Article

PURIFICATION AND CHARACTERIZATION OF β-GLUCOSIDASE FROM GREATER WAX MOTH *Galleria mellonella* L. (LEPIDOPTERA: PYRALIDAE)

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The greater wax moth, Galleria mellonella*, is one of the most ruinous pests of honeycomb in the world. Beta-glucosidases are a type of digestive enzymes that hydrolytically catalyzes the beta-glycosidic linkage of glycosides. Characterization of the beta-glucosidase in* G. mellonella *could be a significant stage for a better comprehending of its role and establishing a safe and effective control procedure primarily against* G. mellonella *and also some other insect pests. Laboratory reared final instar stage larvae were randomly selected and homogenized for beta-glucosidase activity assay and subsequent analysis. The enzyme was purified to apparent homogeneity by salting out with ammonium sulfate and using sepharose-4B-*L*-tyrosine-1-naphthylamine hydrophobic interaction chromatography. The purification was 58-fold with an overall enzyme yield of 29%. The molecular mass of the protein was estimated as ca. 42 kDa. The purified beta-glucosidase was effectively active on* para/ortho*-nitrophenyl-beta-*D*-glucopyranosides* (p-/o-*NPG) with Km values of 0.37 and 1.9 mM and Vmax values of 625 and 189 U/mg, respectively. It also exhibits different levels of activity against* para*-nitrophenyl-*β*-*D*-fucopyranoside* (p-*NPF*),* para/ortho*-nitrophenyl* β*-*D*-galactopyranosides* (p-/o-*NPGal) and* p*-nitrophenyl 1-thio-*β*-*D*-glucopyranoside. The enzyme was competitively inhibited by*

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beta-gluconolactone and also was very tolerant to glucose against p-*NPG as substrate. The Ki and* IC_{50} *values of* δ*-gluconolactone were determined as 0.021 and 0.08 mM while the enzyme was more tolerant to glucose inhibition with IC50 value of 213.13 mM for* p-*NPG.* © *2014 Wiley Periodicals, Inc.*

Keywords: *Galleria mellonella*; β-glucosidase; purification; characterization

INTRODUCTION

β-Glucosidase (β-D-glucoside glucohydrolase, (EC 3.2.1.21) selectively catalyzes the hydrolysis of β-glycosidic bond between glycone residues or of that between glucose and an aryl or alkyl aglycone. The enzyme constitutes a major group among glycoside hydrolases that occur universally in all living organisms from bacteria to humans. β-Glucosidases play key roles in a variety of essential physiological processes and potential biotechnological applications depending on the nature and diversity of the glycone or aglycone moiety of their substrates. Among the mammalian β-glucosidases, the human acid β-glucosidase, commonly known as glucocerebrosidase, catalyzes the degradation of glucosylceramide in the lysosome. The inherited deficiency of the enzyme leads to Gaucher's disease (Woodward and Wiseman, 1982). β-Glucosidases in cellulolytic microorganisms have recently been the focus of much research since cellulose is the most abundant substrate on earth and is very likely to be an important renewable resource of energy in the future (Bisaria and Mishra, 1989; Tomme et al., 1995; Bothast and Saha, 1997). Plant β-glucosidases have been reported to be involved in regulation of the physiological activity of phytohormones by hydrolysis of their inactive hormone–glucoside conjugates (Smith and Staden, 1978; Falk and Rask, 1995), chemical defense against pests (Conn, 1981; Niemeyer, 1988; Poulton, 1990), lignification (Dharmawardhana et al., 1995), β-glucan synthesis during cell wall development and cell wall degradation in the endosperm during germination (Brozobohaty et al., 1993; Leah et al., 1995), and food quality and flavor enhancement (Gunata et al., 1993). Inspite the studies of digestive β-glucosidases in their crude form have been reported from several insect species (Morgan, 1975; Schumaker et al., 1993; Adedire and Balogun, 1995), purification and characterization works have come up short on publication (Marana et al., 2000; Pontoh and Low, 2002). The larvae of *Galleria mellonella* feeds on honeycomb and cause serious economical damage. Due to nutritional and health benefits, honey especially together with honeycomb have been always in high demand. It contains glycosides along with various other compounds (Heath, 1982; Rakhi et al., 2010). Insect β-glucosidases as a type of digestive enzymes catalyze the hydrolysis of these β-glucosides. Hence, the present paper mainly aims to describe purification of the enzyme protein and its characterization as a β-glucosidase, for a better comprehending of its role and establishing a prospective control procedure, in *G. mellonella.*

MATERIALS AND METHODS

Insects

Laboratory colonies of *G. mellonella* have been bred from individuals that were collected from the honeycombs maintained by beekeepers around Balıkesir, Turkey. The larvae were reared on dark honeycomb at 25 ± 1°C, 60 ± 5% of relative humidity, and 12 h

light:12 h dark photoperiod as usual. Caterpillars (300 ± 25 mg in body weight) in the final instar stage were selected for bioassay analysis.

