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Original Contribution

PARTIAL CHARACTERIZATION OF DIGESTIVE CARBOHYDRASES IN THE MIDGUT OF FIG TREE SKELETONIZER MOTH, *CHOREUTIS NEMORANA* HÜBNER (LEPIDOPTERA: CHOREUTIDAE)

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ABSTRACT
Choreutis nemorana (Lepidoptera: Choreutidae) is a generally common pest of fig tree in Iran causing noticeable distortion of the leaves. Application of transgenic plants expressing digestive enzyme inhibitors is one of the safe methods in integrated pest management (IPM) and blocking of carbohydrases by inhibitors represents a safe approach to pest management of *C. nemorana*. So, identification and characterization of the carbohydrases in gut extract of *C. nemorana* is necessary for achieving this goal. In this research, some enzymatic properties of α-amylase, α-/β-glucosidases and α-/β-galactosidases from gut of *C. nemorana* larvae were determined. The optimal pH for α-amylase, α-/β-glucosidases and α-/β-galactosidases was found 11, 6, 4, 5 and 6, respectively. Also, these enzymes reached the highest activity at 55°C for α-amylase, 35 and 45°C for α- and β-glucosidases and 45°C for α- and β-galactosidases, respectively. The zymogram pattern confirmed the presence 3, 2, 4 and 2 bands for α-/β-glucosidases and α-/β-galactosidases, respectively. Among α-/β-glucosidases and α-/β-galactosidases tested, α-galactosidase showed highest thermal stability at 50°C.

Key words: *Choreutis nemorana*; α-amylase; α-/β-glucosidases; α-/β-galactosidases

INTRODUCTION

Carbohydrates are essential for the majority of insects to produce nutrient materials needed for growth, development and for the maintenance of adult survival and reproduction (1). The nutritive value of carbohydrates depends on the availability of digestive enzymes to hydrolyze complex carbohydrates to their constituent monomers which are then absorbed by midgut epithelial cells. Many carbohydrases have been reported from salivary glands and midgut of insects that among them only α-amylase act preferentially on long α-1, 4-glucan chains (2).

This enzyme mediated starch and glycogen hydrolysis and converts them to maltose, maltotriose and maltodextrins (3). Alpha-amylase is found in animals, microorganisms, bacteria, fungi and plants (4). These enzymes play important roles in insect growth and development. Many authors have characterized α-amylases from many different orders of insects including Coleoptera, Lepidoptera and Hemiptera (5-8). In insects, α-amylases are synthesized and secreted by midgut epithelial cells and salivary glands, but these enzymes have been reported also from insect haemolymph (8-11).

In addition to α-amylases, galactosidases and glucosidases are the major enzymes for carbohydrate digestion in herbivorous insects. In insects, digestive glucosidases and galactosidases are important for the hydrolysis of di and oligosaccharides (12). Alpha-glucosidase (EC 3.2.1.20) is an exo-acting hydrolase which

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removes glucose from 1, 4-alpha-glucosidic linkages. This enzyme hydrolyzes sucrose, maltose and maltodextrins. Beta-glucosidase hydrolyzes aryl and alkyl β-glucosides and β1–4 linkages between two glucoses or cellobioses (2). So far, biochemical properties of glucosidases were studied from hypopharyngeal glands of honey bee, the midgut and salivary glands of *Glyphodes pyloalis* Walker (Lepidoptera: Pyralidae) and midgut of *Xanthogaleruca luteola* Müll. (Coleoptera: Chrysomelidae), *Osphrantria coerulescens* (Col.: Cerambycidae), *Rhynchophorus ferrugineus* Olivieri (Col.: Curculionide) (7, 13-16).

Alpha-D-galactosidases (EC 3.2.1.22) are exo-acting hydrolases which catalyze the removal of galactose from 1, 4-α-galactosidic linkages in melibiose, raffinose, stachyose, and gluco- or galactomannans (17). Beta-galactosides available in insect food can be converted to monosaccharides by beta-D-galactosidase (EC 3.2.1.23). Compared to glucosidases, little attention has been paid to the biochemistry of galactosidases in the digestive system of insect (15). Up to now, biochemical properties of α- and ß-galactosidases have been characterized in some coleopteran insects (7, 15, 16).

