Full Length Research Paper

Purification and characterization of *β*-glucosidase from *Reticulitermes flaviceps* and its inhibition by valienamine and validamine

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β-Glucosidase plays a very important role in the carbohydrate metabolism and it is the key enzyme that releases glucose for use as an energy and carbon source for termite. The β-glucosidase from the salivary glands of *Reticulitermes flaviceps* was purified in this study. The molecular mass of the purified enzyme was estimated to be 93.6 kDa based on its mobility in SDS-PAGE. The β-Glucosidase was stable at pH ranging from 5.0 - 6.8 and below 45°C. Its optimal temperature and pH were 35 - 40°C and 5.2 - 6.0, respectively. The β-Glucosidase was competitively inhibited by valienamine and validamine *in vitro*. The inhibition was pH-dependent and dose-dependent. The maximum inhibitory capacity of valienamine and validamine was at the optimal pH of this enzyme. The K_i values of valienamine and validamine were 1.3 and 1.9 mM, respectively, and the IC_{50}s of valienamine and validamine were observed at 1.92 and 2.92 mM, respectively.

Key words: *Reticulitermes flaviceps*; *β*-glucosidase; valienamine; validamine; inhibition.

INTRODUCTION

Termites live in large, socially-structured colonies of workers, soldiers and reproductives with members numbering in the millions (Zimmerman et al., 1982). They play an important role in the breakdown of cellulose in the natural environments. However they are highly destructive pest, causing significant economic losses through damage to timber-in-service such as building, poles, bridges and railway sleepers in addition to forest, fruit and ornamental trees (Su, 2002). The two most effective control options for subterranean termites are soil treatment and baiting. Soil treatments are typically made with large volumes of liquid termiticides that are either neurotoxins or inhibitors of mitochondrial respiration. Baiting, on the other hand, involves recruiting termites to feed on substrates impregnated with a slow-acting chemical insecticide. Both approaches have drawbacks, for example, soil termiticides raise many environmental concerns and baits do not immediately reduce termite populations. In this respect, there is a need for faster-acting bait active in gredients with good environmental

characteristics and broad-spectrum termite's activity (Zhou et al., 2008).

Extensive hydrolysis of cellulose usually requires the activity of enzymes of all three functional classes: endoglucanases (endo-1,4-*β*-glucanases, EC 3.2.1.4); exoglucanases, which include cellobiohydrolase (1,4-*β*-D-glucan cellobiohydrolase, EC 3.2.1.91) and exoglucohydrolase (1,4-*β*-D-glucan glucohydrolase, EC 3.2.1.74); and *β*-glucosidases (EC 3.2.1.21). The endogenous cellulolytic enzymes of termites primarily consist of endo-*β*-1,4-glucanase and *β*-glucosidase (Tokuda et al., 1997; Watanabe et al., 1997; Tokuda et al., 2002; Watanabe et al., 2002; Yang et al., 2004). *β*-Glucosidase is a critical enzyme for cellulose degradation, catalyzing hydrolysis of cellobiose or cello-oligomers to glucose for use as an energy and carbon source for termites (Zhang et al., 2001; Yang et al., 2003; Mo et al., 2004; Sugio et al., 2006). Inhibition on the *β*-glucosidase activity to stop releasing glucose from cellooligosaccharides is sufficient to starve termites. There may be a novel avenue for control of termites by using *β*-glucosidase inhibitors (Zhu et al., 2005).

