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# Molecular Characterization and Potential Synthetic Applications of GH1 β-Glucosidase from Higher Termite *Microcerotermes annandalei*

**Siriphan Arthornthurasuk[1] · Wantha Jenkhetkan[1] · Eukote Suwan[2] · Daranee Chokchaichamnankit[3] · Chantragan Srisomsap[3] · Pakorn Wattana-Amorn[4] · Jisnuson Svasti[3,5] · Prachumporn T. Kongsaeree[1,2]**

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**Abstract** A novel β-glucosidase from higher termite *Microcerotermes annandalei* (MaBG) was obtained via a screening method targeting β-glucosidases with increased activities in the presence of glucose. The purified natural MaBG showed a subunit molecular weight of 55 kDa and existed in a native form as a dimer without any glycosylation. Gene-specific primers designed from its partial amino acid sequences were used to amplify the corresponding 1,419-bp coding sequence of *MaBG* which encodes a 472-amino acid glycoside hydrolase family 1 (GH1) β-glucosidase. When expressed in *Komagataella pastoris*, the recombinant MaBG appeared as a ~55-kDa protein without glycosylation modifications. Kinetic parameters as well as the lack of secretion signal suggested that MaBG is an intracellular enzyme and not involved in cellulolysis. The hydrolytic activities of MaBG were enhanced in the presence of

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✉ Prachumporn T. Kongsaeree
fscippt@ku.ac.th

[1] Department of Biochemistry, Faculty of Science, and Center for Advanced Studies in Tropical Natural Resources, NRU-KU, Kasetsart University, 50 Paholyotin Road, Chatuchak, Bangkok 10900, Thailand

[2] Interdisciplinary Graduate Program in Genetic Engineering, Faculty of Graduate School, Kasetsart University, Bangkok 10900, Thailand

[3] Laboratory of Biochemistry, Chulabhorn Research Institute, Bangkok 10210, Thailand

[4] Department of Chemistry, Center of Excellence for Innovation in Chemistry and Special Research Unit for Advanced Magnetic Resonance, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand

[5] Department of Biochemistry and Center for Excellence in Protein and Enzyme Technology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand

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up to 3.5-4.5 M glucose, partly due to its strong transglucosylation activity, which suggests its applicability in biosynthetic processes. The potential synthetic activities of the recombinant MaBG were demonstrated in the synthesis of *para*-nitrophenyl-β-D-gentiobioside via transglucosylation and octyl glucoside via reverse hydrolysis. The information obtained from this study has broadened our insight into the functional characteristics of this variant of termite GH1 β-glucosidase and its applications in bioconversion and biotechnology.

**Keywords** β-Glucosidase · Glycoside hydrolase family 1 · Higher termite · *Microcerotermes annandalei* · Reverse hydrolysis · Transglucosylation

## Introduction

β-Glucosidases (E.C. 3.2.1.21) are glycoside hydrolases that cleave the β-glucosidic linkage, from the nonreducing end, between two glucose molecules (e.g., β1 → 4 in cellobiose) or glucose-substituted molecules. β-Glucosidases belong to glycoside hydrolase (GH) families 1, 3, 5, 9, 30, and 116 [1, 2]. Under specified conditions, some β-glucosidases also exhibit synthetic activities via reverse hydrolysis or transglucosylation reactions [2]. Industrial applications of both hydrolytic and synthetic activities of β-glucosidases are growing considerably, including conversion of lignocellulosic biomass into bioethanol and other useful biomaterials, improvement of nutritional and organoleptic qualities in food and fruit juice products, production of aromatic compounds from flavorless precursors, and synthesis of oligosaccharides and alkyl-glucosides [3–8].

However, one of the major hindrances of employing the hydrolytic activities of β-glucosidases in biotechnological processes is product inhibition, by which high concentrations of glucose inhibit the hydrolytic activities. Thus, glucose tolerance is a desirable quality in β-glucosidases, and many glucose-tolerant β-glucosidases from various sources have been reported, including bacteria [9–11], fungi [12–20], plants [21], metagenomic libraries [22–25], and insects [26–29]. However, many of the glucose tolerant, and even glucose-enhanced, activities were shown to result from the transglucosylation activity of the enzymes, and potential synthetic activities have only been investigated in terms of the synthesis of glucodisaccharides (such as laminaribiose, cellobiose, sophorose, and gentiobiose) from glucose [29].

Among xylophagous insects, termites (order Isoptera) are known to be the most efficient cellulose decomposers [30], which makes them a good source to screen for valuable glycoside hydrolase enzymes. Recent studies revealed that glycoside hydrolase activities residing in termite body are not solely from gut symbionts but also from endogenous enzymes [31]. Information regarding termite β-glucosidase genes and enzymatic characterizations has been growing markedly [27–29, 32–37]. All of the reported termite β-glucosidases were identified as GH1. Among these, three termite β-glucosidases, from *Nasutitermes takasagoensis*, *Neotermes koshunensis*, and *Coptotermes formosanus*, were reported to be glucose-tolerant [27–29, 37], and only the activity of *Neotermes koshunensis* β-glucosidase was found to be stimulated by glucose [27].

Our study here reports a screening method directly targeting β-glucosidases with a glucose-enhanced activity from termite samples, which has successfully identified a GH1 β-glucosidase from higher termite *Microcerotermes annandalei* (MaBG). The corresponding *MaBG* cDNA was cloned, expressed as a recombinant enzyme, and characterized. The recombinant MaBG exhibited synthetic activities via both reverse hydrolysis and transglucosylation reactions. Our data presented here has helped to shed light on the functional

characteristics of the termite GH1 β-glucosidase and its applications in bioconversion and biotechnology.

## Materials and Methods

### Materials

Worker termites were obtained from various provinces of Thailand, namely Bangkok (2 samples), Karnchanaburi (10 samples), Rachaburi (2 samples), and Uthaithani (5 samples). All chromatographic columns, gel filtration calibration kit (high molecular weight), and Blue Dextran 2000 were purchased from GE Healthcare (Buckinghamshire, UK). The recombinant *Dalbergia nigrescens* β-glucosidase, Dnbglu2, was expressed and purified as described previously [38]. All aryl glycosides and disaccharides were purchased from Sigma (St. Louis, MO, USA). Other chemicals were of analytical grade.

