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Heterologous expression and enzymatic characterization of β-glucosidase from the drywood-eating termite, *Neotermes koshunensis*

Jinfeng Ni,[1,2] Gaku Tokuda,[1,3] Motomi Takehara[1] and Hirofumi Watanabe[1,*]

[1] National Institute of Agrobiological Sciences; Tsukuba, Ibaraki 305–8634, Japan
[2] State Key Laboratory of Microbial Technology, Shandong University; 27 Shandanan Road, Jinan, Shangdong 250100, P. R. China
[3] Center of Molecular Biosciences (COMB), University of the Ryukyus; Nishihara, Okinawa 903–0213, Japan

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Abstract
A β-glucosidase cDNA from the termite, *Neotermes koshunensis*, was successfully overexpressed in *Escherichia coli*, and the product was purified to homogeneity by affinity purification against His-tags. The molecular weight of the recombinant enzyme was 60 kDa. The expressed β-glucosidase preferentially hydrolyzed laminaribiose and cellobiose rather than synthetic substrates such as *p*-nitrophenolic compounds. The K_m value of cellobiose was 3.8 mM and V_{max} was 220 U (μmol of glucose/min)/mg. The optimum pH and thermostability were 5.0 and 45°C, respectively. These enzymatic characters are mostly consistent with the partially-purified β-glucosidase from the salivary glands of *N. koshunensis*. However, the specific activity of the recombinant enzyme was 156.7 U/mg, which is almost 3-folds of that of the partially purified β-glucosidase of *N. koshunensis*. Owing to the successful expression of the termite β-glucosidase in *E. coli*, it may provide an opportunity of termite β-glucosidase for further improvement of the enzymatic properties for potential industrial applications with the aid of bioengineering.

Key words: Termite; *Neotermes koshunensis*; β-glucosidase; heterologous expression

INTRODUCTION

With a shortage of fossil fuel, utilization of cellulose as regenerated energy has attracted general interest, and a broader range of cellulose-decomposing organisms from bacteria to higher animals and plants have been employed as research targets. Termites, as one group of such interested cellulolytic life-forms, are important decomposers of cellulosic materials in tropical and subtropical regions (Yamada et al., 2005). In termite species harboring cellulolytic intestinal flagellates (species except family Termitidae), endogenous (termite-own) enzymes and those from symbiotic flagellates shared roles in cellulose digestion, and each element produced different enzymes in the cellulolytic function (Nakashima et al., 2002a, b; Watanabe et al., 2002).

In cellulose digestion, it is generally accepted that three different enzymes termed cellobiohydrolase (EC 3.2.1.91), endo-β-1,4-glucanase (EC 3.2.1.4) and β-glucosidase (EC 3.2.1.21) play a synergistic role in converting insoluble cellulose to glucose (Beguin and Aubert, 1994). The endogenous cellulolytic enzymes of termites primarily consist of endo-β-1,4-glucanase and β-glucosidase (Tokuda et al., 1997, 2002; Watanabe et al., 1997). So far, endogenous endo-β-1,4-glucanases of termites have been extensively studied (Tokuda et al., 1997; Watanabe et al., 1997, 1998). By contrast, biochemical and molecular biological studies on β-glucosidase of termites are limited. β-Glucosidase is a critical enzyme for cellulose degradation, catalyzing hydrolysis of cellobiose or cello-oligomers to glucose. Among the flagellate-harboring termites, only *Neotermes koshunensis* has been used for purification of the endogenous β-glucosidase that is secreted in the salivary glands (Tokuda et al., 2002). However, since the endogenous β-glucosidase from *N. koshunensis* was characterized using the partially purified enzyme (Tokuda et al., 2002), the real property of this enzyme still re-

* To whom correspondence should be addressed at: E-mail: hinabe@affrc.go.jp
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mains to be elucidated. Purification of digestive enzymes in insects is often inefficient and time-consuming due to their small body sizes and limited availability of the samples, and thus heterologous overexpression using a simple vector-host system would be a powerful tool for characterization and further applications of such enzymes. In the present study we describe the expression and enzymatic characterization of digestive β-glucosidase from the termite, *N. koshunensis*.