Chemicals

Sepharose-4B, L-tyrosine, 1-naphthylamine, *p*-NPG, *o*-NPG, *p*-NPFuc, *p*-/*o*-NPGal, *p*-nitrophenyl1-thio-β-D-glucopyranoside, protein assay reagents and chemicals for electrophoresis were purchased from Sigma-Aldrich (Germany). All other chemicals were of the best available grade.

Enzyme Preparation

Approximately 30 g of 10 larvae of *G. mellonella* were rinsed in cold distilled water and blotted with filter paper. They were ground using a prechilled mortar and pestle in 20 ml of 25 mM Tris-HCl buffer at pH 6.8. The homogenate was subjected to homogenizer using (Art-Miccra D-8, Müllheim) for 2 min, and was pressed through two layers of cheesecloth and the filtrate centrifuged at 6,000 rpm for 15 min at 4°C. The supernatant was used directly as the crude enzymic preparation.

Purification of the Enzyme

All purification steps were performed at 4°C, unless otherwise stated, using the procedures of Kara et al. (2011) with minor modifications. The crude enzyme was treated with solid ammonium sulfate to obtain the 0–70% saturation fraction by centrifuging at 15,000 rpm for 30 min. The precipitate was dissolved in 50 mM sodium phosphate buffer (pH 6.8) and the final saline concentration was adjusted to 1 M ammonium sulfate prior to applying to the sepharose-4B-L-tyrosine-1-naphthylamine column. The column (1.0 cm diameter × 5.0 cm length) was pre-equilibrated with 50 mM sodium phosphate buffer (pH 6.8) including 1 M $(NH_4)_2SO_4$ before loading the enzyme solution. The enzyme was eluted using a linear gradient of 1.0–0.0 M $(NH_4)_2SO_4$ in the same buffer at a flow rate of 30 ml/h; 1 ml fractions were collected. The proteins containing the highest β-glucosidase activity were combined and used as purified enzyme for subsequent studies after confirming homogeneity by gel electrophoresis.

Glucosidase Assay and Protein Determination

During enzyme extraction and purification, β-glucosidase activity was routinely determined using para- and ortho-nitrophenyl-β-D-glucopyranosides (*p*-NPG and *o*-NPG) as substrates. Appropriately diluted 70 μl of enzyme solution in 50 mM sodium acetate, pH 5.5 and 70 μl of substrate were mixed in the wells of a 96-well microtiter plate in quadruplicate. After incubation at 37°C for 30 min, the reaction was stopped by adding 70 μl of 0.5 M Na_2CO_3, and the color that developed as a result of *p*-/*o*-nitrophenol liberation was measured at 410 nm. Enzyme activity was expressed as μmol *p*-/*o*-nitrophenol formed per minute in the reaction mixture under these assay conditions. Protein concentration were determined at each stage of enzyme purification by the Lowry method (Lowry et al., 1951) using bovine serum albumin (BSA) as the standart.

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples were fractionated on 12% SDS-PAGE gels (Laemmli, 1970) using a Minigel system (Bio-Rad Laboratories, USA). Gels were fixed, stained with Coomassie brilliant blue R-250 (Sigma), and destained using standard methods to detect protein bands. For the detection of β-glucosidase activity in a nondenaturing PAGE, the enzyme solution was loaded onto 6% native polyacrylamide gel. After electrophoresis, the gel was equilibrated in two changes of 50 mM sodium acetate buffer, pH 5.5, for 15 min each, and then incubated with the substrate (4-MUG) for 15 min at 37°C. The band corresponding to the enzyme activity was observed and photographed under UV light.

Determination of pH and Temperature Optimum

The effect of varying the pH on *G. mellonella* β-glucosidase activity was examined using 25 mM sodium acetate (3.0–5.8), citrate-phosphate (3.0–7.0), and phosphate (6.0–8.5) buffers. For temperature optimum determination, the enzyme and substrate *p*-NPG solution mixtures were assayed in the temperature range 25–65°C for 30 min.