### Screening for Glucose-Enhanced β-Glucosidase Activities from Termite Crude Extracts

Thirty worker termites from each source were ground in a mortar, in 0.1 M sodium acetate, pH 5.5, containing 1 mM phenylmethylsulfonyl fluoride (PMSF) as a protease inhibitor. The ground termites were sonicated for 30 min and centrifuged at 20,100×*g* for 30 min. The supernatant was collected and incubated with 1 mM *para*-nitrophenyl-β-D-glucopyranoside (*p*NP-Glc) in 0.1 M sodium acetate, pH 5.5, in the presence of 1.55 M glucose at 30 and 50 °C for 30 min, and then the reaction was stopped with 2 M $Na_2CO_3$. The absorbance of *para*-nitrophenol (*p*NP) released from the reaction was measured at 405 nm and compared to a standard curve of *p*NP. One unit of enzyme activity was defined as the amount of enzyme used to release 1 μmol of *p*NP in 1 min. The protein content was measured by using the Bio-Rad protein assay reagent kit (Hercules, CA, USA) and compared to a standard curve of bovine serum albumin.

### Purification and Characterization of the Natural MaBG

A crude enzyme extract was prepared from approximately 16 g of *M. annandalei* worker termites (approximately 4600 insects) as described above. The extract was fractionated by 30–70% (*w*/*v*) ammonium sulfate. The precipitate was redissolved in 0.1 M sodium phosphate, pH 6.0, containing 0.1 M NaCl. The crude enzyme was subjected to gel filtration chromatography on a Sephacryl S-300 column, which was pre-equilibrated with 0.1 M sodium phosphate, pH 6.0, containing 0.1 M NaCl, and eluted with the same buffer, at a flow rate of 1 mL $min^{-1}$. Active fractions were pooled, concentrated by using centrifugal ultrafiltration, and then subjected to anion exchange chromatography on a Hitrap Q HP column, which was pre-equilibrated with 10 mM sodium phosphate, pH 6.0, and washed with the same buffer. The target protein then was eluted with 10 mM sodium phosphate, pH 6.0, containing 1 M NaCl at a flow rate of 1 mL $min^{-1}$. The active fractions were then pooled, concentrated, and further purified by isoelectric chromatofocusing on a Mono P HR column, which was pre-equilibrated with 10 mM sodium phosphate, pH 7.0. The protein was eluted with 1/10 dilution of polybuffer, pH 4.0, at a flow rate of 0.5 mL $min^{-1}$. The protein content and β-glucosidase

activity in the presence of 1.55 M glucose at 50 °C were measured in each fraction before and after loading into each column as described above.

The purity and subunit molecular weight of MaBG was determined by 10% SDS-PAGE. To determine the native molecular weight, the purified MaBG was applied to a Sephacryl S-300 column that was pre-equilibrated with 50 mM sodium phosphate, pH 6.0, 150 mM NaCl, and eluted with the same buffer at a flow rate of 1 mL $min^{-1}$. The native molecular weight of MaBG was calculated by comparing its retention time with those of the standard proteins in the gel filtration calibration kit, comprising ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), and ovalbumin (43 kDa), that were applied to the same column under the same condition. Void volume was determined by using Blue Dextran 2000. Analysis of glycosylation was done by treating the purified MaBG with endoglycosidase $H_f$ (New England Biolabs, Ipswich, MA, USA) at 37 °C for 2 h under denaturing conditions according to the manufacturer's instructions, and cleavage products were examined by 10% SDS-PAGE. The internal amino acid sequence of the MaBG was determined by nanoLC/MS/MS methods. Briefly, a piece of SDS-PAGE gel containing the purified MaBG was digested with trypsin as described previously [39]. The tryptic peptides were fractionated and their mass analyzed by nanoflow liquid chromatography coupled with electrospray ionization (nano ESI MS/MS) quadrupole time-of-flight tandem mass spectrometry (Q-ToF micro; Micromass, UK). The obtained micromass (.pkl) output file was analyzed by Mascot program [40].

## Molecular Cloning of the Complete Coding Sequence of MaBG

Total mRNA was extracted from 20 mg of *M. annandalei* worker termites by using RNeasy kit (Qiagen, Hilden, Germany). First-strand cDNAs were synthesized by using SMARTer RACE Ready Amplification kit (Clontech, Mountain View, CA, USA). The first round of 5′ and 3′ RACE-PCR was performed by using the degenerate primers, Reverse GSP1 and Forward GSP2 (Table 1), respectively, designed based on the conserved nucleotide sequences of other reported β-glucosidases from higher termites, and the Advantage 2 Polymerase Mix (Clontech, Mountain View, CA, USA). Then, specific primers, F1, F2, and R1 (Table 1), designed based on the sequences of the first-round RACE-PCR products, were used in the second round of 5′ and 3′ RACE-PCR. Then, cloning primers, MaBG Forward1 and MaBG Reverse1 (Table 1), designed based on the sequences of the second-round RACE-PCR products, were used to amplify the complete coding sequence of *MaBG*, which was then ligated into pZErO™-2 cloning vector (Invitrogen, Carlsbad, CA, USA) at the *Eco*RV site, transformed into *Escherichia coli* Top10, and sequenced.

The physical and chemical parameters of the deduced amino acid sequence were analyzed using ProtParam program [41]. The presence of the signal peptide and glycosylation sites was predicted by SignalP 4.1 [42], NetNGlyc 1.0 [43], and NetOGlyc 4.0 [44]. The deduced amino acid sequence was blasted against the NCBI database using BLASTp algorithms to search for its homologs [45]. The phylogenetic tree was generated by using MEGA6 [46] with the maximum-likelihood method based on the Poisson correction model [47].

