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Research Article

Biochemical characterization of digestive β-glucosidase from midgut of *Leiptinotarsa decemlineata* (Coleoptera: Chrysomelidae)

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Abstract: The Colorado potato beetle, *Leptinotarsa decemlineata* Say (Col.: Chrysomelidae) is an important pest of potato worldwide and study of its glucosidases is an important step to develop appropriate pest control strategies. In this study, some biochemical aspects of β-glucosidase in the Colorado potato beetle were determined. The results showed that β-glucosidase activity in the midgut of adults was 6.68 Umg^{-1}. Maximum activity of midgut β-glucosidase occurred at pH 4 to pH 5.5; however, the enzyme is active at pH 3 to pH 7 more than 50% of its relative activity. The enzyme was stable at pH 3 to pH 8 for 2 and 8 hours incubation time. According to the results, optimal temperature for the enzyme activity was 50 °C and its stability significantly was reduced at 50 °C during 1 to 8 days incubation time. The enzyme activity decreased with the addition of different concentrations of $MgCl_2$, urea, Tris and $CaCl_2$. Enzyme activity was highly decreased at low concentrations of SDS (1 mM).

Keywords: Biochemistry, midgut, β-glucosidase, *Leptinotarsa decemlineata*

Introduction

The Colorado potato beetle, *Leptinotarsa decemlineata Say.* (Col.: Chrysomelidae), is one of the most serious pests of potato in eastern North America and Europe (Hare, 1990). This pest was also reported from Iran in 1984 and is distributed in many potato production provinces (Nouri Ghanbalani, 2002). Un-controlled populations can completely defoliate potato plants and cause a total yield loss (Hare, 1980, 1990). Synthetic chemical insecticides have been widely used for controlling of the pest but despite their broad applications, the control level was not sufficient and the resistance of many *L. decemlineata* populations has been reported frequently (Forgash, 1981; Gauthier *et al.*, 1981; Harris *et al.*, 1981; Hare, 1990) for this reason, alternative control methods are needed for sustainable management programs of this pest. Host plant resistance to insect pests is one of the most promising ways to reduce pest populations and dependency to pesticides (Pedigo, 1999). Plants produce secondary metabolites such as glucosides that are converted into toxic aglycones in insects' midgut by the activity of digestive enzymes and are thereby defended against herbivore attacks (Wei *et al.,* 2007). This property of plants can be manipulated by genetic engineering to produce resistant plants against herbivorous insects (Mattiacci *et al*. 1995). β-Glucosidase (EC. 3.2.1.21) is an enzyme catalyzing the hydrolysis of glycosidic linkages from the non-reducing terminal of di- and oligo-β-saccharides obtained from the initial digestion of hemicelluloses and cellulose (Terra and Ferreira, 1994). It has been reported that insect β-glucosidases have a key role in insect-plant

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interactions (Terra and Ferreira, 1994). Therefore, study of biochemical characterization of these enzymes and their role in digestive system can be important in the diagnosis of plants resistance to pests. β-Glucosidases are characterized in many insects like *Tenebrio molitor* L. (Col.: Tenebrionidae) (Ferreira *et al.*, 2001), *Bombyx mori* L. (Lep.: Bombycidae) (Byeon *et al.*, 2005), *Rhynchophorus palmarum* L. (Col.: Curculionidae) (Yapi *et al.*, 2009), *Glyphodes pyloalis* Walker (Lep.: Pyralidae) (Ghadamyari *et al.*, 2010), *Apis mellifera* linnaeus (Pontoh and Low, 2002), *Neotermes koshunensis* Shiraki (Iso.: Kalotermitidae) (Tokuda *et al.* 2002). Since there is no information available on the activity of digestive β-glucosidase for the Colorado potato beetle, the purpose of the current study is to characterize some biochemical properties of the midgut-extracted β-glucosidases of *L. decemlineata.*

Materials and Methods

Insect collecting

Colorado potato beetle adults were collected from potato fields (Hamedan province) (2010 to 2011) and maintained on potato leaves under laboratory controlled conditions at 26 ± 1 °C, 70–75% R. H. and a photoperiod of 14L: 10D h.

Sample preparation

The adults of *L. decemlineata* were immobilized on ice and dissected under a stereomicroscope. Their midguts were moved into ice-cold double-distilled water. Samples were homogenized in a pre-cooled hand-held glass homogenizer and resulting homogenates were transferred to new 1.5 ml centrifuge. After that they were centrifuged at 15000g for 10 min at 4 °C. The supernatants were pooled and stored at −20 °C for subsequent analyses.