So far, endogenous endo-*β*-1,4-glucanases of termites have been extensively studied (Tokuda et al., 1997; Watanabe et al., 1997; Watanabe et al., 1998; Watanabe

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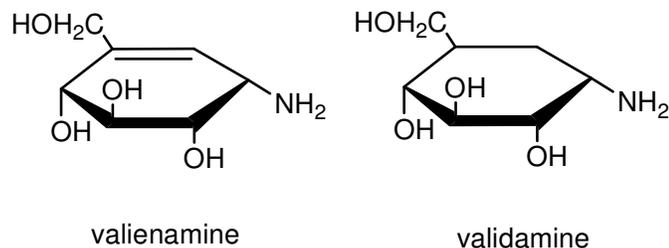
Figure 1. Structures of valienamine and validamine.

et al., 2002). By contrast, biochemical and molecular biological studies on *β*-glucosidase of termites are limited. In the past years, only *Macrotermes muelleri* (Rouland et al., 1992) and *Neotermes koshunensis* (Tokuda et al., 2002) have been used for purification of the endogenous *β*-glucosidase from the termites. A *β*-glucosidase cDNA from the termite of *N. koshunensis*, was successfully overexpressed in *Escherichia coli* (Ni et al., 2007). In the research of termite *β*-glucosidase inhibition, Zhu et al. (2005) have developed an *in vivo* method to test the inhibitory ability of the chemicals to act on *β*-glucosidase and examined the inhibition of *Coptotermes formosanus* *β*-glucosidase *in vivo* by gluconolactone, ferulic acid, sinapinic acid, 1-deoxynojirimycin, and conduritol B epoxide. Zhou et al. (2008) tested three cellulase inhibitors cellobioimidazole, fluoromethyl cellobiose and fluoromethyl glucose against *R. flavipes*. Valienamine [(1S, 2S, 3S, 4R)-1-amino-5-(hydroxymethyl) cyclohex-5- ene-2, 3, 4-triol] and validamine [(1S)-(1, 2, 3, 4, 5)-1-amino-5-hydroxymethyl-2,3,4- cyclohexanetriol] (Figure 1) were two prominent members of C_7N aminocyclitol family of natural products which are increasingly gaining recognition due to their significant biomedical uses. They exhibit α-glucosidase inhibitory and antibiotic activity (Takeuchi et al., 1990; Takeuchi et al., 1990; Chen et al., 2003; Zheng et al., 2005). However, there are few investigations on their inhibition on *β*-glucosidase. Since *β*-glucosidase has a central role in carbohydrate metabolism in termite. *β*-Glucosidase inhibitors can be an important tool for the study of their mechanisms of action, and might assist in the development of potent insecticides. In this paper, we purified the *β*-glucosidase from the salivary glands of *R. flaviceps* and studied its properties and inhibition by valienamine and validamine.

MATERIALS AND METHODS

Materials

R. flaviceps colonies were collected from the Lingping Park (Yuhang, Zhejiang, China) and maintained in glass container with pine wood stick, which were supplied by Yuhang Station of Termite Control (Hangzhou, Zhejiang, China). Termite workers were directly taken out of the nests and then store at –20°C in our laboratory. Valienamine and validamine were prepared according to the me-

thod as described (Zheng et al., 2004). All other chemicals were of analytical grade and commercially available.

Enzyme purification

All procedures for protein purification were conducted at 4°C or under ice condition otherwise noted. The termite salivary glands were collected from 1.81 g worker termites and sonicated (250 W at 5 s intervals for 300 times) in 15 ml of 100 mM sodium phosphate buffer to release the *β*-glucosidase. After sonication, the sample was centrifuged (Avanti J-E, Beckman Coulter Inc., USA) at 48,400×g for 10 min and the supernatant was collected. The volume of raw extract solution was 14 ml. This solution was designated as the raw extract in *β*-glucosidase purification.

