity, where 100% represents the maximum activity for each enzyme.

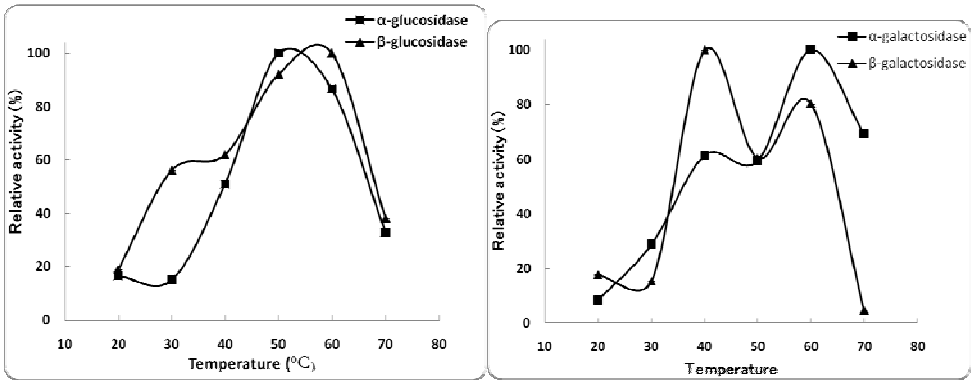


Fig. 3. – Effect of temperature on α-/β-glucosidase and α-/β-galactosidase activities of the digestive system of *Osphranteria coerulescens.* All data were expressed as relative activity, where 100% represents the maximum activity for each enzyme.

ZYMOGRAM ANALYSIS

α-/β-glucosidase and α-/β-galactosidase activity of *O. coerulescens* was analyzed by visualizing it in native PAGE. After colorimetric staining (see Methods), α-/β-glucosidase and α-/β-galactosidase activities indicate the presence of 3, 4, 1 and 6 isoforms of α-/ß-glucosidases and α-/β-galactosidases, respectively, in the digestive system of *O. coerulescens* (Fig. 4). However, the intensity of β-galactosidase bands was higher than other carbohydrases, indicating a higher activity as compared to α-/ß-glucosidases and α-galactosidase in this organism.

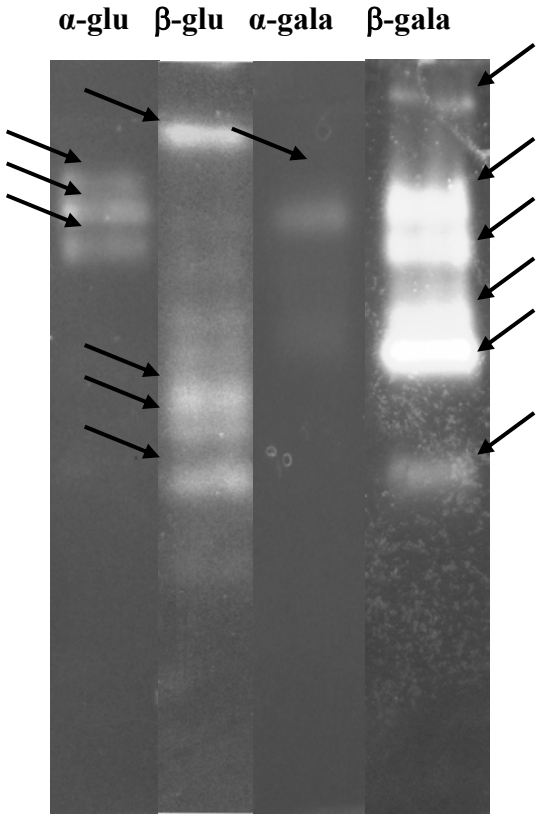


Fig. 4. – Zymogram of α-/β-glucosidase and α-/β-galactosidase activities from the digestive system of last larval instar of *Osphranteria coerulescens*.

DISCUSSION

The present study clearly demonstrated that the larvae of *O. coerulescens* use α-/β-glucosidase and α-/β-galactosidase for polysaccharide digestion. It seems that these enzymes play an important role in the wood and cellulose digestion in this pest. Among the carbohydrases tested, the β-galactosidase activity was the highest. In contrast to our results, Riseh *et al*. (2012) showed that, in the digestive system of *R. ferrugineus* larvae and female adult, the activity of α-glucosidase was higher than that of other carbohydrases. Also, our findings showed that the activity of α-galactosidase is relatively low in the larvae digestive system. The lowest and highest activity in this system corresponded to α-galactosidase and β-galactosidase, respectively. These results correlated well with the α-/β-glucosidase and α-/β-galactosidase specific activities in the digestive system of *X. luteola* last instar

larvae (5). Also, in the digestive system of *R. ferrugineus* female, the activities of α-glucosidase and β-galactosidase were higher than those of β-glucosidase and α-galactosidase (3).

The rank order of the carbohydrase activity, from the highest to the lowest, was β-galactosidase > β-glucosidase > α-glucosidase > α-galactosidase. One study showed that glucosidases activity was low in heteropteran insect, *i.e*., *Coreidae* and *Pentatomidae* (26). But β-glucosidases were active in the midgut and salivary glands of *Brachynema germari* (Hemiptera: Pentatomidae) (27) and in the midgut of *A. acuminate* (28).