Enzyme assay

β-Glucosidase activity was determined by measuring the amount of released p-nitrophenol from p-nitrophenyl- β-D-glucopyranoside (pNβG) as the substrate by the method of Low *et al.* (1986) with slight modifications. The assay mixture included 300 µl of 40 mM citrate-phosphate buffer (pH 5), 10 µl enzyme and 20 µl of 15 mM pNβG. The reaction mixture was incubated for 15 min at 50°C. Enzyme activity was stopped by addition of 700 µl NaOH (2 M). The absorbance was measured at 405 nm after 10 minutes (Bandani *et al*., 2010).

Effect of pH and temperature on the enzyme activity

Optimal pH for enzyme activity was measured using citrate- phosphate buffer at pH 2 to 8 (with 0.5 intervals). Enzyme sample was incubated in citrate-phosphate buffer with different pHs for 2 and 8 h. Residual activity of the treated enzyme sample was measured according to the following section "Enzyme assay" (Bandani *et al.*, 2010).

To determine the optimum temperature for the enzyme activity, the reaction mixture was incubated at different temperatures ranging from 5 °C to 70 °C (with 5 °C intervals) for 35 min followed by section "Enzyme assay". Enzyme stability at different temperatures was measured at 5 °C, 26 °C and 50 °C for 1 to 8 days. Subsequently, residual activity of the enzyme was determined (Bandani *et al*., 2010).

Influence of cations and inhibitors on β -glucosidase activity

To detenmine the effect of different ions on the enzyme activity, different concentrations of chloride salts such as Na^+ (5, 10, 20 and 40 mM), K^+ (5, 10, 20 and 40 mM), Ca^{2+} (5, 10, 20 and 40 mM), Mg^{2+} (5, 10, 20 and 40 mM), sodium dodecylsulfate (SDS; 1 mM), Tris (10 and 20 mM) and Urea (0.4 and 0.8 M) were added to the assay mixture, then relative activity was measured after 35 min (Bandani *et al*., 2010).

Protein determination

Absorbance degree of protein content was measured at 595 nm according to Bradford (1976) using bovine serum albumin as the standard.

Electrophoresis and zymogram analyses of β-glucosidase

Electrophoretic analysis was performed using native-PAGE on 7.5% and 3.5% (w/v)

resolving and stacking polyacrylamide gels, respectively (Laemmli, 1970). Following SDS-PAGE, the gel was washed in 40 mM citrate-phosphate buffer at pH 5 (optimal pH for the enzyme activity) for 20 min at room temperature. Then, the gel was incubated in the buffer including 8 mM fluorogenic substrate 4-methylumbelliferyl-β- D-glucopyranoside at 40 °C for 30 min. Finally, β glucosidase activity was detected by fluorescence under UV illumination.

Statistical analysis

Data were compared by one-way analysis of variance (ANOVA) followed by Tukey's studentized test using the SAS program (SAS Institute, 2004).

Results

β-Glucosidase activity

The specific activity of β-glucosidase from the midgut was 6.68 Umg^{-1} proteins.

Effect of pH and temperature on β glucosidase activity

The effect of pH on the enzyme activity toward pNβG was measured. The enzyme was active from pH 3 to 7 at least 50% of relative activity. Maximum enzyme activity was determined at pH range of 4 to 5.5. β-Glucosidase activity in the midgut was increased steadily from pH 2 to 4 and then decreased with increasing pH values from 6 to 8 (Fig. 1).

Enzyme stability in different pHs for 2 and 8h has been shown in Fig. 2. β-glucosidase from the midgut of *L. decemlineata* was stable at pH 3 to pH 8. The enzyme is not stable at pH 2.

β-glucosidase was active over a broad temperature range. As shown in Fig. 3, the optimal temperature for β-glucosidase activity in *L. decemlineata* midgut was 50 °C. The enzyme activity was increased steadily with increasing temperature from 10 °C to 50 °C. At temperatures above 50 °C, the enzyme activity was gradually decreased until it was nill at 70 °C.