The fig tree skeletonizer moth, *Choreutis nemorana* Hübner (Lepidoptera: Choreutidae) distributed in the Canary Islands and Madeira, through the Mediterranean region and north Africa to Asia (18). This insect is the most destructive pest of fig tree in Guilan province, Iran. The larvae of this insect feed on leaves causing extensive defoliation and at times of severe infestation, damage to fig leaves significantly reduced photosynthesis and led to huge loss of the crop. They are protected by a web of silken threads and feeds on upper epidermal and parenchymal cells (18) and this damage causes the removal of chlorophyll from the leaves reducing photosynthesis. Also, penetration of pesticides in this protected area is considered to be hindered.

Insect control strategies interfering with carbohydrate digestion are known proposed as a practical and safe method for control of herbivorous pest. The plant expressing carbohydrase inhibitors can reduce insects' survival and reproduction and retard their growth. For this reason, many studies have been attempted to characterize the biochemical properties of digestive enzymes and the study of the physiology and biochemistry of digestive carbohydrases in the insect midgut is necessary for achieving this goal. In our previous study, the biochemical characterizations of different type of *C. nemorana* proteases were performed (19). The present study reports some biochemical properties of carbohydrases in the digestive system of *C. nemorana*.

MATERIAL AND METHODS

Chemicals

Triton X-100, bovine serum albumin, 3, 5-dinitrosalicylic acid (DNS) and starch were purchased from Merck (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-α-D-galactopyranoside (4-MUαGa) and 4-methylumbelliferyl-β-D-galactopyranoside (4-MUβGa) were obtained from Sigma (Sigma, St Louis, MO, USA).

Insects

C. nemorana larvae were collected from the fig trees in Guilan province, Iran. Insect rearing was performed on fig leaves at optimum rearing conditions of 25 ± 2 °C, 60 ± 10% RH with a photoperiod of 16 h light and 8 h dark. Last instar larvae were randomly selected 24h after molting for the measuring enzyme activity.

Sample preparation and enzyme assays

300 last instar synchronized larvae (after last molting) were randomly selected for gut extraction. The individuals were immobilized on ice and digestive systems of them (without contents) were removed by dissection under a dissecting microscope in ice-cold saline buffer. Then, tissues were transferred to a freezer (-20 °C). 10 larval guts were put in each tube and considered as one replicate. For measuring enzyme activities, the sample was homogenized in cold double-distilled water using a hand-held glass homogenizer and centrifuged at 10,000 rpm for 10 min at 4°C.

Determination of enzyme activities and protein concentration

Alpha-amylase activity was determined at room temperature (26±2 °C) in 40 mM glycine-phosphate-acetate mixed buffer based on the method of Asadi *et al.* (5). The soluble fraction (10 µl) was added to a tube containing 40 µl of the buffer and 50 µl of 1 % (w/v) starch and incubated for 30 min. The DNS method according to Bernfeld (20) was used for estimation of the reducing sugar obtained from the reaction of amylase. Absorbance was measured at 545 nm with a Microplate Reader Model Stat Fax® 3200 (Awareness Technology Inc.). Control without enzyme including buffer and starch was used in the experiment. Also, separate incubation considered as negative control by adding enzymes to the reaction mixture (buffer + starch) immediately after addition of the DNS.

The activities of α-/β-glucosidases and α-/β-galactosidases were measured with pNαG, pNβG, pNαGa and pNβGa as substrates, respectively based on Ghadamyari *et al.* (14). The gut homogenates were incubated for 30 min at 37 °C with 45 µl of substrate (25 mM) and 115 µl of 40mM glycine-phosphate-acetate buffer. The pNαG, pNβG, pNαGa and pNβGa hydrolysis were stopped by adding 600 µl of NaOH (0.25M) and hydrolysis was measured by colorimetric detection of p-nitrophenol release at 405 nm using a microplate reader (Awareness, Stat Fax 3200, USA) after 10 min. A standard curves using different concentrations of p-nitrophenol were included to enable determination of the amount of p-nitrophenol released during α-/β-glucosidase and α-/β-galactosidase assays. Assays aforementioned were carried out in triplicate, and for all of them, appropriate blanks were run. Protein concentration was determined by the Bradford method (21) using bovine serum albumin as standard.