In vitro Inhibition Studies and Determination of Kinetic Parameters

Various final concentrations of *p*-NPG (0.8–20 mM) and *o*-NPG (0.8–20 mM) were used to estimate the kinetic parameters Km and Vmax. Inhibition experiments were performed using *p*-NPG as substrate and different final concentrations of β-gluconolactone and glucose as possible inhibitors. A double reciprocal Lineweaver–Burk plot was used to calculate the parameters. The activity of β-glucosidase for six different concentrations of each inhibitor was determined by regression analysis. Results are expressed as %, β-glucosidase activity in the absence of an inhibitor was taken as 100%. The inhibitor concentration that reduces the enzymatic activity by 50% (IC50 values) was determined from the plots.

RESULTS AND DISCUSSION

Beta-glucosidase was purified from crude extracts of the greater wax moth (*G. mellonella* L.) to apparent homogeneity by salting out with ammonium sulfate and using the specifically designed sepharose-4B-L-tyrosine-1-naphthylamine hydrophobic interaction chromatography. Upon fractionation of the β-glucosidase active fractions with ammonium sulfate, 45% of the activity was obtained in the fraction saturated with 70% ammonium sulfate. This step removed the greater part of the contaminants and decreased total protein amount from 7.18 to 1.93 mg. The precipitate with β-glucosidase activity was dissolved and saturated with 1 M ammonium sulfate, to improve its efficiency for binding, prior to applying onto the sepharose-4B-L-tyrosine-1-naphthylamine column. Figure 1 shows the typical elution pattern of the enzyme activity on this hydrophobic column. The enzyme activity and total protein concentrations were determined from all fractions collected. The fractions with the highest β-glucosidase activity and the relatively lower protein contents were pooled. This hydrophobic interaction chromatography step purified the enzyme from remaining contaminants to apparent homogeneity, retaining 63.5% of the activity from the previous step; therefore further purification steps were not required. The

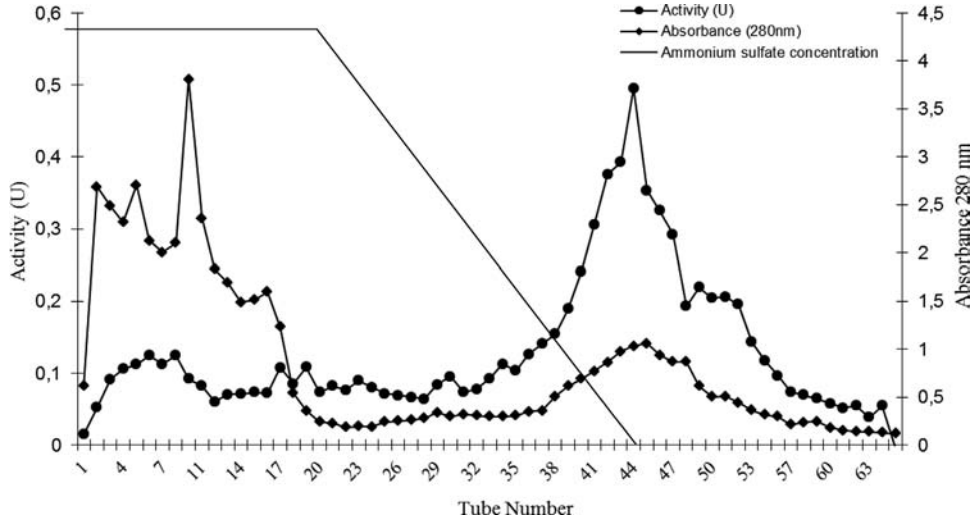

Figure 1. Purification of *Galleria mellonella* L. (Lepidoptera: Pyralidae) β-glucosidase by hydrophobic interaction chromatography. The enzyme activity and total protein concentrations were determined, from all fractions collected, as described in Materials and Methods Section. The enzyme activity was expressed as μmol of *p-*/*o-*nitrophenol liberated per minute in the reaction mixture under the assay conditions.

Table 1. Purification of* Galleria mellonella *L. (Lepidoptera: Pyralidae)* β*-Glucosidase

Step	*Total protein (mg)*	*Specific activity (U/mg)*	*Yield (%)*	*Purification factor (fold)*
Crude extract	7.18	0.42	100	—
Ammonium sulfate	1.93	0.71	45.36	1.68
Hydrophobic chromatography	0.036	24.36	28.77	57.5

β-glucosidase was purified 57.5 fold with an overall enzyme yield of 28.7% and a specific activity of 24.3 U/mg (Table 1).