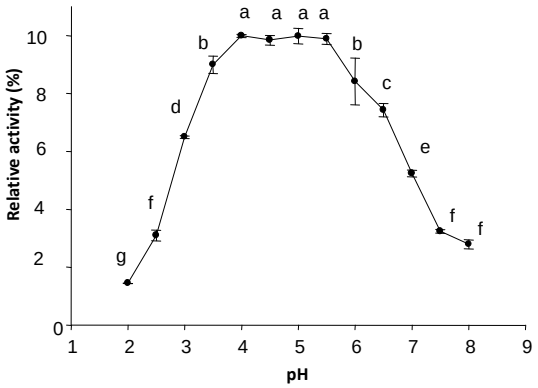


Figure 1 Effect of pH on activity of β-glucosidases extracted from midgut of *Leptinotarsa decemlineata.*

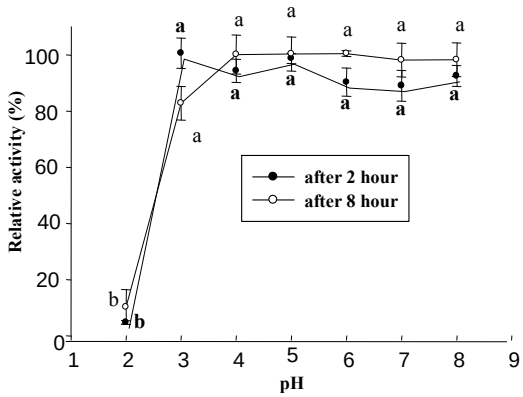


Figure 2 Effect of pH on the stability of β-glucosidase activity from midgut of *Leptinotarsa decemlineata* after 2 and 8 hours.

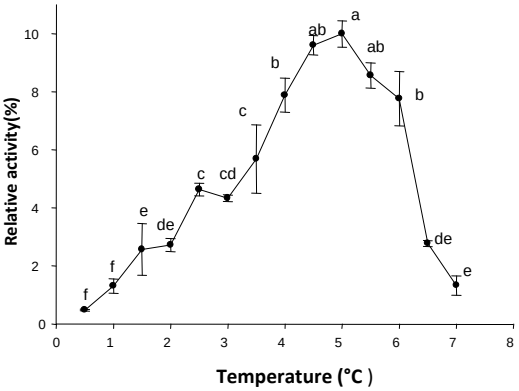


Figure 3 Effect of temperature on β–glucosidases activity extracted from midgut of *Leptinotarsa decemlineata* (incubated for 15 min).

β-glucosidase retained 80% of its original activity after 8 days incubation at 5 °C and 26 °C. The enzyme was unstable at 50 °C after 1 day and lost about 80% of its activity. Enzyme activity was about completely lost at 50 °C after 2 days incubation time (Fig. 4).

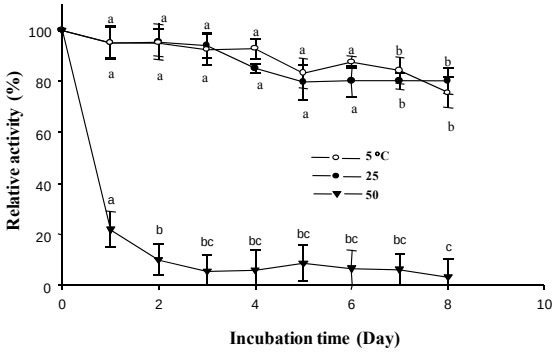


Figure 4 Effect of temperature on stability of midgut β-glucosidase activity of *Leptinotarsa decemlineata* (for 8 days).

Effect of cations and inhibitors on β-glucosidase activity

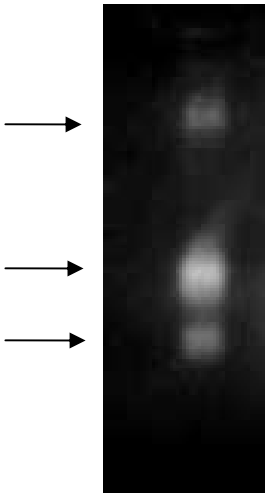
β–glucosidase activity was declined in accordance with increased concentration of $MgCl_2$, $CaCl_2$ and urea. In addition, the results showed that enzyme activity decreased strongly at low concentration of SDS (1 mM). On the other hand, KCl (20 and 40 mM) positively influenced the activity of β-glucosidase. Different concentrations of NaCl did not have any effects on β-glucosidase activity. In addition, Tris (20 mM) inhibited the enzyme activity up to 24% (Table 1).

Zymogram analyses

Further characterization of hydrolytic activity of β-glucosidase of the midgut extract from *L. decemlineata* was performed by incubation of electrophoresed gels containing non-heated enzyme sample with MUaGlc as the substrate. As shown in Fig. 5, three distinct bands related to the enzyme activity were observed.

Table 1 Relative activity of midgut β-glucosidase from *Leptinotarsa decemlineata* at different concentrations of the test compounds.