MATERIALS AND METHODS

Insect. *Neotermes koshunensis* (Isoptera, Kalotermitidae), which are distributed in the subtropical region of Japan, were collected and maintained as previously described (Tokuda et al., 2002).

Bacterial strains, plasmids, and culture conditions. A plasmid vector pAD10α, which was constructed as previously described (Ni et al., 2005), was employed as a cloning vector for *E. coli* DH5α (Toyobo, Osaka, Japan). Overexpression was conducted using the pQE30 vector (Qiagen, Inc., Valencia, CA, USA) with *E. coli* JM109 (Toyobo) as the host cell. *E. coli* cells harboring the pQE30 plasmids were grown at 37°C in Luria broth (LB) medium supplemented with 100 μg/ml of ampicillin. For enzyme production, *E. coli* cells were grown overnight in LB medium at 37°C. An aliquot (100 μl) of the pre-cultured cells was added to 10 ml of LB medium and incubated at 37°C with continuous shaking until the absorbance at 600 nm reached to 1.0. Then, isopropyl-β-D-thiogalactopyranoside (IPTG; final concentration, 1 mM) was added to induce expression and the cells were grown at 26°C for 4 h with continuous shaking.

Preparation of β-glucosidase cDNA from *N. koshunensis* and subcloned into expression vector. The β-glucosidase cDNA from *N. koshunensis* (*NkBG*: AB073638) was obtained according to the methods described previously (Tokuda et al., 2002). The cDNA fragment of the open reading frame (without the sequence encoding the N-terminal signal peptide) was cloned into pAD10α vector. Two primers pQEBGFor (5′ GCT AAA GAA GAA GGG GGA TCC CTC GAG AAA AGA GAC GTA GCC TCC 3′; The underline indicates the *Bam*HI site) and pQEBGRev (5′ TTG TTC TAG AAA GCT TGC GGC CGC CTT ATC TCT GAA GCG CTC AGG 3′; The underline indicates the *Hin*dIII site) were designed to amplify the inserted DNA fragment from the *NkBG*-pAD10α. The amplified fragment was double digested with *Bam*HI and *Hin*dIII and ligated with the pQE 30 vector. The plasmid was then transformed into *E. coli* JM109 and cultured as mentioned above.

Preparation of crude bacterial extracts and purification. After expression, the bacterial cells were recovered by centrifugation and the pellets were lysed using CelLytic^{TM} B Cell Lysis Reagent (Sigma-Aldrich, St. Louis, MO, USA). The supernatants were recovered and are referred to as crude extracts. The recombinant enzyme was purified from the crude extracts using the MagExtract His-tag fusion protein purification kit (Toyobo) according to the manufacturer's instruction. Enzyme purity was assessed by SDS-PAGE, which was performed using Ready Gel E-R520L (5–20% gradient; ATTO, Japan). Broad range prestained SDS-PAGE standards (Bio-Rad, Hercules, CA, USA) were used as the molecular weight markers.

Enzyme and protein assays. β-Glucosidase activity was measured using cellobiose (Nakalai Tesque, Kyoto, Japan) as the substrate. An aliquot (25 μl) of the enzyme solution was added into 100 μl of 2% cellobiose (w/v) in 0.1 M sodium acetate buffer (pH 5.5). The reaction mixture was then incubated at 37°C for 5 min and liberated glucose was detected by adding 1 ml of the glucose oxidase-mutarotase reagent (Glucose CII Test Wako; Wako Pure Chemical Co., Tokyo, Japan). The amount of glucose produced was determined by measuring absorbance at 505 nm. One unit (U) of enzyme activity was defined as the amount of enzyme which produced 1 μmol of glucose per min. Protein concentrations were determined using Quick Start Bradford dye reagent (Bio-Rad) with bovine serum albumin as the standard. Western blotting was carried out as previously described (Sugimura et al., 2003) with the anti His-tag antibody that was conjugated with horseradish peroxidase (Qiagen).