Then the *β*-glucosidase was separated by columns employed a Biologic Duo Flow system (Bio-Rad Laboratories, USA). Firstly, the sample was applied to an anion-exchange column (High Q, 20 ml, Bio-Rad laboratories, USA). The column was washed with 100 ml of starting buffer (50 mM Tris-HCl, pH 7.5) and the absorbed proteins were eluted employing the same buffer with a linear gradient of NaCl from 0 to 500 mM at a flow rate of 1.0 ml/min. Active fractions were concentrated and desalted by dialysis in 50 mM sodium acetate buffer containing 10% glycerol at pH 4.6 with two changes of fresh buffer. The desalted enzyme solution was applied to a weak acid cation column of CM IEC (Bio-Rad laboratories, USA) that had been equilibrated with 50 mM sodium acetate buffer containing 10% glycerol at pH 4.6. The column was washed with the same buffer at a flow rate of 1.0 ml/min and the absorbed proteins were eluted from the column employing a mobile phase of the same buffer with stepwise linear gradient from 50 to 500 mM at flow rate of 1.0 ml/min. The active fraction was pooled and desalted by dialysis in 10 mM sodium phosphate at pH 6.8. The enzyme solution was concentrated using vacuum freeze-drying plant (Labconco, USA), and then were dissolved in 2 ml of 10 mM sodium phosphate buffer (pH 6.8). Finally, the enzyme solution chromatographed on a 70 × 1.5 cm column (Bio-Rad laboratories, USA) packed with 200 ml Sephadex G-100 and proteins were eluted with 10 mM sodium phosphate buffer (pH 6.8) at a flow rate of 0.4 ml/min. Homo-geneous *β*-glucosidase obtained from active fractions was pooled for latter analysis.

SDS-PAGE analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed using a Mini-gel system (Bio-Rad Laboratories, USA) with a 5% acrylamide stacking gel (pH 6.8) and 12% separating gel (pH 8.8) (Laemmli, 1970). The protein stan-dards used were as follows: rabbit phosphorylase B, 97.4 kDa; bovine serum albumin, 66.2 kDa; rabbit actin, 43.0 kDa; bovine carbonic anhydrase, 31.0 kDa; and trypsin inhibitor, 20.1 kDa (Shanghai Sangon Co. Ltd.). The gel was stained with coomassie brilliant blue R-250.

Enzyme assays

β-glucosidase activity against *p*-nitrophenyl-*β*-D-glucopyranoside (*β*-PNPG) was determined employing a modification of the Low method (Low et al., 1986). A selected volume of sample was diluted with its sample buffer to a total volume of 0.1 ml and was added to 4.9 ml of sodium phosphate buffer (0.1 M, pH 6.0) containing *β*-PNPG (20 mM final). The reaction mixture was incubated at 37°C for 10 min. To this solution was added 1 ml of 1 M Na_2CO_3. After cooling to room temperature, liberated *p*-nitrophenol (pNP) in this mixture was assayed by spectrophotometry at 400 nm. One *β*-glucosidase activity unit (U) was defined as the quantity of enzyme
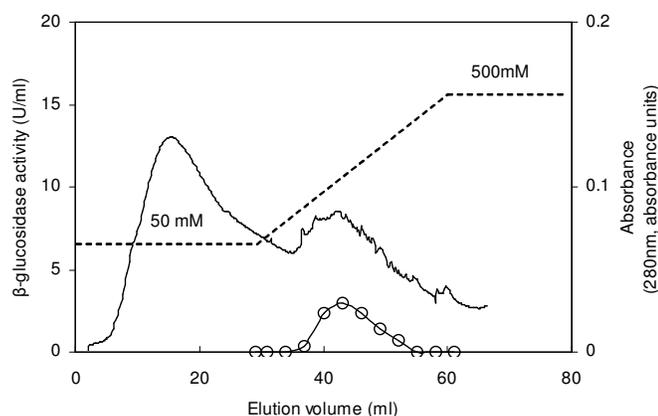
Figure 2. CM IEC cation-exchange chromatography. (—) protein content measured by absorbance at 280 nm; (O) β-glucosidase activity (U/ml); (- - -) NaAc gradient (50 - 500 mM).

Figure 3. Sephadex G-100 gel filtration chromatography. (—) protein content measured by absorbance at 280 nm; (O) β-glucosidase activity (U/ml).

required produce 1.0 µmol of *p*-nitrophenol per minute under these experimental conditions.