Compounds	Concentration (mmol/L)	Relative activity (%)
Control	-	100
NaCl	5	97.24 ± 0.04
	10	99.63 ± 0.04
	20	93.81 ± 0.006
	40	99.80 ± 0.05
KCl	5	89.55 ± 0.08
	10	91.97 ± 0.02
	20	100.67 ± 0.05
	40	100.37 ± 0.12
$CaCl_2$	5	87.50 ± 0.05
	10	69.41 ± 0.02
	20	52.54 ± 0.13
	40	51.28 ± 0.04
$MgCl_2$	5	81.92 ± 0.06
	10	72.86 ± 0.008
	20	59.47 ± 0.04
	40	40.56 ± 0.01
Urea	400	89.62 ± 0.11
	800	67.48 ± 0.15
Tris	10	94.75 ± 0.10
	20	76.05 ± 0.05
SDS	1	5.00 ± 0.02



Figurer 5 Zymograms of β-glucosidase in the midgut of *Leptinotarsa decemlineata.* Arrows show three distinct bands related to the enzyme activity.

Discussion

β-glucosidases are widely present in plants, fungi, bacteria and other animals especially major orders of insects (Esen, 1993). These enzymes hydrolyze cellobiose and other cello-oligosaccharides to glucose that can be absorbed by midgut epithelial cells. Because β-glycans are major dietary components of many insect species, thus β -glucosidase has key role in carbohydrate digestion within insect digestive system (Terra and Ferreira, 1994). Yapi *et al.* (2009) expressed that β-glucosidases in *R. palmarum* hydrolyze cellobiose, cellodextrins, laminaribiose, sophorose, gentiobiose and p-nitrophenyl- β-D-glucopyranoside. Moreover, Ferreira *et al.* (1998) reported that *Abracris flavolineata* Degeer (Orth.: Acrididae), *T. molitor* and *Scaptotrigona bipunctata* Lepeletier (Hym.: Apidae) displayed higher β-glycosidase specific activities. In contrast, predaceous insects such as *Pheropsophus aequinoctialis* L. (Col.: Carabidae) and *Pyrearinus termitilluminans* Costa (Col.: Elateridae) have low β-glucosidase activity. Midgut β-glucosidases of *L. decemlineata* have optimal activity in pH range between 4-5.5, which is consistent with other observations; e.g. Yapi *et al.*(2009) showed that maximal β-glucosidase activity of the palm weevil *R. palmarum* was at pH 5, while the optimum pH and thermostability of *N. koshunensis* β-glucosidase were 5 and 45 °C (Ni *et al.* 1985). In addition, midgut β–glucosidase of *Rhodnius prolixus* Stål had optimal pH value at 4.5 (Terra *et al.* 1988). A digestive enzyme is affected by pH of gut contents (Terra and Ferreira, 1994) and it seems that there is a correlation between enzyme pH and luminal pH of insect's gut (Applebaum, 1985). Some beetle families such as Chrysomelidae have acidic pH value across their midgut (Terra and Ferreira, 1994) which can explain the acidic activity of β-glucosidase in the midgut of *L. decemlineata*. The reason for broader pH range of β-glucosidases activity could be due to the presence of three isozymes in the midgut of *L. decemlineata*. In many cases, the pH-dependency of enzyme activity is reversible, and the enzyme incubated in weakly acid or alkaline condition of the titration curve regains its maximum activity when shifted to the optimum pH. So, a pH stability curve is usually broader than the reversible pH optimum curve (Bisswanger, 2004). This wide pH range stability of β-glucosidases from the midgut of *L. decemlineata* is consistent with Pontoh and Low (2002), who reported that β-glucosidases from the ventriculus and honey sac in *A. mellifera* were active in pH 3.5 to 9.5 and 4.5 to 9.0, respectively. Our results also showed that β-glucosidase of the Colorado potato beetle has an optimal activity at 50°C and its activity increased steadily from 15–50 °C but it is unstable at 50 °C (Fig. 5). α-glucosidase and β-glucosidase of most insects have optimal temperature ranging from 20 to 50 °C (Huber and Mathison, 1976; Takenaka and Echigo, 1978; Ghadamyari *et al.*, 2010). The optimal temperature of β-glucosidase activity of *R. palmarum* was at 55 °C (Yapi *et al.*, 2009). Digestive enzymes are protein structures that catalyze biological reactions and each enzyme has a temperature range for its optimal activity. Temperatures above this range disrupt three-dimensional structure of enzyme that may be irreversible (Price and Stevens, 1989). It could be concluded that the instability of β-glucosidase of *L. decemlineata* at 50 °C is a consequence of its three-dimensional structure change.