## Expression and Purification of the Recombinant MaBG

For expression in *E. coli*, the *MaBG* gene was subcloned into the *Nde*I site of the pET15b expression vector (Novagen, Madison, WI, USA), which was then transformed into the expression host, *E. coli* BL21 DE3. For protein expression, the bacterial culture ($O.D._{600}$

**Table 1** Nucleotide sequences of the cloning primers

| Primer names | Oligonucleotide sequences (5′ → 3′) |
|---|---|
| Reverse GSP1 | NTANNCNTTNGCNATCCAGTTCAG |
| Forward GSP2 | CAAGATCTNGGNGGATGGNCNAAT |
| F1 | CGTCTTAGCGTGATGGCTGCTCTCG |
| F2 | TGCCACAGCCTCTACAAGATCTTGG |
| R1 | CTCCTCCTTGGTGAAACTGGGCAG |
| MaBG Forward1 | TTTGTACATATGGCTGCTCTCGAATTCC[a] |
| MaBG Reverse1 | AAAAAACATATGTCAACCCTCAGCAAATTC[a] |
| MaBG Forward2 | TAAAAACTGCAGCTGCTCTCGAATTCCC[a] |
| MaBG Reverse2 | AAAAACTCTAGATCAACCCTCAGCAAATTC[a] |

[a] The underlined sequences are restriction sites of *Nde*I in MaBG Forward1 and MaBG Reverse1, *Pst*I in MaBG Forward2, and *Xba*I in MaBG Reverse2

0.8) grown in LB broth containing 50 μg $mL^{-1}$ ampicillin, at 37 °C, 220 rpm, was induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside for 2 h. The cell pellet was harvested by centrifugation, resuspended in lysis buffer [20 mM Tris-HCl, pH 8.0, 50 mM KCl, 0.5% (*v*/*v*) Tween20, 0.1% (*v*/*v*) Triton-X 100, 1 mM ethylenediaminetetraacetic acid, and 1 mM PMSF], and disrupted by sonication. The soluble and insoluble fractions were separated by centrifugation, and then subjected to 10% SDS-PAGE analysis. β-Glucosidase activity assay was measured against 1 mM *p*NP-Glc in the presence of 1.55 M glucose at 50 °C as described above.

For expression in *Komagataella pastoris*, the *MaBG* gene was amplified from the pZErO-MaBG plasmid by using the primer pair, MaBG Forward2 and MaBG Reverse2 (Table 1), and subcloned into the pPICZαBNH8 expression vector [48]. The pPICZαB-MaBG vector was then transformed into *K. pastoris* Y11430. A single yeast colony harboring pPICZαB-MaBG was grown in BMGH supplemented with $4 \times 10^{-5}$% (*w*/*v*) biotin, at 30 °C, 200 rpm, overnight, and changed into BMMH supplemented with $4 \times 10^{-5}$% (*w*/*v*) biotin and 0.5% (*w*/*v*) casamino acid, at 30 °C, 200 rpm. Methanol 0.5% (*v*/*v*) was added daily to maintain the inducing condition for 1 week. The recombinant MaBG was purified from culture media by hydrophobic interaction chromatography followed by immobilized metal-ion affinity chromatography as described previously [48]. Protein content and β-glucosidase activity toward 1 mM *p*NP-Glc in the presence of 1.55 M glucose at 50 °C were measured in each fraction before and after loading into each column as described above.

## Effects of Temperature, pH, and Glucose Concentrations on the Activities of the Natural and Recombinant MaBG

The standard assay for measuring enzyme activity was performed by incubating the purified natural or recombinant MaBG (3 mU) with 15 mM *p*NP-Glc in 0.1 M sodium phosphate, pH 6.0, in the presence of 1.55 M glucose, at 50 °C for 5 min, and stopped with 2 M $Na_2CO_3$. The absorbance of *p*NP released from the reaction was measured at 405 nm and compared to a standard curve of *p*NP. To determine the optimal temperature, the reactions were incubated at temperatures of 30–90 °C. To determine the optimal pH, the reactions were incubated in either 0.1 M sodium citrate or 0.1 M sodium phosphate, at pH 3–8 . To determine the thermal stability, the enzyme was preincubated in 0.1 M sodium acetate, pH 5.0, at temperatures of 4–90 °C for 30 min, before its activity was measured at its optimal temperature and pH. To determine pH stability, the

enzyme was preincubated in 50 mM sodium citrate, pH 3–6, or 50 mM sodium phosphate, pH 6–8, at 4 °C for 30 min, before its activity was measured at its optimal temperature and pH.

The purified enzyme (3 mU) was reacted with 3, 6, and 9 mM *p*NP-Glc in the presence of 0–4.8 M glucose in 50 mM sodium phosphate, pH 6.0, at 50 °C for 5 min. The reaction was stopped by adding 2 M $Na_2CO_3$. The absorbance of *p*NP released from the reaction was measured at 405 nm and compared to a standard curve of *p*NP.

## Kinetic Analysis of the Recombinant MaBG

The purified enzyme (3 mU) was reacted with various concentrations of substrates in 0.1 M sodium phosphate, pH 6.0, at 50 °C for 5 min. The reactions with aryl glycosides, namely *p*NP-Glc, *para*-nitrophenyl-β-D-fucopyranoside (*p*NP-Fuc), *para*-nitrophenyl-β-D-galactopyranoside (*p*NP-Gal), *para*-nitrophenyl-β-D-mannopyranoside (*p*NP-Man), and *para*-nitrophenyl-β-D-xylopyranoside (*p*NP-Xyl), were stopped by adding 2 M $Na_2CO_3$. The absorbance of *p*NP released from the reaction was measured at 405 nm and compared to a standard curve of *p*NP. The reactions with lactose, sucrose, cellobiose, gentiobiose, and laminaribiose were stopped by heating at 100 °C for 5 min, and the released glucose was then reacted with 50 μL of 2 mg $mL^{-1}$ 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (Roche, Indianapolis, IN, USA) and 100 μL of glucose oxidase reagent (Sigma, St. Louis, MO, USA) at 37 °C for 15 min. The absorbance was measured at 405 nm and compared to a standard curve of glucose. Kinetic parameters were determined from the Michaelis-Menten equation by using KaleidaGraph program (Synergy Software). In cases where the concentrations of the substrate were low compared with the $K_m$ value, the Lineweaver-Burk equation was used.