Characterization of the recombinant β-glucosidase. Kinetic constants (K_m and V_{max}) were determined using cellobiose varying from 1 to 20 mM in 0.1 M sodium acetate buffer (pH 5.5) on the basis of Lineweaver-Burk plot. The effect of temperature on the recombinant β-glucosidase activity was measured at 20 to 75°C with 5°C intervals. Ther-

mostability was determined by measuring the residual enzyme activity after pre-incubation of the diluted enzyme for 30 min at 20 to 75°C at 5°C intervals. The effect of pH was analyzed with McIlvaine's buffer (di-sodium phosphate–citric acid) (McIlvaine, 1921) ranging from pH 3.0 to 9.0 at 0.5 intervals. For pH stability, the enzyme was pre-incubated at pH 3.0–9.0 at 0.5 intervals at ambient temperature for 30 min. After enzymatic reactions, the enzymatic activities were measured as aforementioned.

Substrate specificities. Substrates for the experiments were purchased from Sigma-Aldrich unless otherwise indicated. To analyze substrate specificity, β-glucosidase activity was determined by measuring the release of glucose from various substrate solutions and suspensions (in 2% [w/v] in 0.1 M sodium acetate buffer [pH 5.5] unless specially mentioned). The substrate used for the specificity test were: gentibiose, melibiose, palatinose, sophorose, lactose, laminarin, salicin, cellulose (sodium carboxymethylcellulose, 1%, w/v), laminaribiose (Seikagaku Corp., Tokyo, Japan), cellotriose (30 mM, Nakalai Tesque), cellotetraose (30 mM, Nakalai Tesque), and cellopentose (15 mM, Nakalai Tesque). The condition of the enzymatic assays was the same as the quantitative assay aforementioned with cellobiose. β-Glucosidase activity was also determined by measuring the release of *p*-nitrophenol from 10 mM *p*-nitrophenyl-β-D-glucoside (glucopyranoside) (*p*NPβGlu) (Nakalai), 10 mM *p*-nitrophenyl-β-D-galactopyranoside (*p*NPβGal), 10 mM *p*-nitrophenyl-β-D-fucopyranoside (*p*NPβFuc), or *p*-nitrophenyl-β-D-mannopyranoside (*p*NPβMan) in 0.1 M sodium acetate buffer (pH 5.5) as described by Inoue et al. (2005). For the *p*-nitrophenyl compounds, 25 μl of enzyme were added into 100 μl of the substrate solution and incubated for 5 min at 37°C. The reaction was terminated by addition of 1 ml of 0.6 M Na_2CO_3 solution and the amount of *p*-nitrophenol librated was measured by the absorbance at 410 nm. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of released *p*-nitrophenol per min.

RESULTS

Overexpression of the β-glucosidase gene and purification of the enzyme

The endogenous β-glucosidase cDNA of *N. koshunensis* was cloned into pQE30 and successfully overexpressed in *E. coli* JM109. The homogeneous protein band corresponding to the molecular weight of 60 kDa was observed after the His-tag-based affinity purification from the cell free crude extract (Fig. 1a). Western blot analysis using anti His-tags antibody confirmed that the inserted β-glucosidase cDNA was overexpressed in *E. coli* (Fig. 1b). Table 1 summarizes the purification results of the recombinant β-glucosidase. The expressed protein had apparent β-glucosidase activity and was purified to 6.4 fold from the crude extracts with 20% recovery. Approximately 0.1 mg of the purified enzyme was obtained from 10 ml of cell culture.