The purification factor and the enzyme yield with the above procedure have been relatively high as compared with previous β-glucosidase purification studies from different sources. Romero-Segura et al. (2009) reported the purification of the enzyme with eightfold purification factor and enzyme yield of 8.3%. The purification factor and the enzyme yield values of β-glucosidase from vanilla bean and soybean have been reported as 7.2-fold and 8.4%, and 20-fold and 20%, respectively (Hsieh and Graham, 2001; Odoux et al., 2003). Employing least number of sequential steps (two in this case) is an important strategy to obtain maximum enzyme yield. Also, the structure and characteristics of matrix and ligand of hydrophobic interaction chromatography are critically important. In our study 1-napthylamine, which is a hydrophobic group, was added to sepharose-4B gel matrix with the extension of L-tyrosine arm. Having an aryl-structured hydrophobic 1-napthylamine in the hydrophobic gel have been shown to increase the purification factor of the enzyme. Since aryl ligands have hydrophobic and aromatic characters, our results also suggest that the enzyme shows both hydrophobic and aromatic interactions with these residues (Kara et al., 2011).

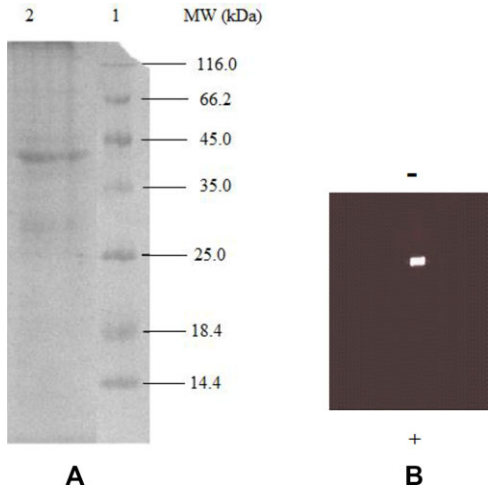

Figure 2. (A) SDS-PAGE of the purified β-glucosidase from *Galleria mellonella* L. (Lepidoptera: Pyralidae). The enzyme was electrophoresed at pH 8.3 on a 12% polyacrylamide gel and stained with Coomassie brilliant blue R-250. Lanes: 1, molecular weight standards (β-galactosidase,116 kDa; bovine serum albumin, 66.2 kDa; egg albumin, 45 kDa; lactate dehyrogenase, 35 kDa; Rease Bsp981 (*Escherichia coli*), 25 kDa; β-lactoglobulin, 18.4 kDa; Lysozyme, 14.4 kDa; 2, purified β-glucosidase. (B) Native-PAGE (6%) gel zymogram of β-glucosidase developed with the fluorogenic substrate 4-MUG.

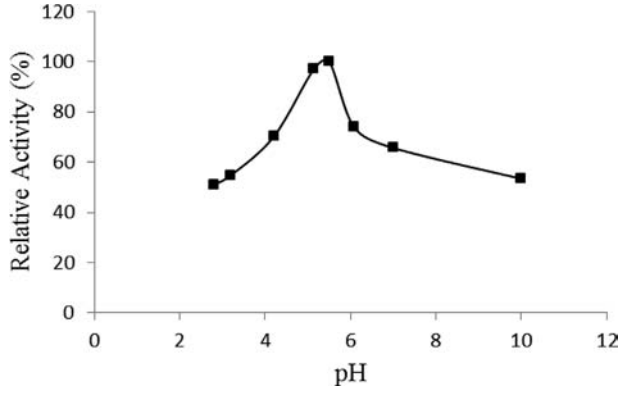

Figure 3. Effect of pH on activity of purified β-glucosidase from *Galleria mellonella* L. (Lepidoptera: Pyralidae).

SDS-PAGE analysis of the purified enzyme showed the presence of a single band with an apparent molecular mass of ca. 42 kDa, when stained with Coomassie brilliant blue (Fig. 2A). The estimated subunit molecular mass of the protein is different to β-glucosidases from various insect sources, such as 72 kDa from honey bees (*Apis mellifera*) (Pontoh and Low, 2002) and 57 kDa from the silkworm (*Bombyx mori*) (Gyeong et al., 2005). In order to confirm the activity data of native protein with spectrophotometric assays, native-PAGE zymogram assay was performed. The result showing an activity band of beta-glucosidase is shown in Fig. 2B.