## Synthetic Activities of the Recombinant MaBG

The transglucosylation reaction was performed by reacting the recombinant MaBG (3 mU) with 50 mM *p*NP-Glc in 0.1 M sodium phosphate, pH 6.0, at 30 °C for 24 h. Aliquots (3 μL) of the reaction mixture and the glucoside standards were spotted on a TLC plate (Silica gel 60 F254, Merck, Darmstadt, Germany), which was then developed twice with a mixture of *n*-butanol:acetic acid:water (8:2:1) for 5.5 cm. Then, the TLC plate was dipped into 20% sulfuric acid in ethanol and charred at 120 °C. To isolate the reaction products, the reaction mixture was then lyophilized, resuspended in methanol, and separated by RP-HPLC. The fractions containing the compound of interest were then lyophilized and resuspended in methanol for mass spectrometric analysis, or in 50 mM sodium phosphate, pH 6.0, for treatment with 0.5 U of Dnbglu2 enzyme [38] at 30 °C for 1 h to release the disaccharide moiety. The samples and the standard commercial reagents were analyzed by TLC as described above.

The reverse hydrolysis reaction was performed by reacting the recombinant MaBG (0.1 U) with 250 mM glucose in buffer-saturated octanol at 30 °C [49]. Aliquots (3 μL) of the reaction mixture and the glucoside standards were spotted on TLC plate, which was developed twice the mixture of chloroform:ethanol (3:1) for 5 cm and then twice with a mixture of isopropanol:ethanol:water (5:1:2) for 2 cm, and visualized as described above.

## Accession Number of MaBG

The coding sequence of *MaBG* was deposited to GenBank with accession no. KU170546.

## Results

### Screening for Glucose-Enhanced β-Glucosidase Activities from Termite Crude Extracts

Seven species of termite samples were collected, namely *Coptotermes anandalei* (three samples), *C. gestroi* (one sample), *Globitermes sulphureus* (one sample), *Hypotermes* sp. (two samples), *Microcerotermes* sp. (seven samples), *M. annandalei* (four samples), and *M. crassus* (one sample). The activity screening revealed that, in the presence of 1.55 M glucose, the crude extracts of *C. gestroi* and *G. sulphureus* had relatively low β-glucosidase activities (lower than 0.5 U $mg^{-1}$), whereas the crude extracts of *Hypotermes* sp., *C. anandalei*, and *M. crassus* had intermediate levels of β-glucosidase activities (0.5–1.0 U $mg^{-1}$). High levels of β-glucosidase activities (higher than 1.0 U $mg^{-1}$) were found in the crude extracts of *M. annandalei* and *Microcerotermes* sp. The β-glucosidase activities at 50 °C were higher than those at 30 °C for all species. Thus, *M. annandalei*, which is a higher termite, was chosen to be the source of the potentially glucose-tolerant β-glucosidase, namely MaBG.

### Purification and Characterization of the Natural MaBG

MaBG in the crude extract of *M. annandalei* was precipitated with 30–70% ammonium sulfate and purified to homogeneity by gel filtration, anion exchange chromatography, and isoelectric chromatofocusing. Purification fold (4.4-fold) and percent recovery (1%) were rather low with a specific activity of 2.4 U $mg^{-1}$ (Table 2). The obtained MaBG appeared as a single protein band of approximately 55 kDa on a 10% SDS-PAGE (Fig. 1), which was similar to other termite β-glucosidases previously reported (52–60 kDa) [37]. It probably existed as a dimer with a native molecular weight of approximately 81.5 kDa as judged by gel filtration (Fig. S1). Treatment with endoglycosidase $H_f$ indicated that the natural MaBG possessed no glycosylation modification (Fig. 2a).

### Determination of the Primary Sequence of MaBG and Bioinformatic Analysis

The internal amino acid sequences of the purified MaBG, determined by LC/MS/MS analysis, matched with four regions of a GH1 β-glucosidase from a higher termite *Odontotermes formosanus* (GenBank accession ADD92156.1) (results not shown). Following the cloning procedures, a full-length, 1,419-bp *MaBG* cDNA was obtained. An open reading frame of *MaBG* encodes a polypeptide of 472 amino acids with a calculated MW of 54,135.2 Da and p*I* of 5.69. (Fig. S2). A homology model of MaBG, based on the template structure of *N. koshunensis* β-glucosidase (PDB code 3VIF), exhibited the expected $(\alpha/\beta)_8$ barrel structure (not shown) [50]. The catalytic acid/base and the catalytic nucleophile of MaBG are likely to be E168 and E375 in the NE/DP and I/VTENG motifs, respectively, that are conserved in all GH1 β-glucosidases [2].

Peptide analysis predicted the absence of both the signal peptide cleavage site and the N-linked and O-linked glycosylation sites on the MaBG, which is consistent with the result from the endoglycosidase $H_f$ treatment of the natural MaBG (Fig. 2a). Based on BLASTp analysis, the deduced amino acid sequence of MaBG is most similar to β-glucosidase from *O. formosanus* (GenBank accession ADD92156.1) with 81% identity and 91% homology and CfGlu1C from *Coptotermes formosanus* (GenBank accession AEW67361.1) with 80%

**Table 2** Summary of purification steps of the natural MaBG

| Purification step | Total volume (mL) | Activity (U)[a] | Protein (mg) | Specific activity (U $mg^{-1}$) | Recovery (%) | Fold |
|---|---|---|---|---|---|---|
| Crude enzyme | 39.0 | 80.1 | 147.5 | 0.5 | 100 | 1.0 |
| 30–70% $(NH_4)_2SO_4$ | 12.0 | 74.0 | 46.9 | 1.6 | 92 | 3.2 |
| Sephacryl S-300 | 64.0 | 67.7 | 30.4 | 2.2 | 85 | 4.4 |
| Hitrap Q HP | 17.0 | 31.8 | 9.8 | 3.2 | 40 | 6.4 |
| Mono P | 3.5 | 1.2 | 0.5 | 2.4 | 1 | 4.4 |

[a] The β-glucosidase activity was determined against 1 mM *p*NP-Glc in 0.1 M sodium acetate, pH 5.5, in the presence of 1.55 M glucose at 50 °C for 30 min

identity and 89% homology [51], whose sequences contain no obvious signal peptide or glycosylation sites. On the other hand, MaBG is distantly similar (33–57% identity) to termite β-glucosidases exhibiting high cellobiase activity, such as that from *Neotermes koshunensis* (PDB code 3AHZ_A) [33]. Available full-length amino acid sequences of termite β-glucosidases at the time were retrieved for multiple sequence alignment and phylogenetic analysis. Phylogenetic analysis confirmed that MaBG is more related to *O. formosanus* β-glucosidase and *C. formosanus* CfGlu1C, than those other secretory termite β-glucosidases which are glycosylated and show obvious cellobiase activity (Fig. S3).