Effect of pH and temperature on enzyme activities

The pH profile of amylase, α-/β-glucosidases and α-/β-galactosidases were determined at room temperature using 40 mM glycine-phosphate-acetate buffer adjusted to various pHs (pH 3.0 to 12) by adding HCl or NaOH for acidic and basic pH values, respectively (5, 15). The activities of the enzymes were determined by incubating the reaction mixture at different temperatures ranging from 15 to 65 ºC for 30 min. Enzyme activity was measured by the standard assay method as previously described.

Thermostability

Thermal stabilities of alpha/beta glucosidases and alpha-beta galactosidases were determined at their optimal pH by incubating the enzymes at 45, 55, 50 and 50 ºC, respectively for series of time intervals, followed by cooling on ice, and determining residual activity under above assay conditions.

Polyacrylamide gel electrophoresis and zymogram analysis

Non-denaturing polyacrylamide gel electrophoresis (PAGE) for galactosidases and glucosidases was carried out at constant voltage (100V) at 4 °C. For activity staining for α-/β-glucosidase and α-/β-galactosidase, the samples were mixed with sample buffer and applied onto a polyacrylamide gel (4 and 10 % polyacrylamide for the stacking and resolving gels, respectively). After the electrophoresis is complete, the gel was immersed in 3 mM 4-MUαG, 4-MUβG, 4-MUαGa and 4-MUβGa in 0.1 M sodium acetate (pH 5.5) for 10 min at room temperature to develop colored bands indicating α-/β-glucosidase and α-/β-galactosidase activities, respectively. The blue-fluorescent bands appear in a few minutes under UV exposure and photographed with gel documentation apparatus (Uvitec Cambridge).

Statistical analysis

All experiments were performed in triplicate and the data were compared by one-way analysis of variance (ANOVA) followed by Tukey's test (22).

RESULTS

Enzyme activities

The activities of alpha-amylase, α-/β-glucosidase and α-/β-galactosidase were assessed in crude extracts of *C. nemorana* larvae. The specific activity of these enzymes is presented in **Table 1**.

Effect of pH and temperature on enzyme activities

The α-amylase from the gut of *C. nemorana* larvae is active in a wide range of alkaline pHs (pH 7.0–12) with its maximum activity at pH 11

(Figure 1). The enzyme activity retained more than 60% of its maximal activity in this pH range. Also, maximum activities of α-glucosidase and α-galactosidase in the digestive system of *C. nemorana* were observed at pH 6.0 and 5.0, respectively, whereas, the optimum pH values for β-glucosidase and β-galactosidase were 4.0 and 6.0, respectively **(Figure 1)**. The optimum temperatures for the α-amylase and the α-glucosidase are 55 and 35 °C, respectively and other enzymes are optimally active at 45 °C **(Figure 2)**.

The irreversible thermo inactivation of the enzymes were investigated at different temperatures **(Figure 3)**. Among these enzymes, α-galactosidase and β-glucosidase showed the highest and β-galactosidase the lowest thermostability.

