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# Functional analyses of the digestive β-glucosidase of Formosan subterranean termites (*Coptotermes formosanus*)

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ABSTRACT

The research was to elucidate the function of the β-glucosidase of Formosan subterranean termites in *vitro* and in *vivo*. The gene transcript was detected predominantly in the salivary gland tissue, relative to the midgut and the hindgut of the foraging worker caste, indicating salivary glands were the major expression sites of the β-glucosidase. Using recombinant β-glucosidase produced in *Escherichia coli*, the enzyme showed higher affinity and activity toward cellobiose and cellotriose than other substrates tested. In assessing impacts of specific inhibitors, we found that the β-glucosidase could be irreversibly inactivated by conduritol B epoxide (CBE) but not gluconolactone. Termite feeding assays showed that the CBE treatment reduced the glucose supply in the midgut and resulted in the body weight loss while no effect was observed for the gluconolactone treatment. These findings highlighted that the β-glucosidase is one of the critical cellulases responsible for cellulose degradation and glucose production; inactivation of these digestive enzymes by specific inhibitors may starve the termite.

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## 1. Introduction

Wood-feeding termites, such as *Coptotermes formosanus*, have twofold impacts on our society and economy: they play an important role in natural biomass recycling and can seriously damage wood structures and landscaping trees. Understanding catalytic enzymes that are responsible for efficient wood degradation in these creatures would also have twofold implications: application for converting wood-cellulose to glucose for biofuel production and development of cellulase-specific inhibitors as termiticides to starve the termite.

Genes encoding wood degradation enzymes in termites have been sought via metagenomics (such as Warnecke et al., 2007) or metatranscriptomics (such as Tartar et al., 2009). These data revealed rich reservoirs of wood processing enzymes/proteins based upon sequence similarity and prediction. Concurrently, functional analyses of individual candidate genes via heterologous expression systems have shed light on the true enzymatic properties, some of which include endogenous endo-β-1,4-glucanases in *C. formosanus* (Zhang et al., 2009, 2011), in *Nasutitermes takasagoensis* (Hirayama et al., 2010) and in *Reticulitermes flavipes* (Zhou et al., 2010); symbiont endoglucanase in *Reticulitermes speratus* (Todaka et al., 2010); endogenous β-glucosidases in *Neotermes koshunensis* (Ni et al., 2007), in *Nasutitermes takasagoensis* (Tokuda et al., 2009), in *C. formosanus* (Zhang et al., 2010) and in *R. flavipes* (Scharf et al., 2010). These recombinant cellulolytic enzymes are useful for studying cellulose metabolism in termites and have potentials to be engineered as new biocatalysts for cellulose-based biofuel production.

Previously, we demonstrated that the recombinant endogenous β-glucosidase in glycoside hydrolase family 1 (GH1) can completely digest the hydrolytic products of filter-paper cellulose catalyzed by an endogenous endo-β-1,4-glucanse in GH9 family (Zhang et al., 2010). Following this finding, the objectives of this study were to evaluate the coding gene expression levels in different digestive tissues of *C. formosanus*, characterize the enzymatic kinetics of the recombinant protein, and assess the impacts of specific inhibitors on enzyme activity and termite survival. The study would provide informative results for better understanding the function of the enzyme *in vitro* and *in vivo*.

## 2. Materials and methods

### 2.1. Termites

Termites of *C. formosanus* were collected from field traps placed in the City Park of New Orleans, Louisiana, USA and maintained in plastic containers with slats of spruce wood in a laboratory incubator (26 ± 1 °C, in dark).

### 2.2. β-glucosidase gene analysis

Three digestive tissues (salivary gland, foregut, midgut and hindgut) were dissected from worker caste. Remaining carcass

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(without gut and head) was saved as control. A pool of 50 individuals each was subjected to total RNA isolation using NucleoSpin RNA II reagents and protocols (Clontech, Mountain View, CA). An aliquot of 1 μg RNA from each sample was reverse transcribed to cDNA using Advantage RT-for-PCR kit (Clontech), in which mixed primers were used (oligo-dT: random hexamer = 6: 4). Quantitative real-time PCR (qRT-PCR) was performed as described previously (Zhang et al., 2011). Briefly, the reaction contained 5 μl cDNA, 2 μl primer pairs (10 μM each), 5.5 μl de-ionized water, and 12.5 μl iQ5 SYBR Green supermix (Bio-RAD, Hercules, CA). cDNA concentration applied to PCR was optimized by 10-fold dilution. The sequences (5′ → 3′) of the primers were as follows: tggctgcgaggatatactga (forward) and tcgtagcgccttgaatatga (reverse), which had been determined to produce a single PCR band with expected amplicon size (165 bp). 18S Ribosomal RNA was used to normalize variations of cDNA concentration using primers S18rtf and S18rtr (Zhang et al., 2011). PCR signals were recorded and analyzed using MJ Opticon Monitor software (Bio-RAD). All individual PCRs had three replicates; data of threshold cycle (Ct) with efficiencies between 90–110% were used to compare relative abundance of the gene transcript. Data of ΔCt, normalized to 18S ribosomal RNA, were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

### 2.3. Recombinant C. formosanus β-glucosidase

Recombinant β-glucosidase was produced in *Escherichia coli* and purified using His-Mag agarose beads (Novagen, Madison, WI) as described previously (Zhang et al., 2010). Following purification, buffer was exchanged to store the purified protein in 100 mM sodium acetate (NaAc), pH 5.6, with 10% glycerol, using an Ultra-10K centrifugal filter unit (Millipore, Cork, Ireland). The protein concentration was estimated with Bradford reagent (Sigma, St. Louis, MO) using BSA as the protein standard. The protein with greater than 90% homogeneity, determined by SDS–PAGE, was used to perform enzymatic assays.