## Expression and Purification of the Recombinant MaBG

The ORF of *MaBG* gene was firstly expressed in *E. coli* BL21 DE3 as a recombinant MaBG with an N-terminal $His_6$ tag that appeared as a 55-kDa protein band on a 10% SDS-PAGE.

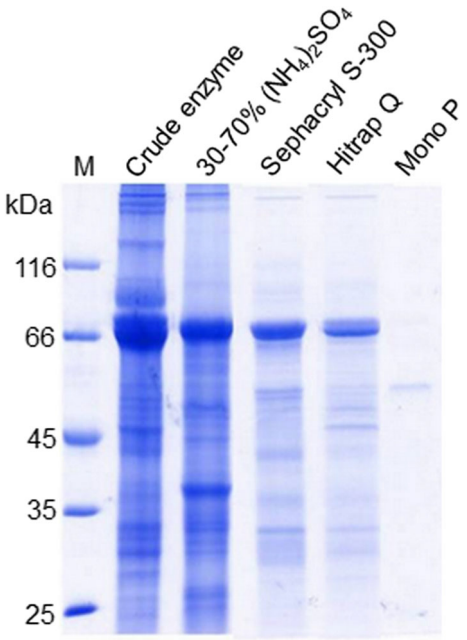


**Fig. 1** Purification of MaBG from the crude extract of *M. annandalei*. The purification steps are indicated above the lanes. Lane M is protein size markers

However, the bacterially expressed recombinant MaBG mostly formed an inclusion body, which was not well solubilized (Fig. S4), and had no detectable β-glucosidase activity. Therefore, the *MaBG* cDNA was expressed in yeast *K. pastoris*, which yielded an active enzyme of approximately 55 kDa (Fig. 2b). The recombinant MaBG was then purified from the culture broth, with a purification fold of 19.0, percent recovery at 4.3%, and a specific activity of 6.3 U $mg^{-1}$ (Table 3). Treatment with endoglycosidase $H_f$ also showed no glycosylation modification on the recombinant MaBG, consistent with the result from the natural MaBG (Fig. 2b, compared with Fig. 2a) and the glycosylation prediction.

## Effects of Temperature, pH, and Glucose Concentrations on the Activities of the Natural and Recombinant MaBG

The optimal temperature and pH for the natural and recombinant MaBG in the hydrolysis of *p*NP-Glc in the presence of 1.55 M glucose were 50–55 °C (Fig. 3a) and pH 5–6 (Fig. 3b). Both enzymes were stable between 0 and 40 °C and at pH 4–8, with approximately 70% activity remaining after incubation at these temperatures and pH, respectively, for 30 min, but completely lost its activity after incubation at 60 °C or below pH 4. The thermostability of MaBG was much lower in the absence of glucose (Fig. 3a).

The activities of the natural and recombinant MaBG toward *p*NP-Glc were determined in the presence of 0–4.8 M glucose (Fig. 4). The results showed that the activities of both MaBG forms were enhanced in the presence of up to 3.5–4.5 M glucose, depending on the starting concentration of *p*NP-Glc, compared with the activity in the absence of added glucose. Further increase in glucose concentration resulted in decreased *p*NP-Glc hydrolyzing activities.

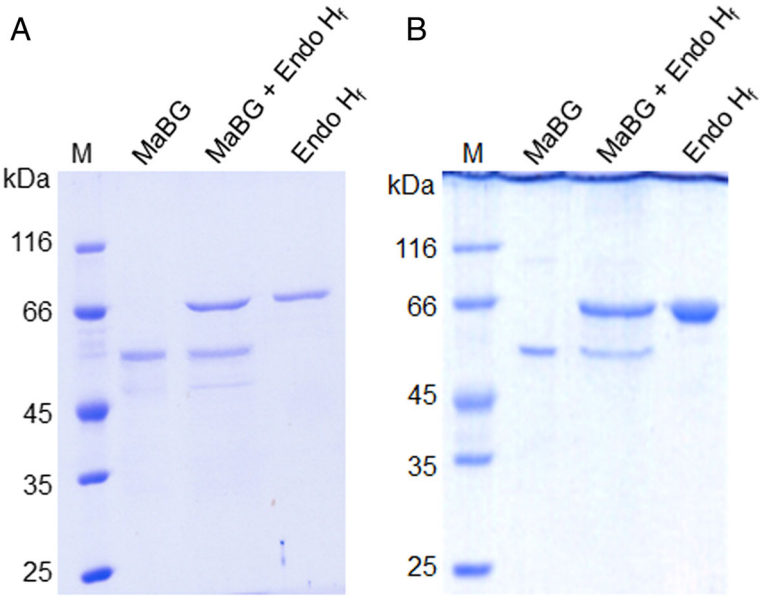


**Fig. 2** Glycosylation analysis of the natural MaBG (**a**) and recombinant MaBG (**b**). The protein samples were treated with endoglycosidase $H_f$ (Endo $H_f$) as indicated above the lanes. Lane M is protein size markers

**Table 3** Summary of purification steps of the recombinant MaBG

| Purification step | Total volume (mL) | Activity (U)[a] | Protein (mg) | Specific activity (U $mg^{-1}$) | Recovery (%) | Fold |
|---|---|---|---|---|---|---|
| Culture media | 600 | 12.2 | 36.5 | 0.3 | 100 | 1.0 |
| Ultrafiltration | 30.0 | 7.2 | 17.1 | 0.4 | 59.3 | 1.3 |
| Adding 1 M $(NH_4)_2SO_4$ | 29.5 | 6.5 | 13.9 | 0.5 | 53.4 | 1.4 |
| Phenyl sepharose | 4.5 | 3.8 | 3.2 | 1.2 | 31.6 | 3.6 |
| $Ni^{2+}$-sepharose | 2.0 | 0.9 | 0.1 | 6.4 | 7.5 | 19.1 |
| Ultrafiltration | 0.5 | 0.5 | 0.1 | 6.3 | 4.3 | 19.0 |