The β-glucosidase activity was determined by assaying the liberated glucose for the study of substrate specificity. Enzyme solution (0.1 ml) and the substrate of various concentrations were added to 4.9 ml of sodium phosphate buffer (0.1 M, pH 6.0). The reaction mixtures were incubated at 37°C for 10 min. And then 1 ml of 1 M Na_2CO_3 was added to this solution. After cooling to room temperature, liberated glucose was determined according to the method as described (Zhan et al., 2003). K_m values were determined for the various substrates from Lineweaver-Burk double reciprocal plots.

Protein assays

Protein concentrations were determined at each stage of enzyme purification by the Bradford method (Bradford, 1976) with bovine serum albumin (BSA) as the standard. Protein concentrations in the column effluents were monitored by measuring A_{280}.

Inhibition of β-glucosidase by valienamine and validamine

Kinetics of enzyme inhibition: The enzyme reactions were performed according to the above reaction condition with inhibitors of various concentrations (0, 1.5 and 3.0 mM). The reactions were started by adding different concentrations of substrate (β-PNPG) to the enzyme/inhibitor solutions. After incubation in a 37°C water bath for 10 min. The reaction was stopped by adding 1.0 ml of 1.0 M Na_2CO_3 solution. From the above reactions, Lineweaver-Burk double reciprocal plots were used to determine the type of inhibition and the inhibition constants (K_i).

Effect of pH on the binding of the inhibitors: The effect of pH on the binding of the inhibitors was estimated by measuring the inhibition constants at different pH values ranging from 3.2 to 8.0, 200 mM sodium citric acid was used as buffer solutions. At each pH value, Dixon plots of 1/v versus the concentration of the inhibitor were used to determine the inhibition constants. Then plots of 1/K_i versus pH value were used to determined relationships of pH value to β-glucosidase inhibition by valienamine and validamine.

Effect of inhibitors concentrations on enzyme activity: Various amounts of valienamine or validamine were added to assay mixtures containing the β-glucosidase, 20 mM β-PNPG in 100 mM sodium phosphate buffer (pH 6.0). The mixtures were then incubated in a 37°C water bath for 10 min. The residual activity was
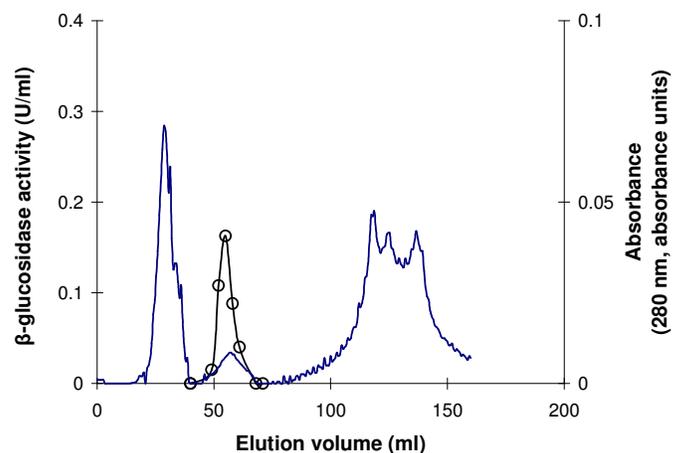
determined. IC_{50} value is concentration of inhibitor necessary to inhibit β-glucosidase activity by 50%.

RESULTS

Purification of β-glucosidase from *R. flaviceps*

Through high Q anion-exchange chromatography, CM cation-exchange chromatography (Figure 2), and Sephadex G-100 Gel filtration (Figure 3), β-glucosidase from termite was purified approximately 48.83-fold, and the final yield was approximately 8.34%. Table 1 presents the effects of each purification step on total protein, activity, specific activity, and fold of purification from the termite. A homogeneous protein was obtained according to the criteria of SDS-PAGE with a molecular mass of around 93.6 kDa.