### 2.4. Substrate specificity and kinetics of recombinant β-glucosidase

Substrates tested were listed in Table 1, including di-/tri-saccharides, glycosides and poly-saccharides. Each substrate was serially diluted with 50 mM NaAc (pH 5.6), ranging from 0.475 to 57.0 mM. For polysaccharides, serial dilutions were made from 0.5 to 15 mg/ml in the same buffer. Eight concentrations were tested. Reactions were set up in a 96-well PCR plate with each well containing 95 μl of substrate and 5 μl of purified β-glucosidase (0.2 μg). The plate was incubated at 37 °C in a PCR thermal cycler for 10 min and then cooled to 22 °C for 5 min. Aliquots of 15 μl reaction from individual wells were then transferred to a 96-well microplate with individual wells containing 185 μl Glucose (HK) Assay Reagent (Sigma). The microplate was incubated with mixing in a microplate reader (xMark, Bio-RAD) at room temperature for 15 min before absorbance at 340 nm was read. Serial dilutions of glucose, ranging from 2 to 120 nmol in 15 μl, were included in the assay as standards. Each reaction had 3 replicates. Data were validated where correlation coefficients ($r^2$) of glucose standard curves were equal or greater than 0.985 in this assay. The Michaelis constant ($K_m$) and the maximal relation rate ($V_{max}$) were determined by the Lineweaver–Burk plot method.

### 2.5. Thermostability and optima of temperature and pH of recombinant β-glucosidase

The thermostability of recombinant β-glucosidase were assessed by incubating aliquots of the enzyme at temperatures ranging from 37 to 60 °C for 30 min. Aliquots of 5 μl treated samples (0.2 μg) were then mixed with 45 μl of 10 mM cellobiose (in 50 mM NaAc, pH 5.6). The mixtures were incubated at 37 °C for 30 min. The effect of different temperatures on recombinant β-glucosidase activity was measured by incubating the same composite reaction above (less pretreatment of enzyme) over a range of temperatures from 25 to 60 °C for 30 min. The effect of pH on activity was determined by incubating the enzyme with 10 mM cellobiose in different pH buffers (3.8–7.8) at 37 °C for 30 min. All buffers were made up of 50 mM NaAc and pH values were adjusted with 6 M NaOH or 5 N HCl. All assays had three replicates. Glucose yielded in each reaction was determined using Glucose (HK) Assay Reagent as described above.

**Table 1**
Substrate specificity and kinetics of recombinant β-glucosidase.[a]

| Substrate | $K_m$ (mM) | $V_{max}$ (μmol/min/mg) |
|---|---|---|
| *Di- & tri-saccharides* | | |
| Cellobiose (98%) | 2.3 | 462.6 |
| Cellotriose (95%) | 4.9 | 375.5 |
| Gentiobiose (85%) | 26.6 | 123.2 |
| β-Lactose (99%) | 5.4 | 28.9 |
| Laminaribiose (95%) | 58.0 | 30.2 |
| Maltose (98%) | ND | ND |
| Trehalose (99%) | ND | ND |
| Sophorose(98%) | ND | ND |
| Melibiose (99%) | ND | ND |
| *Glycosides* | | |
| Salicin (99%) | 11.8 | 323.2 |
| Aesculin (98%) | 13.5 | 228.9 |
| Amygdalin (99%) | 35.6 | 209.9 |
| Stevioside (98%) | ND | ND |
| Arbutin (98%) | ND | ND |
| *Polysaccharides* | | |
| Cellulose (20 micron) | ND | ND |
| Levan | ND | ND |
| Curdlan | ND | ND |

[a] Each assay had three replicates; data shown were the average of two independent assays. Purities of individual substrates were shown in parentheses (All were purchased from Sigma except laminaribiose from Megazyme). ND = Activity not detected.

### 2.6. Effect of glucose on recombinant β-glucosidase activity

The tolerance of recombinant β-glucosidase to glucose (the end product of cellulose digestion) was assessed as follows: An aliquot of 100 μl of glucose at concentrations from 0.2 to 2.0 M in 50 mM NaAc (pH 6.5) was mixed with 2 μl of enzyme (1 μg) and incubated at 37 °C for 30 min. Then, 98 μl of 25 mM *p*-nitrophenyl-β-glucopyranoside (in 50 mM NaAc, pH 6.5; Sigma) were added and mixed; the mixture was incubated at 37 °C for another 30 min before absorbance at 410 nm was read with the xMark microplate reader. The amount of *p*-nitrophenol released from control (without glucose in pre-incubation) was taken as 100% activity. Each individual treatment had four replicates. The differences of activities among treatments were analyzed with ANOVA.

### 2.7. Effect of inhibitors on recombinant β-glucosidase activity

Two general β-glucosidase inhibitors, conduritol B epoxide (CBE) and gluconolactone (Sigma), were tested for their ability to inhibit the activity of the recombinant termite β-glucosidase. Aliquots of 5 μl recombinant β-glucosidase (0.2 μg) were pre-incubated with 20 μl of various concentrations of each inhibitor (in 50 mM NaAc, pH 6.5) at room temperature for 20 min. Following pre-incubation, 25 μl of 12 mM cellobiose in 50 mM NaAc (pH 6.5) were added to each reaction. The mixtures were incubated at 37 °C for 30 min and then aliquots of 5 μl were sampled for glucose concentration