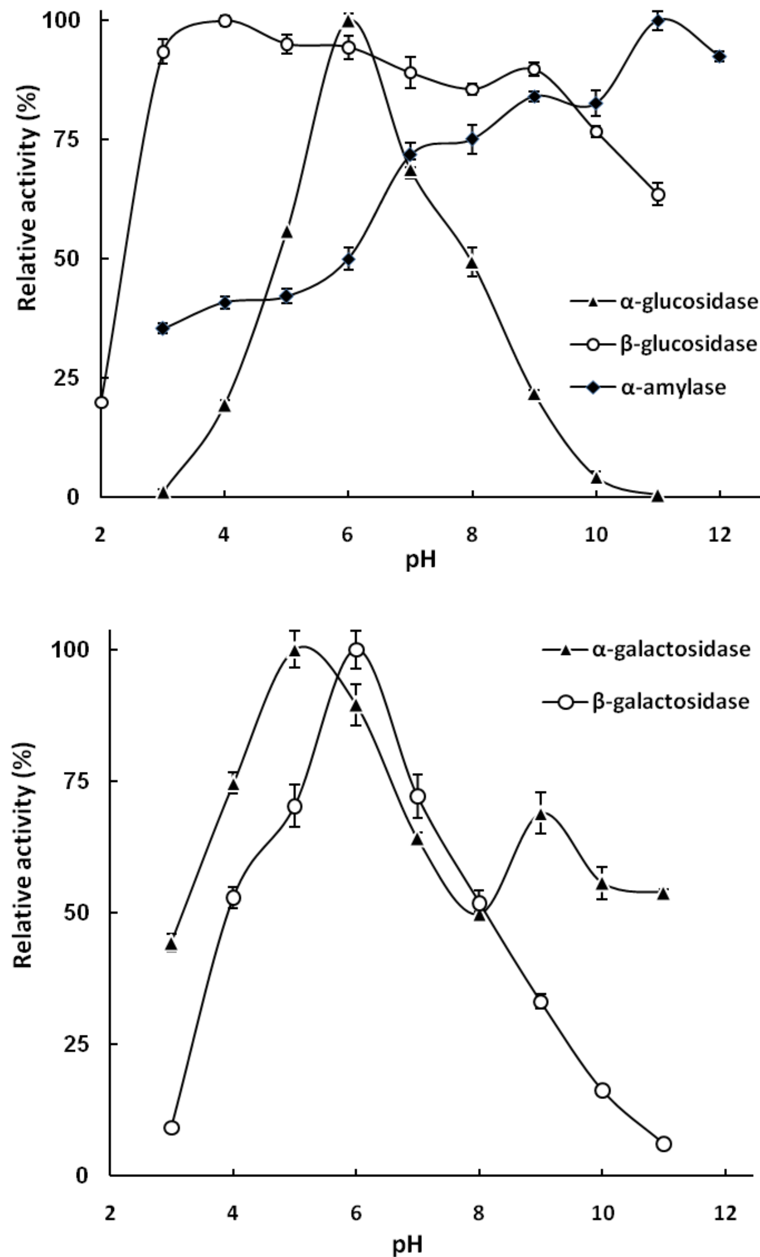

Figure 1. The effect of pH on the mean activities (±SE) of α-amylase, α-/β-glucosidases and α-/β-galactosidases extracted from the digestive system of *C. nemorana.*