Characterization of purified β-glucosidase

Effect of temperature and pH

Optimum temperatures were determined using 10 min incubation on 0.02 M β-PNPG in 100 mM sodium phosphate buffer (pH 6.0) at a temperature ranging from 20 - 50°C. Optimum pH was determined using 10 min incubations at 37°C. Thermostability was determined after 30 min incubations in 0.1 M sodium phosphate buffer (pH 6.0) at 20 - 50°C. The pH stabilities were determined after 30 min incubations in universal buffer from 3.6 - 8.0 at 37°C. The residual enzyme activity was determined under standard conditions described above. The buffers used for these experiments were 0.1 M glycin-hydrochloric acid for pH 3.0, 0.1 M sodium acetate buffer for pH 4.0 - 6.0, and 0.1 M sodium phosphate buffer for pH 6.0 - 8.0, respectively.

Table 1. Summary of purification of β-glucosidase from *R. flaviceps.*

Step	Activity (µmol/min)	Protein (mg)	Specific activity (U/mg)	Yield (%)	Fold of purification
Crude extract	189.88	410.00	0.46	100.00	1.00
High Q	108.26	63.3	1.71	57.01	3.69
CM IEC	43.31	8.55	5.10	22.81	11.00
Sephadex G-100	15.83	0.7	22.61	8.34	48.83
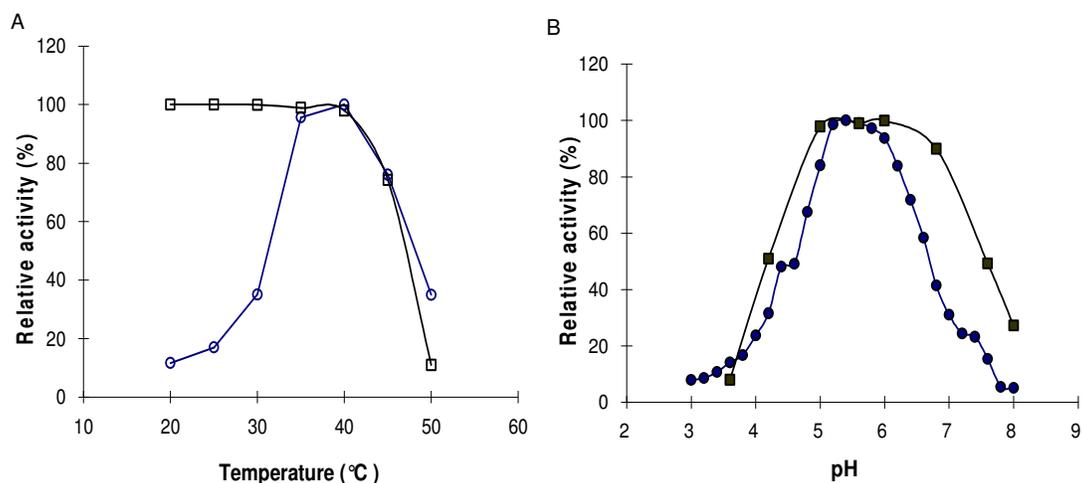
Figure 4. Effect of temperature (A) and pH (B) on the activity and stability of β-glucosidase measured on 20 mM β-PNPG. Temperature optimum (○); pH optimum (●), temperature stability (□); pH stability (■)., The buffers used were 0.02 M glycin-hydrochloric acid for pH 3.0, 0.02 M sodium acetate buffer for pH 4.0 - 6.0, 0.02 M sodium phosphate buffer for pH 6.0 - 8.0.

No peculiar property could be observed during the studies of effect of pH and temperature on the maximum activity and stability of β-glucosidase (Figure 4). The optimal pH value was between 5.2 and 6.0 and optimal temperature was between 35 and 40 °C. The enzyme was stable at pH ranging 5.0 - 6.8 and below 45 °C. During storage at 50 °C for 30 min, the enzyme lost about 65% of its activity. The enzyme was stable at a pH ranging 5.0 - 7.0.