Characterization of the purified recombinant β-glucosidase

Table 2 shows the comparison of the recombinant β-glucosidase with the partially purified β-

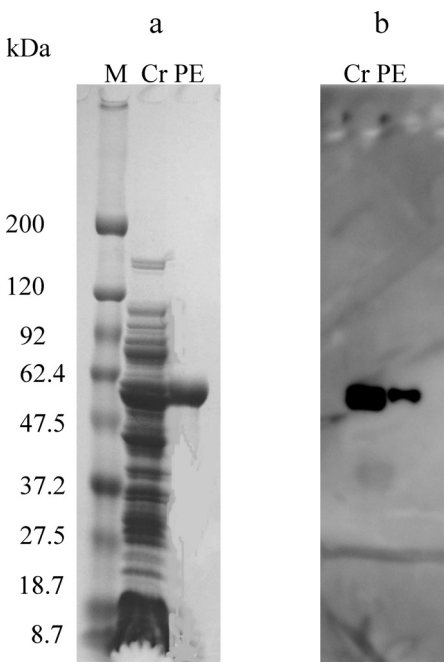

Fig. 1. Overexpression of *NkBG* in *E. coli* JM109. (a) SDS-PAGE analysis of the expression of the β-glucosidase, (b) Western blot analysis using anti His-tag antibody. M: molecular weight markers; lane Cr: crude extract; PE: purified recombinant enzyme (NkBG).

Table 1. Purification of the recombinant β-glucosidase from *E. coli*

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Crude extract	3.13	76.7	24.5	100	1
Purified enzyme	0.095	15.57	156.7	19.9	6.4

One unit (U) is the amount of enzyme which produced 1 μmol of glucose/min.

Table 2. Comparison of the native termite salivary and recombinant β-glucosidases[a]

Enzymes	Specific activity (U/mg)	K_m value (mM)	V_{max} value (U/mg)	Optimal temperature (°C)	Thermostability (°C)	Optimal pH
Salivary β-glucosidase[b]	55.3	2.5	nd	nd	~45	5.6
Recombinant β-glucosidase	156.7	3.8	220	50	~45	5.0

[a] Values shown are the means of triplicate experiments with cellobiose as substrates.
[b] Data reported by Tokuda et al. (2002). nd: not determined.