[a] The β-glucosidase activity was determined against 1 mM *p*NP-Glc in 0.1 M sodium acetate, pH 5.5, in the presence of 1.55 M glucose at 50 °C for 30 min

## Kinetic Analysis of the Recombinant MaBG

Kinetic parameters of the recombinant MaBG for hydrolysis of various aryl glycosides and disaccharides were determined at 50 °C and pH 6 (Table 4). The $k_{cat}$ values were calculated per subunit rather than per native molecule. The kinetic parameters of the natural MaBG were not determined due to limited amounts of the purified enzyme. Compared with the results from previous studies, the $K_m$ value of the recombinant MaBG toward *p*NP-Glc was within the range of 0.12–1.66 mM reported for other termite β-glucosidases [27, 28, 33–35, 52]. The recombinant MaBG showed higher efficiency in hydrolyzing *p*NP-Fuc than other *p*NP glycosides tested, which is consistent with some other GH1 β-glucosidases from termites [27, 28, 36, 53, 54], but is contrary to that from *Reticulitermes flavipes* [34]. Among the disaccharides tested, the recombinant MaBG could hydrolyze laminaribiose and gentiobiose, with relatively high $K_m$ (14.9 ± 1.2 and 38.7 ± 5.30 mM, respectively), but not cellobiose or lactose or sucrose at all. Notably, among all the substrates tested, MaBG showed the highest $k_{cat}$ value toward laminaribiose (69.3 ± 2.4 $s_{-1}$), which is about 8-fold higher than that toward *p*NP-Glc (8.98 ± 1.00 $s^{-1}$).

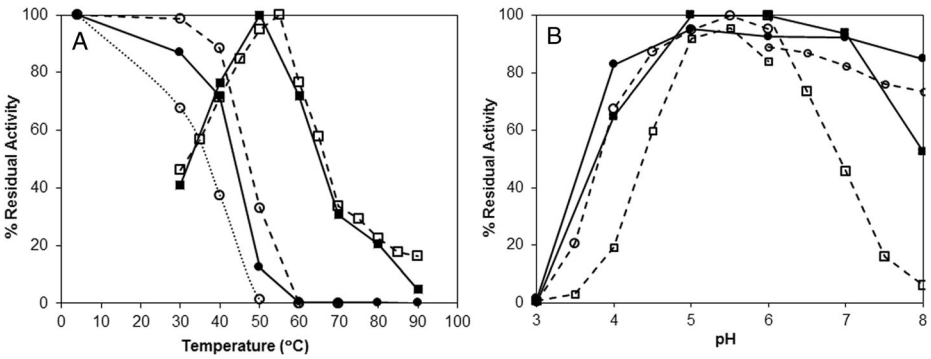


**Fig. 3** Effects of temperature (**a**) and pH (**b**) on the activity of MaBG. The optimal condition and stability are shown as squares and circles, respectively. The activities of the natural and recombinant MaBG are shown as closed symbols with solid lines and open symbols with dashed lines, respectively. The temperature stability of the recombinant MaBG in the absence of glucose is shown as open circles with a dotted line

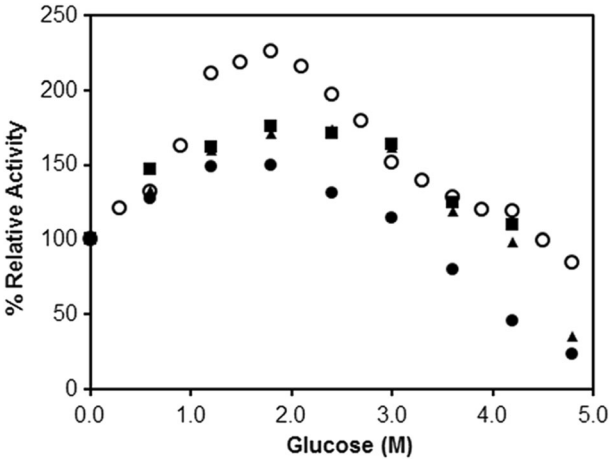


**Fig. 4** Effect of glucose concentrations on the activity of MaBG. The activities of the natural (closed symbols) and recombinant (open symbols) MaBG were determined toward 3, 6, and 9 mM *p*NP-Glc (represented by circles, triangles, and squares, respectively) in 0.1 M sodium phosphate, pH 6.0, in the presence of 0–4.8 M glucose, at 50 °C for 5 min

## Synthetic Activities of the Recombinant MaBG

The transglucosylation activity of the recombinant MaBG in the presence of 50 mM *p*NP-Glc was observed by TLC. Between 10 and 120 min of incubation, *p*NP-Glc (the remaining starting material) and two unknown products were present as the major constituents, while, surprisingly, glucose (the hydrolysis product) could not be observed in the reactions at all (Fig. 5a). The major unknown product, pX, whose bands were between those of *para*-nitrophenyl-β-D-cellobioside (*p*NP-Cel) and glucose, was isolated by HPLC (Fig. S5), and its molecular mass was found to be 498 *m*/*z*, which was equivalent to the mass of *p*NP-diglucoside $[Mass+^{35}Cl]^-$ (result not shown). It was later identified as *para*-nitrophenyl-β-D-gentiobioside (*p*NP-Gen), after treating it with Dnbglu2, which is capable of hydrolyzing disaccharides (Fig. 5b). After 24 h, the reaction was found to contain *p*NP-Glc (the remaining starting material), glucose (the hydrolysis product), *p*NP-Gen (the transglucosylation product), as well as a small amount of the second unknown product, whose bands were between those of *p*NP-Glc and *p*NP-Cel.

The reverse hydrolysis activity of the recombinant MaBG was also investigated in the reaction between free glucose and buffer-saturated octanol [49]. TLC analysis of the reaction products showed the presence of octyl glucoside from day 2 of the reaction (Fig. 6), indicating that a long-chain alkyl alcohol could also act as a glucosyl acceptor.