The pH optimum for β-glucosidase activity was 5.5 (Fig. 3), and the enzyme retained over 50% of the original activity between pH 4.5 and 6.5. This pH optimum is in agreement with the previously determined optimum pH values of β-glucosidases from various insect

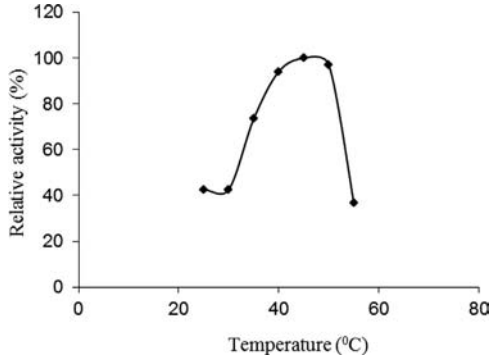

Figure 4. Effect of temperature on activity of purified β-glucosidase from *Galleria mellonella* L. (Lepidoptera: Pyralidae).

Table 2. Relative Activity of* Galleria mellonella *L. (Lepidoptera: Pyralidae)* β-*Glucosidaseon Various Substrates

Substrate	*Relative activity, %*
p-Nitrophenyl β-D-glucopyranoside (*p*-NPG)	100
o-Nitrophenyl β-D-glucopyranoside (*o*-NPG)	109
p-Nitrophenyl β-D-galactopyranoside	41
o-Nitrophenyl β-D-galactopyranoside	35
p-Nitrophenyl β-D-fucopyranoside	455
p-Nitrophenyl 1-thio-β-D-glucopyranoside	1

sources (Ferreira et al., 1998; Pontoh and Low, 2002; Marana et al., 2000; Gyeong et al., 2005).

The enzyme displayed maximal activity at 42°C (Fig. 4). Similar temperature optima of β-glucosidases have been reported from an insect rice striped stem borer (Zibaee et al., 2009) and from several other organisms (Saha and Bothast, 1996a; Hernandez et al., 2003; Turan and Zheng, 2005), but also found lower than for some insect and other sources of β-glucosidases that show the highest enzymatic activity at around 50°C (Chen and Halkier, 1999; Pontoh and Low, 2002; Bernardi et al., 2003; Li et al., 2005). Increased catalytic activity at higher temperatures, such as 50°C, is not physiologically meaningful because the activity is lost due to thermal denaturation in a very short incubation time (Li et al., 2005; Turan and Zheng, 2005).

The reaction kinetics of the purified β-glucosidase were determined from Lineweaver–Burk plots with artificial substrates *p-/o*-nitrophenyl-β-D-glucopyranosides (*p-/o*-NPG). The enzyme was effectively active on *p-/o*-NPG with K_m values of 0.37 and 1.9 mM and Vmax values of 625 and 189 U/mg, respectively. Affinity of the enzyme for *p*-NPG was considerably higher than for *o*-NPG. The higher β-glucosidase affinities for *p*-NPG have also been reported from different sources (Hsieh and Graham, 2001; Odoux et al., 2003; Li et al., 2005). The enzyme also exhibits different levels of activity against various other substrates (Table 2). Rate of hydrolysis of *para*-nitro-phenyl-β-D-fucopyranoside (*p*-NPF) surprisingly was 4.55, relative to *p*-NPG. Similarly, higher activity rates of β-glucosidases from vanilla bean, butter bean, and wheat seedlings for *p-/o*-NPF have been reported (Itoh-Nashida et al., 1987; Sue et al., 2000; Odoux et al., 2003), however, these fucopyranosides