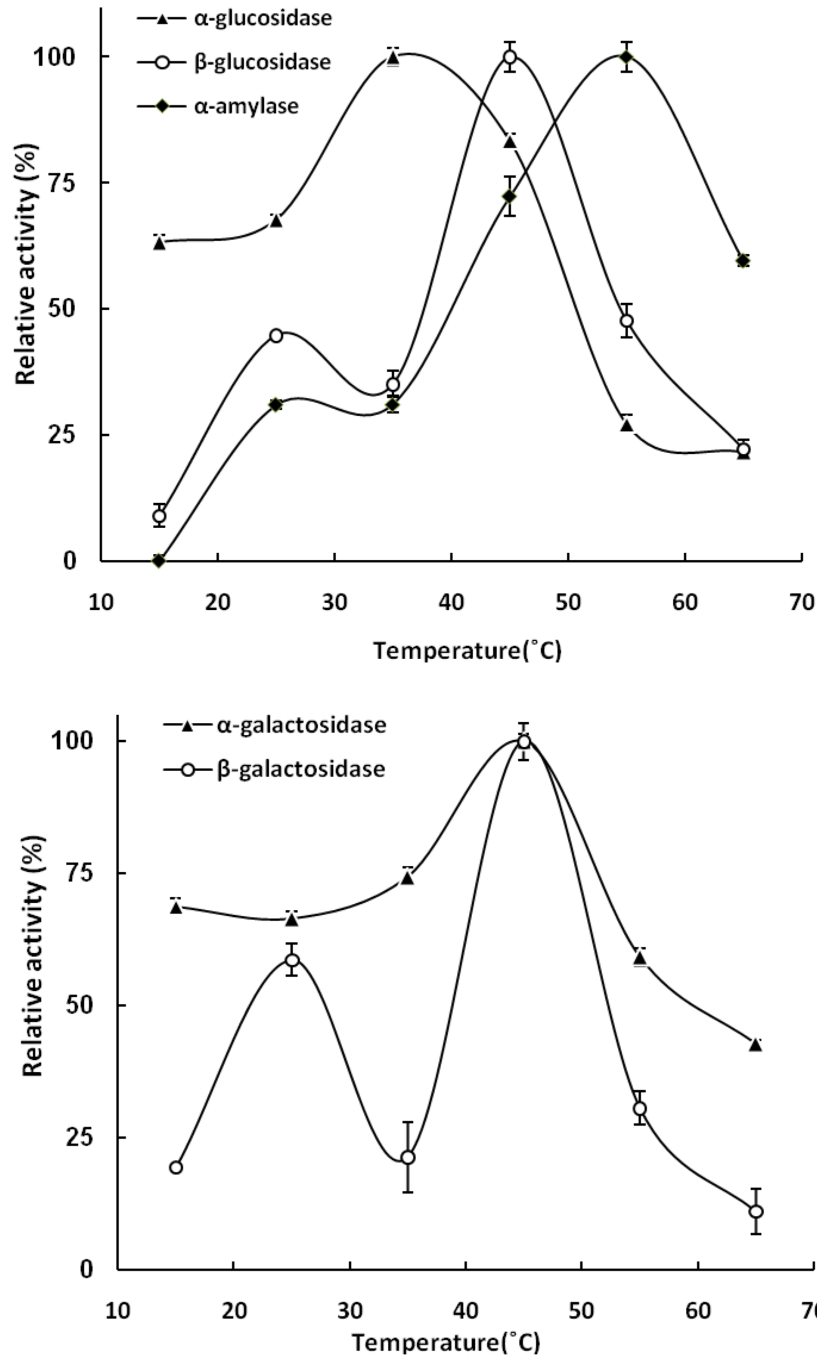

Figure 2. The effect of temperatures on the mean activities (±SE) of α-amylase, α-/β-glucosidases and α-/β-galactosidases extracted from the digestive system of *C. nemorana* at their optimal pH.

Zymogram analysis

The zymogram patterns on native PAGE indicated the presence of different isozymes for each carbohydrase in the digestive system of *C. nemorana*. The results revealed the presence of at least 3, 2, 4 and 2 isozymes for α-/β-glucosidase and α-/β-galactosidase, respectively **(Figure 4)**.