**Table 4** Kinetic parameters of the recombinant MaBG

| Substrate | $K_m$ (mM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($s^{-1}$ $mM^{-1}$) |
|---|---|---|---|
| *p*NP-Glc | 1.47 ± 0.05 | 8.98 ± 1.00 | 6.12 ± 0.76 |
| *p*NP-Fuc | 0.49 ± 0.04 | 8.71 ± 0.13 | 17.7 ± 1.5 |
| *p*NP-Gal | 4.64 ± 0.45 | 1.09 ± 0.04 | 0.24 ± 0.02 |
| *p*NP-Man | 0.43 ± 0.08 | 0.10 ± 0.004 | 0.23 ± 0.04 |
| *p*NP-Xyl | 2.48 ± 0.21 | 0.18 ± 0.003 | 0.07 ± 0.01 |
| Gentiobiose | 38.7 ± 5.3 | 0.83 ± 0.11 | 0.02 ± 0.004 |
| Laminaribiose | 14.9 ± 1.2 | 69.3 ± 2.4 | 4.64 ± 0.39 |

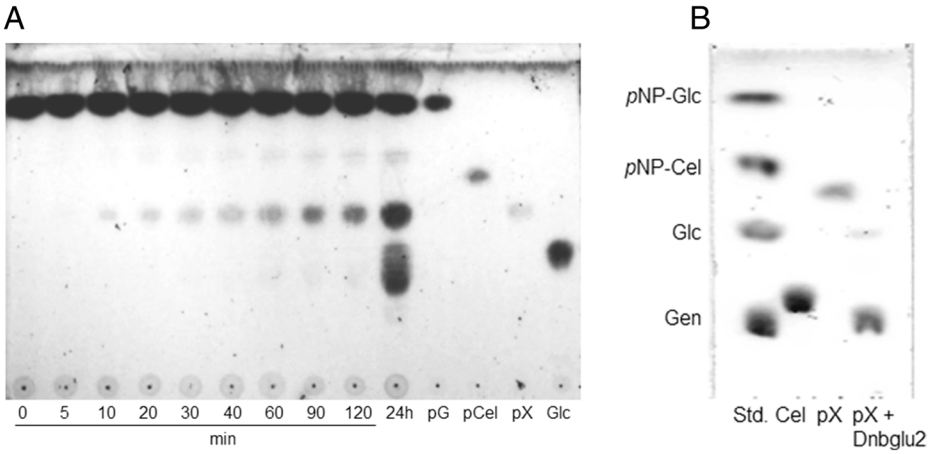


**Fig. 5** TLC analysis of the transglucosylation products of the recombinant MaBG. **a** The reaction was performed between 3 mU enzyme and 50 mM *p*NP-Glc at 30 °C for 24 h and analyzed in a time-course manner. The commercial standards were 20 nmol *p*NP-Glc (pG), 10 nmol *p*NP-Cel (pCel), and 20 nmol glucose (Glc), and the in-house standard is *p*NP-Gen (pX). **b** Comparison of the HPLC-isolated pX, before and after treatment with Dnbglu2, as well as 20 nmol of each commercial standard: *p*NP-Glc, *p*NP-Cel, glucose, cellobiose (Cel), and gentiobiose (Gen)

## Discussion

Termites are important decomposers of cellulose in tropical and subtropical regions, harboring both cellulase and β-glucosidase activities. While the glycoside hydrolase activities were thought to be mainly attributed to their gut symbionts, endogenous enzymes have recently been discovered. Furthermore, at least three termite β-glucosidases were reported to be glucose-tolerant [27–29, 37], which were likely due to the transglucosylation activity of the enzymes, and yet their synthetic applications have never been explored. Many termite β-glucosidases have been reported in the past, but they often lack information on the kinetic

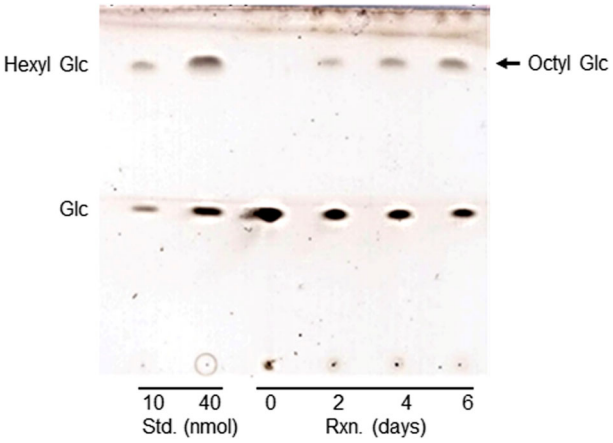


**Fig. 6** TLC analysis of the reverse hydrolysis products of the recombinant MaBG. The reaction was performed between 0.1 U enzyme and 250 mM glucose in buffer-saturated octanol at 30 °C for 6 days and analyzed in a time-course manner. The commercial standards were 10 and 40 nmol of glucose (Glc) and hexyl glucoside (Hexyl Glc). The spots of octyl glucoside (Octyl Glc) are indicated by an arrow

parameters or the coding sequences. So, in this study, we have characterized MaBG, a glycoside hydrolase family 1 β-glucosidase from a higher termite *M. annandalei*, in full details, including isolation of the natural enzyme, delineating its complete coding sequence, examining its hydrolytic properties, as well as investigating its potential biosynthetic applicability via transglucosylation and reverse hydrolysis.