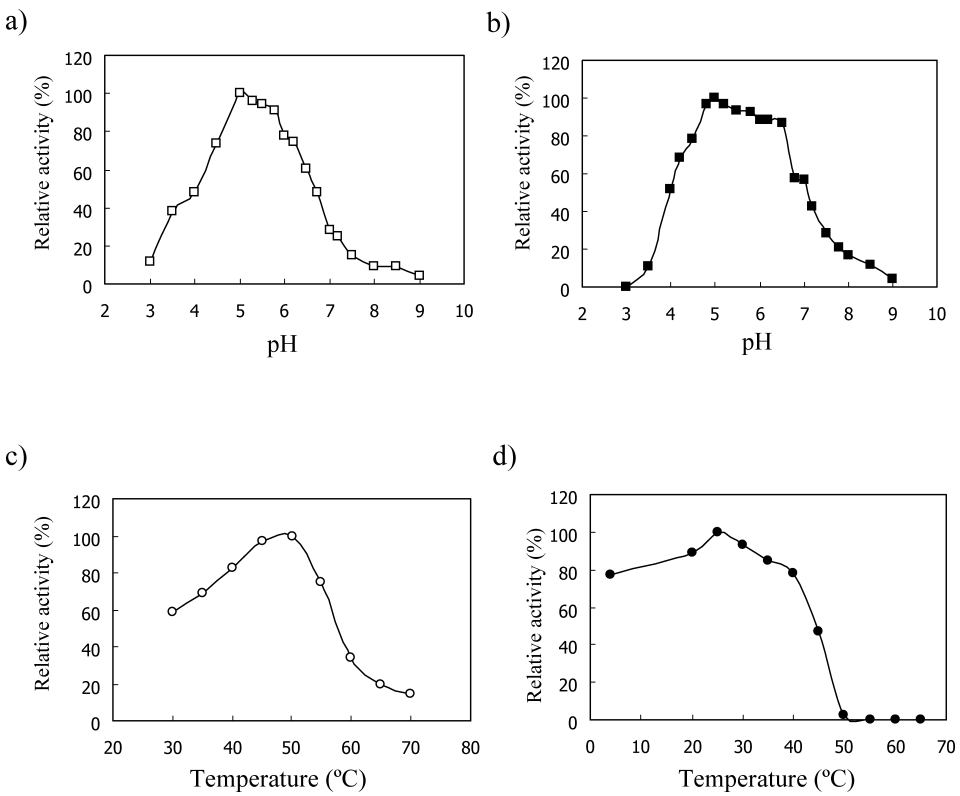

Fig. 2. Effects of pH and temperature on the recombinant β-glucosidase activity and stability of the recombinant termite β-glucosidase. (a) Effect of pH on the β-glucosidase activity. (b) Effect of pH on the enzyme stability. (c) Effect of temperature on the β-glucosidase activity. (d) Effect of temperature on the stability. The relative activities are shown as the means of at least three measurements.

glucosidase from the salivary glands of *N. koshunensis* (Tokuda et al., 2002). The recombinant enzyme exhibited pH optimal at 5.0, and was stable (i.e. retaining over 50% of the maximal activity) from pH 4 to 7 (Fig. 2a and b). The same enzyme showed maximum activity at 50°C in the re-

action for 5 min, and retained the activity above 80% of the maximum value during the preincubations at 40°C and below for 30 min, but almost completely lost activity at 50°C (Fig. 2c and d). The specific activity of the purified recombinant β-glucosidase against cellobiose was 156.7 U/mg, which is about three times higher than that of salivary β-glucosidase partially purified from *N. koshunensis* (55.3 U/mg; Tokuda et al., 2002). The K_m and V_{max} for cellobiose were 3.8 mM and 220 U/mg, respectively (Table 2).

Substrate specificities

Among the substrates tested, the recombinant β-glucosidase exhibited the maximal activity against laminaribiose. When cellobiose was used as the substrate, it showed 80% of activity as compared with laminaribiose. The recombinant β-glucosidase also hydrolyzed cellotriose and cellotetraose to some extent, and weakly degraded lactose and laminarin (Fig. 3a). No activity was observed against other natural compounds used as substrates (Fig. 3a). Among the five synthetic chromogenic substrates, the recombinant β-glucosidase showed the maximal activity against *p*NPβFuc (66.5 U/mg), and the activity against *p*NPβGlu was about 20% of the activity against *p*NPβFuc (Fig. 3b). Compared with the activity against cellobiose (156.7 U/mg), the purified recombinant enzyme showed considerably lower activity against the synthetic analog *p*NPβGlu (13.6 U/mg). The enzyme showed weak activity against *p*NPβGal, and it did not hydrolyze *p*NPβMan and *p*NPβXyl (Fig. 3b).