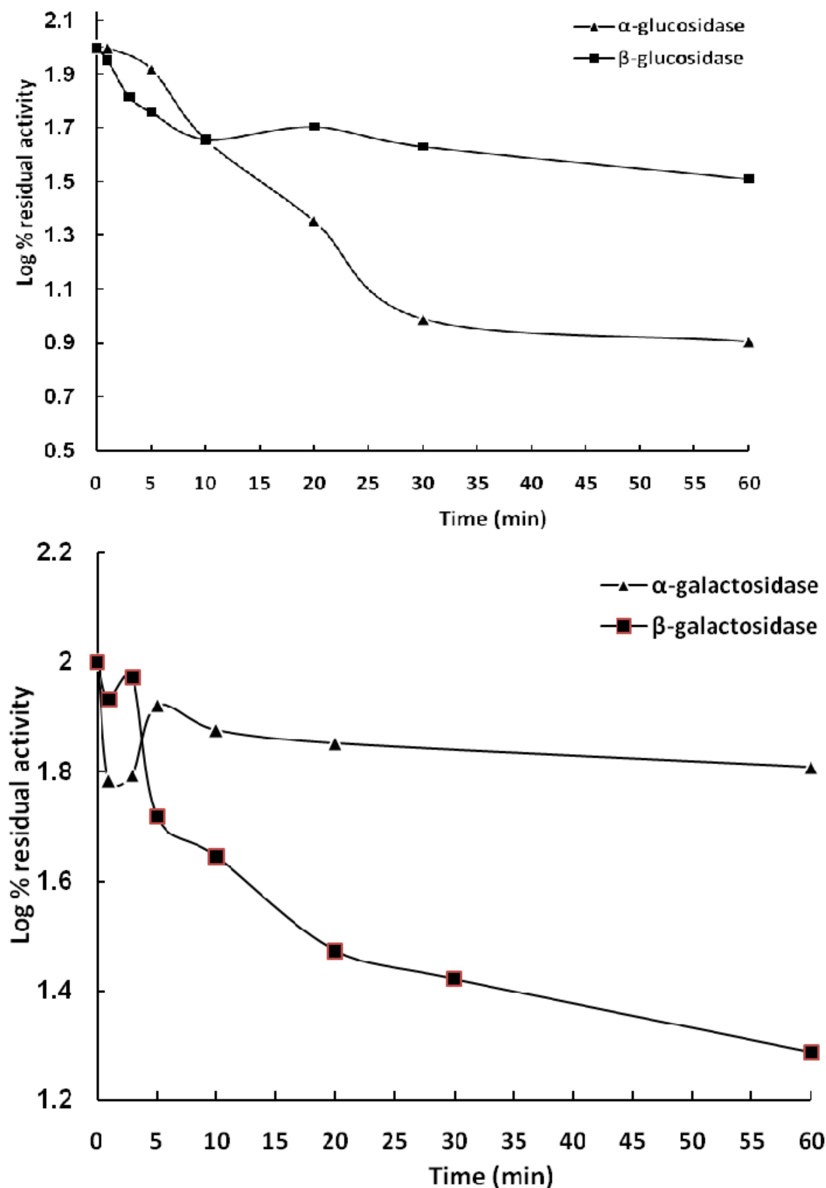

Figure 3. Effect of temperatures on stability of gut α-glucosidase (45 °C) β-glucosidases (55 °C) and α-/β-galactosidases (50 °C) activities of *C. nemorana* at their optimal pH.

DISCUSSION

The specific activity of α-amylase in the gut of *C. nemorana* was obtained as 7.68±0.16 $\mu mol.min^{-1}.mg^{-1}$ protein **(Table 1)** and the activity of this enzyme in room temperature (26 ± 2 °C) and 35 °C was the same **(Figure 2)**. The activity of α-amylase in the midgut of *Naranga aenescens* L. (Lep.: Noctuidae) (at room temperature) and *G. pyloalis* (at 35 °C) were reported as 2.4 and 0.011 $\mu mol.min^{-1}.mg^{-1}$ protein, respectively (5,23). Also, activity of α-amylase in *Helicoverpa armigera* Hubner (Lep.: Noctuidae) was obtained as 0.034 $\mu mol.min^{-1}.mg^{-1}$ protein (24). Activity of *α*-amylase in midgut of other insects ranges from 0.0673 to 1.2 $\mu mol.min^{-1}.mg^{-1}$ protein (25-28). It seems the α-amylase activity in digestive system of *C. nemorana* was higher than *N. aenescens, G. pyloalis* and *H. armigera.*

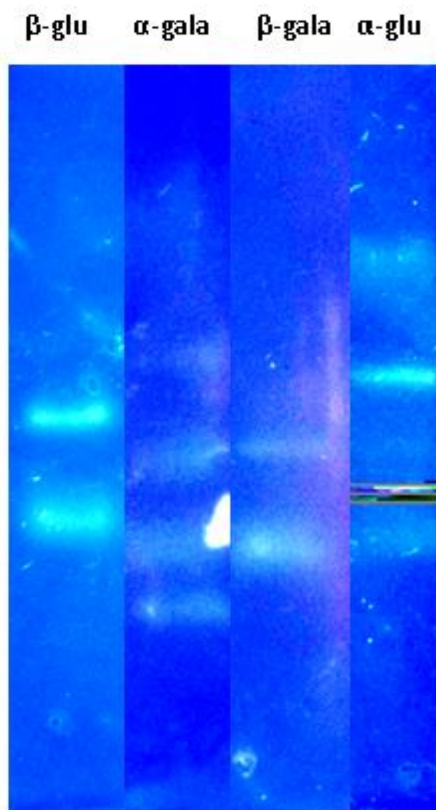

Figure 4. Zymogram of α-/β-glucosidases (α-glu, β-glu) and α-/β-galactosidases (α-gala, β-gala) extracted from the digestive system of *C. nemorana*.