In this study, the midgut of *O. coerulescens* was divided into three main sections (M_1-M_3) (Fig. 1). The activity of α-/β-glucosidase and α-/β-galactosidase in M1 was higher than in M2 and M3. Riseh *et al*. (2012) have also divided the digestive system of *R. ferrugineus* into three parts (V1-V3) and showed that there was a significant difference in the activity of α-/ß-glucosidases and β-galactosidases in V1, V2 and V3 of last larval instar, and the rank order of the β-galactosidase activity, from the highest to the lowest, was V1> V2 >V3, whereas the α-/β-glucosidase activities in V2 were much higher than in V1 and V3 (3). Sharifi *et al*. (2011) showed that α-galactosidase activity in the midgut of *X. luteola* was higher than that found in the foregut and hindgut of last larval instar. Also, in this pest, the β-galactosidase activity in the midgut was 2.12-fold and 3.71-fold higher than its activity in the foregut and hindgut of last larval instar, respectively. Nakashima *et al*. (2002) reported that the activities of ß-glucosidase in the midgut of *Odontotermes formosanus* and *Coptotermes formosanus* were higher than in the foregut and hindgut. These results indicate that the midgut of these two termites has also a cellulase secretion function (29). In fact, the ß-glucosidase secreted by midgut of *C. formosanus* may play an important role for the termite in digestion of cellulosic wood ingested (30).

The ratios of ß-glucosidase/ß-galactosidase were 0.25 for larvae of *O. coerulescens*, when the activities were measured in the whole digestive system, whereas the ratio of α-glucosidase/α-galactosidase was 3.0. The ratios of β -glucosidase/ β -galactosidase in the digestive system of last instar larvae of *R. ferrugineus* were 0.67, whereas for α-glucosidase/α-galactosidase, the reported ratio was 42.95. These results showed that the β-galactosidase and α-glucosidase activities in the digestive system of the *R. ferrugineus* larvae were higher than the β-glucosidase and α-galactosidase activities (3). The ratios of β-glucosidase/ β-galactosidase in other insects was reported as 88.5 in the case of fungus gnat, *Rhynchosciara americana* Wiedemann, Diptera: Sciaridae (31), 105 for the dog fly (32), 58 in the digestive system of triatomid bug*, Rhodnius prolixus* (33), and 2.5 in spotted cowpea bruchid (34). The reported ratios of α-glucosidase/α-galactosidase in the digestive system of *X. luteola* last larval instar were 3.5 (5).

The effect of pH on the enzymatic activity using pNαG, pNßG, pNαGa and pNßGa as substrates showed that β-glucosidase and α-/β-galactosidase had optimal

activities at pH 4. Also, the maximum activity for α-glucosidase was obtained at pH 5 (Fig. 2). In the digestive system of *X. luteola*, the optimum pH for α- glucosidase and α- galactosidase activities was reported at pH 5 and 4, respectively, whereas the optimum pH for β-glucosidase and β-galactosidase activities was 6 and 3, respectively (5). Similarly, Gatehouse and Anstee (1983) showed that α-glucosidase of *C. maculatus* reached the highest activity at pH 5.6. Overall, the studies on coleopteran insects indicated an optimal pH for α-glucosidase in slightly acid region. Highest activity for sucrose hydrolyzing *α*-glucosidase from the midgut of *Dermestes maculatus* adults was reported at pH 6.3 (35).

In this study, α-/β-glucosidase and α-/β-galactosidase activities of *O. coerulescens* were measured at different temperatures ranging from 20°C to 80°C. The optimal temperatures of α- and β-glucosidases were 50°C and 60°C and those for α- and β-galactosidases were 60°C and 40°C, respectively. The highest activity of α-/β-glucosidases and α-/β-galactosidases in *R. ferrugineus* at various temperatures were reported at 50, 50-60, 40-60 and 40°C, respectively (3), which is consistent with the values of α- and ß-glucosidases activities in *O. coerulescens.* The results of Ramzi and Hosseininaveh (2010) showed that α- and β-glucosidase of the midgut and salivary glands of *Brachynema germari* Kolenati (Hemiptera: Pentatomidae) had the highest activity at 30°C and 35°C, respectively. The *X. luteola* α- and β-glucosidases activities have an optimum temperature at 60 and 50°C, respectively. Also, the optimal temperature for α- and β-galactosidase in the digestive system of this pest was 60 and 40°C, respectively (5).

The activity of α-/β-glucosidases and α-/β-galactosidases of *O. coerulescens* was also surveyed by zymogram analysis. The results showed the presence of 3, 4, 1 and 6 major activity bands for α-/ β-glucosidases and α-/β-galactosidases in the larvae digestive system, respectively. In this experiment, the activity of β-galactosidase from the digestive tract of last larval instar was much higher than that of α- and β-glucosidases and α-galactosidase. Also, as shown in Fig. 4, the intensity of the bands indicating the α-galactosidase activity was less than that for α- & ß-glucosidases and ß-galactosidases. The zymogram pattern showed that β-glucosidase and β-galactosidase from the digestive system of *X. luteola* had three and one isoforms, respectively (5), and the activity of β-glucosidase was much higher than that of β-galactosidase. Also, glucosidases activity in native PAGE in the midgut and salivary glands extract of the pistachio red seed bug *Lygaeus pandurus* (Hemiptera: Lygaeidae) showed one isoform (36). Riseh *et al*. (2012) showed the presence of at least 2, 3, 1 and 1 major isoforms for α- and β-glucosidases and α- & β-galactosidases in the digestive system of *R. ferrugineus*, respectively, and the intensities of the bands corresponding to glucosidase activities from the digestive system of last larval instar were much higher than those of galactosidases (3).

In conclusion, glucosidases and galactosidases play an important role in digestion of wood and cellulose in *O. coerulescens*. Since the chemical control of this pest is considered impractical, one area that could be considered in the development of new insecticidal agents is based on the physiology and biochemistry of carbohydrases from the digestive system of this pest. In this study, we showed that the lowest and highest carbohydrase activity in the digestive system of *O. coerulescens* was related to α-galactosidase and β-galactosidase, respectively. Therefore, the discovery of novel inhibitors for β-galactosidases available in plants could contribute to managing this pest *via* pest-resistant transgenic plants.

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