Most of the other reported termite β-glucosidases appeared to have the signal peptide cleavage site and at least one position of N-linked glycosylation, which supports their functions as secretory enzymes participating in cellulose degradation. However, our phylogenic analysis found MaBG to be distantly similar (33–56% identity) to those termite β-glucosidases with obviously high cellobiase activity [27, 28, 34, 55], while the peptide analysis predicted the absence of both the signal peptide cleavage site and the N-linked and O-linked glycosylation sites on the MaBG sequence. Furthermore, the lack of cellobiase activity of MaBG was shown experimentally by kinetic analysis (Table 4) and the lack of the N-glycosylation by treatment with endoglycosidase $H_f$ (Fig. 2a). Even when expressed as a recombinant enzyme in *K. pastoris*, MaBG did not appear to contain any glycosylation modifications (Fig. 2b). The lack of hydrolytic activities of MaBG toward cellobiose was in contrast with the reported 72.5–638 μmol/min/mg cellobiase activities of other termite β-glucosidases, which suggested their roles in cellulose degradation in the digestive system. Together, these results suggested that MaBG is an intracellular enzyme, not involved in cellulolysis. Accordingly, the sequence of MaBG is highly similar to β-glucosidase from *O. formosanus* (GenBank accession ADD92156.1) and CfGlu1C from *C. formosanus* (GenBank accession AEW67361.1), which also lack both signal peptide and glycosylation sites. While the functional characterization of the *O. formosanus* β-glucosidase has not been reported, CfGlu1C was shown to exhibit a synergistic effect with two other *C. formosanus* β-glucosidases (CfGlu1B and CfGlu1D) in lactose hydrolysis, but by itself hydrolyzed lactose, sucrose, and cellobiose with lower activities when compared with CfGlu1B [32]. Furthermore, MaBG also shares moderate similarity with a myrosinase 1-like protein and lactase-phlorizin hydrolase-like proteins from the termite *Zootermopsis nevadensis*, which might be involved in plant defense against herbivores [56] and the production of some pheromonal compounds in insects [57], respectively (Fig. S3). Intracellular β-glucosidases are believed to exhibit broad specificities and have multiple functions inside eukaryotic cells such as in plants and in porcine species, or play a role in the regulation of cellulase gene expression in *Trichoderma ressei* and *Penicillium decumbens* [58–61]. Despite our attempt to report the novel characteristics of an intracellular β-glucosidase from termites, the exact biological roles of this group of enzymes have yet to be clarified.

While MaBG appeared to lack glycosylation modification, it seemed to require other cytosolic chaperone machinery found only in eukaryotic cells [62] to assume proper folding and activity. The failure to express MaBG as an active recombinant protein in *E. coli*, despite the lack of glycosylation modification, was in contrast with some previously reported termite β-glucosidases that were fully active when expressed in *E. coli* [33, 35–37, 50, 53, 55]. The temperature and pH conditions for activity and stability of MaBG were similar to those reported for other termite β-glucosidases [27, 33–35, 37, 52, 53]. However, when compared with the glucose-tolerant β-glucosidase from *N. takasagoensis* [28], MaBG displayed a lower optimal temperature and temperature stability. Nonetheless, glucose was found to exert a stabilizing effect on MaBG, presumably by its occupation in the substrate-binding pocket that helped to stabilize the enzyme structure.

MaBG exhibited a high transglucosylation activity, as evidenced by the glucose-stimulating effect on its *p*NP-Glc hydrolyzing activity (Fig. 4). When glucose was present up to 3.5–4.5 M in the reaction, MaBG appeared to preferentially utilize glucose, rather than water, as an acceptor for the glycosyl moiety from the glucosyl-enzyme intermediate [63, 64]. However, *p*NP-Glc hydrolyzing activities were decreased when the glucose concentrations were higher than 3.5 M, which was probably due to the competitive inhibition effect of glucose. Similarly, a glucose-stimulating effect was also found, but in a lower extent, in a termite β-glucosidase from *Neotermes koshunensis* [27]. Furthermore, in the presence of high *p*NP-Glc concentration (50 mM), MaBG displayed synthetic activity of the β1 → 6 bond formation (in *p*NP-Gen) (Fig. 5), as the first *p*NP-Glc molecule acted as a glucosyl donor and the second *p*NP-Glc molecule acted as a glucosyl acceptor that outcompeted a water molecule for the glucosyl moiety covalently bound to the enzyme. The formation of the β1 → 6 glucosidic bond in transglucosylation is consistent with the observed hydrolytic activity toward gentiobiose. Only when *p*NP-Glc was depleted in later hours of the transglucosylation reaction (due to being used up as the glucosyl donor and glucosyl acceptor), the water molecule could then compete with *p*NP-Glc in taking up the glucosyl moiety from the glucosyl-enzyme intermediate, producing glucose as a hydrolysis product. On the other hand, high concentrations of both glucose and octanol led to a thermodynamically driven reversal of hydrolysis, producing octyl glucoside as a product (Fig. 6). Note that more enzyme was needed to drive the reverse hydrolysis reactions than the hydrolysis or transglucosylation reactions, due to a lower reverse hydrolysis activity. It is possible that a large amount of enzyme could cause a secondary hydrolysis reaction, in which some of octyl glucoside (produced in the reverse hydrolysis reaction) was hydrolyzed back to octanol and glucose. Nonetheless, the presence of octyl glucoside from the reverse hydrolysis reaction indicated that the reverse hydrolysis activity was dominant than the hydrolysis activity under the assay conditions. Transglucosylation and reverse hydrolysis activities have been reported for many GH1 enzymes [58, 65–68]. However, this is the first report of transglucosylation and reverse hydrolysis activities of an intracellular GH1 β-glucosidase from termite and suggests its potential applications in enzymatic synthesis of useful glucoside products. In particular, synthetic reactions can be achieved via reverse hydrolysis without the need of an expensive glucosyl donor such as *p*NP-Glc.

In conclusion, a novel GH1 β-glucosidase with a potential synthetic activity, MaBG, was successfully isolated from *M. annandalei*, expressed recombinantly in yeast *K. pastoris*, and enzymatically characterized. MaBG seemed to be an intracellular enzyme due to the lack of both the signal peptide cleavage site and glycosylation modifications. Its actual physiological role is still unknown, but unlikely to be participating in lignocellulose digestion. It displayed a broad specificity characteristic and a capability in catalyzing transglucosylation and reverse hydrolysis reactions with various types of glucosyl acceptors. Further studies would be of interest in exploring the synthetic activities of MaBG for possible industrial applications. Also, further study in terms of the active site structure in relation with substrate specificity and catalytic capabilities will help improve our insight in exploiting GH1 enzymes in both hydrolytic and synthetic manners.

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**Compliance with Ethical Standards**

**Conflict of Interest** The authors declare that they have no conflicts of interest.

**Ethical Statement** This article does not describe any studies on human participants or animals performed by any of the authors.

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