Table 1. *The specific activities (µmol min^{-1} mg protein^{-1}) of digestive carbohydrases in last larval instar of C. nemorana at their optimal pH*

Enzyme	Activity (µmol min^{-1} mg protein^{-1})*
α-glucosidases (EC 3.2.1.20)	9.9 ± 0.06
β-glucosidases (EC 3.2.1.21)	0.7 ± 0.004
α-galactosidases (EC 3.2.1.22)	1.6 ± 0.041
β-galactosidases (EC 3.2.1.23)	3.2 ± 0.06
α-amylase (EC 3.2.1.1)	7.6 ± 0.1

*Results are the mean ± SE of ten individual insect tissues (n=3), each with triplicate analysis

α-/β-glucosidases and α-/β-galactosidases activities were measured in the digestive system of last larval instar of *C. nemorana* using *p*NαG, *p*NβG, *p*NαGa and *p*NβGa as substrates **(Table 1**). Our results showed that there are significant differences in the specific activities of α-/β-glucosidases and α-/β-galactosidases in digestive system of last instar *C. nemorana*. Previous works on the phytophagous lepidopterans glucosidases showed the presence of α- and β-glucosidase in the salivary glands and in the midgut (14, 29- 31). In this study, α-glucosidase showed the highest activity among the tested carbohydrases. In contrast to our results, Aghaali *et al.* (15) showed that, in the digestive system of *O. coerulescens* larvae, β-galactosidase activity was higher than that of other glucosidases and galactosidases. Also, in the digestive system of

R. ferrugineus larvae and female adult, α-glucosidase activity was higher than that of other glucosidases and galactosidases (16).

The carbohydrase specific activities increase in the order of α-glucosidase> α-amylase> β-galactosidase > α-galactosidase > β-glucosidase. α- and β-glucosidase activity at 35 °C in midgut of *G. pyloalis* were reported as 0.195 ± 0.004 and 1.07 ± 0.003 $\mu mol.min^{-1}.mg^{-1}$ protein, respectively (14). Also, the presence of *α*- and β-glucosidase activity in other phytophagous lepidopterans has been reported (29- 32), but our knowledge about the galactosidses is nugatory. The specific activity of β-glucosidase in *C. nemorana* was higher than lepidopterian insects such as *G. pyloalis* and *N. aenescens* (11, 14). Also, similar to our results, the specific activity of β-glucosidase in *R. palmarum* was reported as 0.69 $\mu mol.min^{-1}.mg^{-1}$ protein. The high β-glucosidase activity is found in some insects belonging to different orders such as *Abracris flavolineata* (Orthoptera: Acrididae) (foliar feeder), *Tenebrio molitor* (Col.: Tenebrionidae) (stored product feeder) and *Scaptotrigona bipunctata* (pollen feeder) (Hym.: Apidae). In contrast, low glucosidases activities is found from predaceous insects such as *Pheropsophus aequinoctialis* (Col.: Carabidae) and *Pyrearinus termitilluminans* (Col.: Elateridae) (33). Also, low β-glucosidase activity is reported in the decomposed plant material feeder, *Rhynchosciara americana* (Diptera: Sciaridae) and in herbivorous feeders, *S. frugiperda* (Lep.: Noctuidae), *E. ello* and *D. saccharalis* (Lep.: Pyralidae) (33). So far, very high β-glucosidase activity was measured in Apollo butterfly, *Parnassius apollo*. *Frankenbergeri* that allows this pest to hydrolyze the compounds containing β-glycosidic bonds in its food (34). It seams the activities of glucosidases and galactosidases vary depending on the availability of compounds containing β-glycosidic bonds in diet and insect species. The literature showed the glucosidase activities involved in digestion of carbohydrate and interactions between herbivorous insect and host plant and heme detoxification in blood-sucking insect such as *Rhodnius prolixus* (Hemiptera: Reduviidae) (15, 35).