Effect of metal ions and chemicals

The effects of several metal ions and EDTA were examined (Table 2). A sodium acetate buffer (pH 6.0) was used as the reaction medium in place of a citrate: phosphate buffer, since some ions formed insoluble salts in a buffer containing phosphate. The β-glucosidase activity was not affected by EDTA (1.0 and 2.0 mM). Of all the metal ions tested, Ag^+ and Hg^{2+} completely inhibited the enzyme activity at a concentration of 1.0 mM. Mn^{2+} and Ni^{2+} reduced the activity of β-glucosidase by 20% or more at a concentration of 2.0 mM, and Cu^{2+} decreased the activity to 45.1% of its maximum at a concentration of 2.0 mM. Zn^{2+} and Co^{2+} had no obviously

Table 2. Effect of EDTA and metal cations on β-glucosidase activity.

Metal cation	Concentration (mM)	Reactive activity (%)
Control		100
EDTA	1	100
	2	100
Ag^+	1	None[a]
	2	None
Zn^{2+}	1	97.4
	2	95.7
Mn^{2+}	1	88.2
	2	72.4
Hg^{2+}	1	None
	2	None
Ni^{2+}	1	87.9
	2	74.5
Co^{2+}	1	96.5
	2	101.3
Cu^{2+}	1	61.1
	2	45.1

[a] No β-glucosidase activity was observed

Table 3. Michaelis-constants of the purified enzyme for various substrates.

Substrate	K_m (mM)
p-nitrophenyl-*β*-D-glucopyranoside	9.3
p-nitrophenyl-*α*-D-glucopyranoside	None[a]
Cellobiose	1.9
Cellotriose	7.4
Lactose	12.1
maltose	None
Gentiobiose	43.4
Validamycin A	9.1

[a] No *β*-glucosidase activity was observed.

effect on enzyme activity.

Substrate specificity

The affinity of the enzymes was determined for several substrates. The results were shown in Table 3. The *β*-glucosidase exhibited hydrolytic activity toward cellobiose, cellotriose, lactose, *p*-nitrophenyl-*β*-D-glucopyranoside, gentiobiose and validamycin A. Among all the substrates tested in this study, the most favorable was found to be cellobiose. The K_m value for cellobiose was 1.7 mM. The enzyme showed weak activity against gentiobiose. No activities were observed against maltose and *p*-nitrophenyl-*α*-D-glucopyranoside.

Inhibition of β-glucosidase by valienamine and validamine

Kinetics of enzyme inhibition

To characterize the mechanism of inhibition of valienamine and validamine, kinetic studies were performed at pH 6.0. When *β*-PNPG was used as substrate, K_m value of *β*-glucosidase was 9.3 mM. The result showed that valienamine and validamine were competitive inhibitor of the *β*-glucosidase (Figure 5). By calculation, the K_i values for valienamine and validamine were about 1.3 and 1.9 mM, respectively. The difference of K_i values indicates that the enzyme recognizes the difference of structures in valienamine and validamine (a double bound of carbon in valienamine).

Effect of pH on the binding of the inhibitors

The effect of pH on the binding of the inhibitor was estimated by measuring the inhibition constants at different pH values ranging from 3.2 to 8.0. Then plot of $1/K_i$ versus pH value was used to determine the effect of pH on *β*-glucosidase inhibition. Termite *β*-glucosidase presents a pH dependence of the inhibition by valiena-
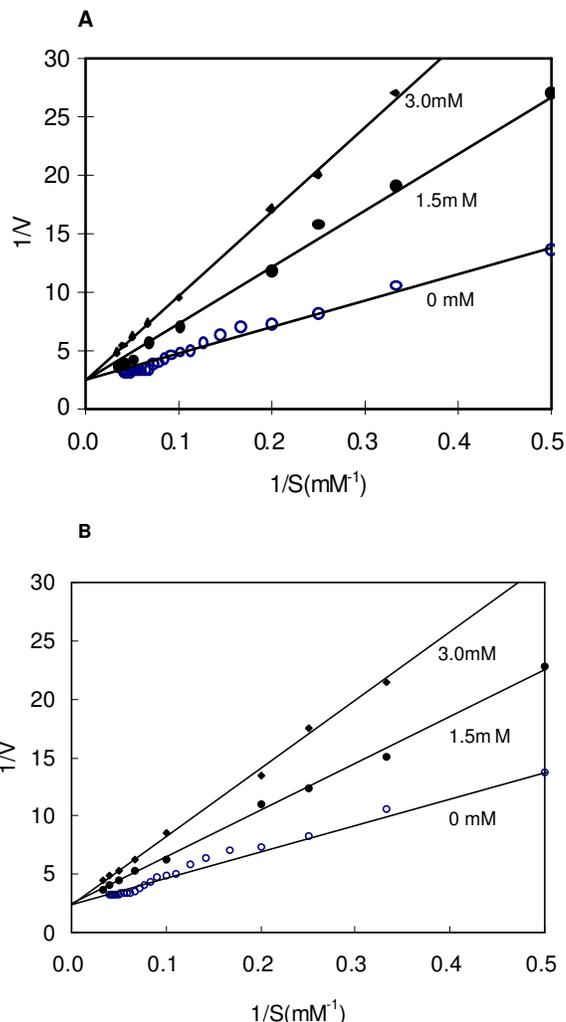
Figure 5. Lineweaver-Burk double reciprocal plots of *β*-glucosidase activity in the absence or presence of various concentration of valienamine (A) or validamine (B).