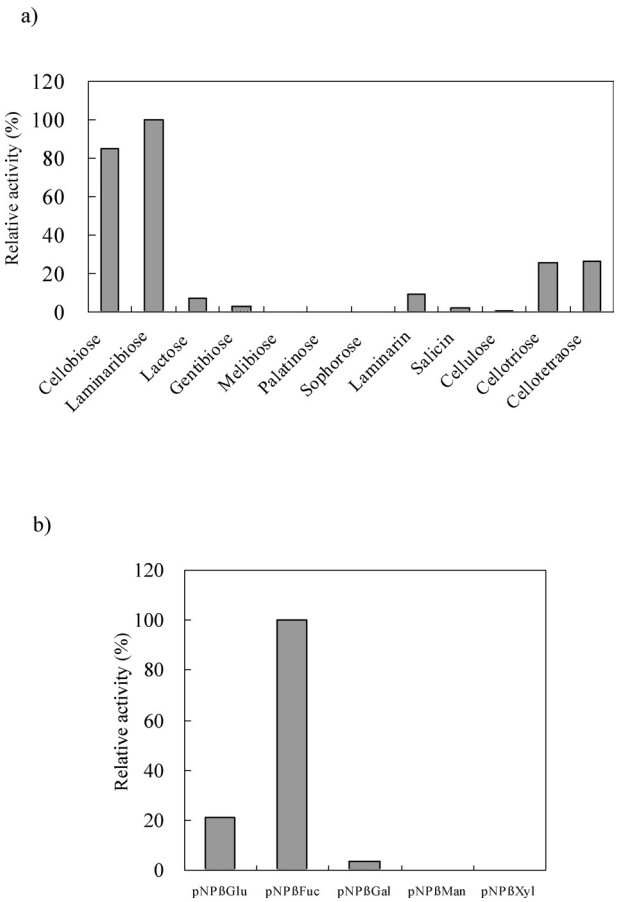

Fig. 3. Substrate specificities of the recombinant β-glucosidase against natural substrates (a) and synthetic substrates (b). Relative activities are shown as the means of three assays, which were calculated in comparison with the activities against laminaribiose (186.4 U/mg; a) or *p*NPβFuc (66.5 U/mg; b) (shown as 100%).

DISCUSSION

The present study showed the successful overexpression of termite β-glucosidase in *E. coli*. Although a number of microbial β-glucosidase genes have been expressed in either *E. coli*, yeast or fungi (Pandey and Mishra, 1997; Saloheimo et al., 2002; Li et al., 2004; Hong et al., 2007) studies on heterologous expressions of insect β-glucosidases are still limited (Marana et al., 2002; Byeon et al., 2005).

The enzymatic properties and substrate specificities of the expressed β-glucosidase were mostly consistent with those of the native β-glucosidase from the salivary glands of *N. koshunensis* (Tokuda et al., 2002). As is the case of the partially purified β-glucosidase, this enzyme preferentially hydrolyzes β-1,3 glycosidic bonds in addition to β-1,4 glycosidic linkages because of the strong activity against laminaribiose. The recombinant β-glucosidase also hydrolyzed *p*-nitrophenolic compounds to some extent, confirming that the termite β-glucosidase is affiliated with Class 1 enzymes, which hydrolyze both native and synthetic substrates (Terra et al., 1996). The present study employed cellotriose and cellotetraose as short cellulosic substrates in addition to cellobiose, and the recombinant enzyme showed more active hydrolysis of cellobiose than cellotriose or cellotetraose. Since the endogenous endo-β-1,4-glucanases of termites are able to hydrolyze cello-oligosaccharides (Tokuda et al., 1997; Watanabe et al., 1997), the present β-glucosidase would primarily partici-

pate in cleavage of cellobiose or some other hemicellulosic disaccharides to produce glucose. However, its inactiveness against *p*NPβXyn suggests that the termite β-glucosidase is not involved in degradation of the major hemicellulosic compounds such as xylan and its derivatives.

As previously reported, the present β-glucosidase has a potential N-glycosylation site near the C-terminus (Tokuda et al., 2002). Considering the consistency in the molecular weights and the enzymatic properties between the recombinant β-glucosidase of the current study and the partially purified (native) β-glucosidase from the salivary glands of *N. koshunensis* (Tokuda et al., 2002), the latter is likely functional without glycosylation *in vivo*. However, it is noteworthy that there are a few small differences in enzymatic properties between the recombinant and the native β-glucosidases. The recombinant enzyme revealed threefold higher specific activity than that of the native enzyme (Tokuda et al., 2002). This difference might be caused by impurity of the native enzyme, and the V_{max} value of the recombinant β-glucosidases would reflect the real property of the catalytic velocity of the salivary β-glucosidase of *N. koshunensis*.

In the previous paper, we showed a mass-production system of termite endo-β-1,4-glucanases using the same vector-host combination as described in the present study (Ni et al., 2005). The present result provokes expectations for further exploration and characterization of β-glucosidases not only in flagellate-harboring termites but also in flagellate-free termites that have diverse feeding preferences (Waller and Fage, 1987). Furthermore, an accumulation of DNA sequence data of the termite enzymes will provide an opportunity for improvement of the β-glucosidase properties using bioengineering methods such as DNA shuffling like the case of the termite endo-β-1,4-glucanases (Ni et al., 2005), which enables modifications of the enzymes of insect origins to be adequate for industrial applications or biomass conversions.

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