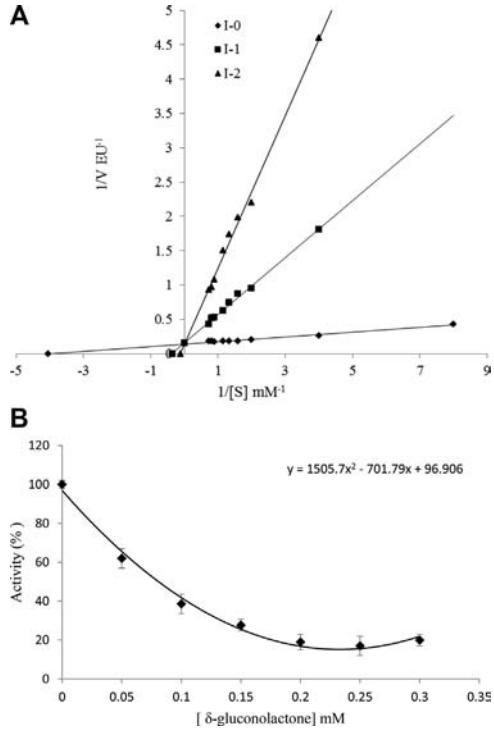

Figure 5. Inhibition of the purified β-glucosidase from *Galleria mellonella* L. (Lepidoptera: Pyralidae) by δ-gluconolactone. (A) Lineweaver–Burk plot with various substrates (*p*-NPG) and inhibitor (δ-gluconolactone) concentrations for determination of K_i and inhibition type. The intercept of plots indicates competitive inhibition for δ-gluconolactone. (B) Activity (%) curve of the β-glucosidase in the presence of different δ-gluconolactone concentrations.

competitively inhibited β-glucosidase activity from the yeast *P. pastoris* against *p*-NPG, *o*-NPG, and 4-MUG as substrates (Turan and Zheng, 2005).

The inhibition kinetic experiments of the enzyme were performed using *p*-NPG as substrate, while δ-gluconolactone and glucose as inhibitors. The enzyme was inhibited by both inhibitors investigated. δ-Gluconolactone was the most effective (competitive) inhibitor of the enzymatic activity with K_i value of 0.021 mM (Fig. 5A) and IC_{50} of 0.27 mM (Fig. 5B). This strong inhibitory effect of δ-gluconolactone is in agreement with the previous reports regarding the inhibition of β-glucosidases from various sources (Dharmawardhana et al., 1995; Odoux et al., 2003; Kara et al., 2011). Based on the published reports we have scanned, this is the first study regarding glucose inhibition of β-glucosidase activity from insects. However, the inhibition kinetics of β-glucosidases from several plant and especially microorganism sources have been extensively studied using glucose as an inhibitor, since glucose inhibition of β-glucosidases is undesirable if the enzymatic hydrolysis of cellulose is performed as an industrial process. β-Glucosidases from *Arabidopsis thaliana* and vanilla bean were not inhibited by glucose up to 250 mM and 2 M, respectively (Odoux et al., 2003; Turan, 2008), while the enzyme from orange fruit was effectively inhibited by lower concentrations (Cameron et al., 2001). Highly glucose tolerant β-glucosidases have been reported from yeasts *Candida sake, Pichia etchellsii, D. vanrijiae vanrijiae*, and *Candida peltata* with K_i values of 0.2, 0.3, 0.44, and 1.4 M, respectively (Saha and Bothast, 1996b; Pandey

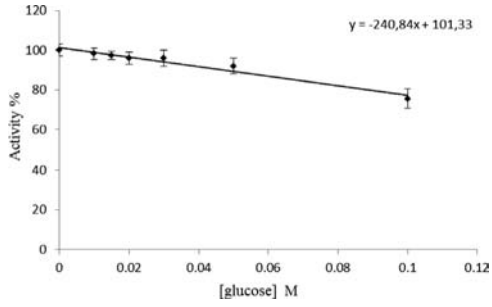

Figure 6. Inhibition of the purified β-glucosidase from *Galleria mellonella* L. (Lepidoptera: Pyralidae) by glucose. Activity (%) curve of the β-glucosidase in the presence of different glucose concentrations.

and Mishra, 1997; Gueguen et al., 2000; Belancic et al., 2003). According to Saha and Bothast (1996a), β-glucosidase activities from some yeast strains were even stimulated by glucose. However, most microbial β-glucosidases are strongly inhibited by glucose with the inhibition constants ranging from 0.6 to 10 mM (Gunata et al., 1993). Interestingly, similar to various organism β-glucosidases summarized above, the *G. mellonella* L. (Lepidoptera: Pyralidae) β-glucosidase activity was also highly tolerant to glucose with IC_{50} value of 213.13 mM towards *p*-NPG as substrate (Fig. 6).

In conclusion, the β-glucosidase from *G. mellonella* L. (Lepidoptera: Pyralidae) was first time purified to apparent homogeneity and then characterized for a better comprehending of it's role and establishing a prospective control procedure, in *G. mellonella*. The purification was only in two sequential steps, by salting out with ammonium sulfate and using the specifically designed sepharose-4B-L-tyrosine-1-naphthylamine hydrophobic interaction chromatography. This is an important factor to obtain the higher protein yield since the necessary protein might be lost or decomposed during the traditional prolonged sequential purification steps.

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