The optimum pH for α-amylase activity in midgut of *C. nemorana* was 11.0 **(Figure 1)**. Majority of research on lepidopteran α-amylase showed the optimum pH at 8.0-12.0. For example, the maximum activity for *α*-amylase reported 8.0 and 9.0 in the midgut of *N. aenecens* (5) and *H. armigera* (24), respectively. Also, the optimal pH for α-amylase activity was 9.2 in *Antheraea mylitta* (Lep.: Bombycidae), 12.0, 10.8, 11.3 and 10.8 in midgut lumen of *Acherontia atropos* (Lep.: Sphingidae), *Lasiocampa quercus* (Lep.: Lasiocampidae), *Manduca sexta* (Lep.: Sphingidae) and *Lichnoptera feline* (Lep.: Noctuidae), respectively (26, 36). Alpha-amylases in lepidopteran insect are generally most active in the neutral to alkalin pH condition, whereas, in coleopteran and hemipteran insect are active in neutral to acidic pH condition (2, 5, 6, 7, 9).

Maximum activity of α-glucosidase and α-galactosidase in midgut of *C. nemorana* was observed at pH 6.0 and 5.0, respectively, whereas, the optimal pH for β-glucosidase and β-galactosidase activity were attained as 4.0 and 6.0, respectively. Our result is consistent with report of Ghadamyari *et al.* (14) when they worked on glucosidases of *G. pyloalis* and found α and β-glucosidase in midgut of this insect were generally most active in neutral to slightly acidic pH conditions. It seems the pH value in digestive system of this pest is alkali such as other lepidopteran insect; however the gut pH in this pest was not studied.

These diversities in optimal pH of insect α-amylase, α- and β-glucosidase and α- and β-galactosidases may reflect insect's phylogenetic relation or response to different diets (34). Also, the origin of the α- and β-glucosidase, i.e., digestive system or salivary glands may justify these differences. Because the deduced amino acid sequences of α- and β-glucosidase from the midgut of one species are different to those of the salivary α- and β-glucosidase (37).

Most insect α- and β-glucosidase exhibit optimal temperature ranging from 20 to 50 ºC (13). Optimal temperature for ß-glucosidase in *Zygaena trifolii* (Lep.: Zygaenidae) was reported as 40 °C (29). Stability of the enzymes activity of *C. nemorana* at high temperature showed that among α-/β-glucosidases and α-/β-galactosidases tested α-galactosidase showed highest stability at 50 °C.

The crude extracts of *C. nemorana* midgut were analyzed by native PAGE. The zymogram analysis indicated that at least 3, 2, 4 and 2 isozymes were presented for α-/β-glucosidase and α-/β-galactosidase, respectively in *C. nemorana* midgets **(Figure 4)**. The carbohydrases produced by several insect sources show different number of isozymes. The number of isozymes for the most of these earlier reported enzymes is in the range of one to four (7, 11, 15, 16).

CONCLUSION

Defoliator pest feed on the plant leaves and sometimes caused the plant death. Plants utilize secondary metabolites such as antibiotics, alkaloids, terpenes, cyanogenic glucosides, lectins and enzyme inhibitors as defense mechanism against pests. The enzyme inhibitors can impair food digestion through their inhibition on pest midgut digestive carbohydrases or proteases. These inhibitors can retard the insect growth and development. An alternative method for pest control is development of new insecticidal agents based on the biochemistry of carbohydrases exists in the insect midgut. Our results showed that the specific activity of α-amylase and α-glucosidase was highest among the tested carbohydrases. Therefore, these carbohydrases have important role in digestion of fig leaves in *C. nemorana*. So, control of this pest could be achieved by producing plant cultivars that express the carbohydrase inhibitors.

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