mine and validamine. The pH dependence for the binding ($1/K_i$) of the two compounds to *β*-glucosidase is illustrated in Figure 6. The pH profile shows that the maximum inhibitory capacity of valienamine and validamine was at the optimal pH of this enzyme.

Effect of inhibitors concentrations on enzyme activity

The dose-dependent inhibition of *β*-glucosidase activity by valienamine and validamine is shown in Figure 7. IC_{50}s of valienamine and validamine were 1.92 and 2.92 mM, respectively.

DISCUSSION

β-Glucosidases are widely distributed in the living world and play pivotal roles in many biological process, such as
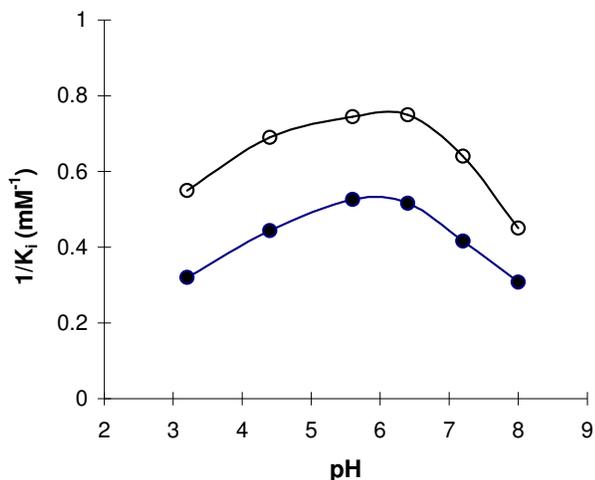
Figure 6. pH dependence for the binding of valienamine (○) and validamine (●) to β-glucosidase.
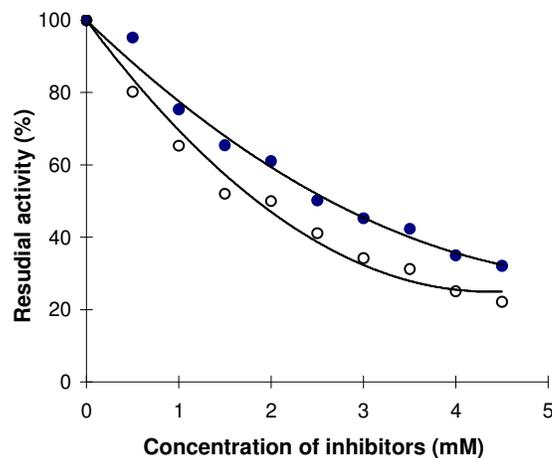
Figure 7. Effect of valienamine (○) and validamine (●) concentration on the β-glucosidase activity, IC_{50} value is concentration of inhibitor necessary to inhibit β-glucosidase activity by 50%.