Based on obtained results, SDS, $MgCl_2$, $CaCl_2$, urea and Tris decreases β-glucosidase activity significantly. Zeng and Cohen (2001) reported that Cu^{2+} had the highest effect (118%) and Mg^{2+} the least effect (96%) on α- and β-glucosidase of *Lygus hesperus* Fabricius (Hem.: Miridae). Similarly, Yapi *et al.* (2009) showed that $CuCl_2$, $ZnCl_2$, $FeCl_3$ decreased β-glucosidase activity in digestive fluid of the palm weevil larvae, *R. palmarum*, whereas $BaCl_2$, $MgCl_2$, $MnCl_2$, $SrCl_2$ and $CaCl_2$ had no effect on the enzyme activity. Ghadamyari *et al.* (2010) demonstrated that the $CaCl_2$ (40 mM) decreased β-glucosidase activity and increased

α-glucosidase activity in pyralid *G. pyloalis*. They also illustrated that urea (4 mM) and SDS (8 mM) significantly decreased digestive β-glucosidase activity. Mahboobi *et al.* (2011) showed that activity levels of midgut β-glucosidase in *Aelia acuminata* L. (Hem.: Pentatomidae) was increased with increasing concentrations of NaCl, $MgCl_2$, $CaCl_2$, KCl, whereas, its activity was decreased in the presence of sodium dodecylsulfate, urea and Tris. Biochemical characterization revealed that *L. decemlineata* midguts have three β-glucosidase isoforms. Previous studies with other insect midgut β–glucosidase showed at least three, four or even five distinct bands (Azevedo *et al*., 2003).

Plants produce a wide variety of secondary metabolites used as defensive agents against herbivores. These include cyanogenic alkaloids, triterpenoid, glycosides, phenols, flavonoids and non-protein amino acids. Among those compounds, it seems that glycosides have important role in plant resistance to insects (Hsiao, 1988). Most plant glycosides are β-linked o-glycosyl compounds that have hydrophobic aglycone therefore; they are hydrolyzed by insect glycosylceramidases and β–glucosidases that have a glycosylceramidase-like activity (Terra and Ferreira, 1994). Aglycons released of β-glucosidase activities are usually more toxic than the glycosides themselves (Yu, 1989).

Several plants have been identified to produce glycosides which are feeding deterrents (Klun *et al.*, 1967; Elliger *et al.* 1981) or have antifeedant activity for phytophagous insects (Montgomery and Arn, 1974). Recognizing these compounds in plants and application of genetic engineering techniques to transfer genes producing toxic compounds to target plants can be appropriate strategy for developing host plant resistant to insects.

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توصیف ویژگی‌های بیوشیمیایی آنزیم بتاگلوکوزیداز معده میانی سوسک کلرادوی سیب‌زمینی

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چکیده: سوسک کلرادوی سیب‌مینی *Leptinotarsa decemlineata* Say یکی از مهم‌ترین آفات سیب‌زمینی در دنیاست و مطالعه گلوکوزیدازهای آفت گام مهمی در راستای تدوین راهبردهای کنترل مناسب آن می‌باشد. در این تحقیق برخی از ویژگی‌های بیوشیمیایی آنزیم بتاگلوکوزیداز موجود در معده میانی سوسک کلرادوی سیب‌زمینی تعیین شد. نتایج به‌دست آمده نشان داد میزان فعالیت این آنزیم در معده میانی حشرات کامل آفت ۶/۶۸U/mg بود. حداکثر فعالیت آنزیم بتاگلوکوزیداز معده میانی در محدوده pH بین ۵/۵-۴ به‌دست آمد؛ هر چند در محدوده pH بین ۷-۳ آنزیم به‌میزان ۵۰ درصد حداکثر فعالیت نسبی‌اش از خود فعالیت نشان داد. آنزیم در دامنه pH بین ۸-۳ به مدت دو و هشت ساعت پایدار بود. دمای بهینه برای فعالیت آنزیم ۵۰ درجه سلسیوس بود و پایداری آن به‌صورت معنی‌داری در مدت ۸-۱ روز در این دما کاهش یافت. فعالیت آنزیم با افزودن غلظت‌های مختلف کلرید منیزیم، اوره، تریس و کلریدکلسیم کاهش نشان داد اما افزودن غلظت‌های ناچیز سدیم دودسیل سولفات (SDS) سبب بازدارندگی شدید روی فعالیت آنزیم بتاگلوگوزیداز شد.

واژگان کلیدی: بیوشیمی، معده میانی، بتاگلوکوزیداز، *Leptinotarsa decemlineata*