degradation of cellulosic biomasses, hydrolysis of glyco-lipids, cyanogenesis, and modification of secondary metabolites. They are found widely in fungi (Peralta et al., 1997; Saibi et al., 2007), yeast (Ohnishi and Tanaka 1996; Villena et al., 2007), bacteria (Nunoura et al., 1996), plants (Akiyama et al., 1998) and insects (Pontoh and Low 2002). Many β-glucosidases have been purified and their biological properties were characterized and reported. Termites play an important role in the break-down of cellulose in natural environments, and β-glucosidase is the key enzyme that releases glucose from cellulose for use as an energy and carbon source for termites. The research of β-glucosidase from termites is interesting and important. In numerous species of termites, the *R. flaviceps*, which is commonly distributed in warm temperate and subtropical regions, is one of the major damaging termite pests (Stansly et al., 2001; Zhang et al., 2001). The salivary gland is the one of the major site of β-glucosidase production in *R. flaviceps* (Yang et al., 2003). In the present study, through high Q anion-exchange chromatography, CM cation-exchange chromatography and Sephadex G-100 gel filtration, β-glucosidase from the salivary glands of *R. flaviceps* was purified to homogeneity by SDS-PAGE with a molecular mass of 93.6 kDa. The enzyme may be in the monomeric form and it is different as compared with β-glucosidase from N. koshunensis (60 kDa) which is predicted to be monomeric (Tokuda et al., 2002). β-Glucosidase from *R. flaviceps* exhibited broad substrate specificity. It is active not only on cellobiose or lactose but also *p*-nitrophenyl-β-D-glucopyranoside, confirming that the termite β-glucosidase is affiliated with class 1 enzymes, which hydrolyze both native and synthetic substrates according to the classification of Terra and Ferrira (1994). The substrate specificities did not show any significantly diffe-rences with that of *M. muelleri* (Rouland et al., 1992) and *N. koshunensis* (Tokuda et al., 2002). β-Glucosidase

activity of *R. flaviceps* was not affected by EDTA, sug-gesting that metal ions are not essential for the enzyme activity. Silver and mercury ions completely inhibited the enzyme activity at a concentration of 1.0 mM, suggesting that sulfhydryl groups may play an essential role in enzyme activity.

Cellulases are considered as a potential target site for novel and more environmentally friendly termite-specific insecticides. Searching for and designing inhibitors, which inhibit β-glucosidase is a novel approach for the potential management of termites. Zhu et al. (2005) have developed an *in vivo* method to test the inhibitory ability of the chemicals to act on β-glucosidase and examined five prototype β-glucosidase inhibitors against *C. formosanus* after feeding in a prototype enzyme activity assay. One inhibitor was found completely ineffective (sinapinic acid) while four others (conduritol B epoxide, 1-deoxynojirimycin, ferulic acid and gluconolactone) pro-vided between 27 and 65% inhibition. Zhou et al. (2008) observed three cellulase inhibitors cellobio-imidazole, fluoromethyl cellobiose and fluoromethyl glucose against *R. flavipes*. *In vitro*, fluoromethyl glucose showed virtually no inhibition of β-glucosidase, cellobio-imidazole and fluoromethyl cellobiose both inhibited β-glucosidase activity (I_{50}s in nM and mM range, respectively). In this study, valienamine and validamine competitively inhibited β-glucosidase activity from the salivary glands of *R. flaviceps in vitro*. Moreover, the inhibition was pH-dependent and dose-dependent. The maximum inhibitory capacity of valienamine and validamine was at the optimal pH of this enzyme. The K_i values of valienamine and validamine were 1.3 mM and 1.9 mM respectively. The IC_{50}s were in mM range which was similar with cellobio-imidazole (Zhou et al., 2008). To our knowledge, this is the first investigation of valienamine and valida-mine as β-glucosidase inhibitors in insect. The findings

mentioned above may provide some useful information about the *β*-glucosidase and help to design new potent inhibitors for the termite *β*-glucosidase to control termites.

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