Insect science

Insect Science (2010) 17, 245–252, DOI 10.1111/j.1744-7917.2010.01321.x

ORIGINAL ARTICLE

Hydrolysis of filter-paper cellulose to glucose by two recombinant endogenous glycosyl hydrolases of *Coptotermes formosanus*

Dunhua Zhang[1], Alan R. Lax[1], John M. Bland[1], Jiujiang Yu[2], Natalie Fedorova[3,4] and William C. Nierman[4]

[1]*Formosan Subterranean Termite Research Unit, Southern Regional Research Center, ARS, USDA, New Orleans,* [2]*Food & Feed Safety Research Unit, Southern Regional Research Center, ARS, USDA, New Orleans,* [3]*Department of Biochemistry and Molecular Biology, The George Washington University School of Medicine, Washington, DC, and* [4]*The J. Craig Venter Institute, Rockville, MD, USA*

Abstract Genes encoding for glycosyl hydrolases (GH) in multiple families were recovered from an expression sequence tag library of *Coptotermes formosanus*, a xylophagous lower termite species. Functional analyses of these genes not only shed light on the mechanisms the insect employs to successfully use cellulosic materials as energy sources, which may serve as strategic targets for designing molecular-based bio-pesticides, but also enrich discoveries of new cellulolytic enzymes for conversion of biomass into biofuel. Our study demonstrated that cellulose could be converted to glucose by two recombinant endogenous glycosyl hydrolases (endo-β-1,4 glucanase in GH9 and β-glucosidase in GH1). While the former cleaved cellulose to cellobiose and cellotriose, the resulting simple cellodextrins were digested to glucose. Both of the *Escherichia coli*-expressed recombinant proteins showed properties that could be incorporated in a glucose-based ethanol production program.

Key words biofuel, cellulose digestion, endo-β-1,4 glucanase, β-glucosidase, termite

Introduction

The Formosan subterranean termite, *Coptotermes formosanus* Shiraki, is one of the most destructive and costly wood-feeding insects in many parts of the world (Su & Scheffrahn, 2000; Lax & Osbrink, 2003; Archicentre, 2003; Tsunoda, 2003). Clarifying the molecular mechanisms that enable the wood-degrading insect to utilize lignocellulose as a sole food source is thus a tantalizing area of research in which to explore the potential of developing cellulolytic enzyme-specific biopesticides (Zhu *et al.*, 2005; Zhou *et al.*, 2008) and optimizing recombinant cellulolytic enzyme production for biomasss conversion (Ni *et al.*, 2005, 2007a, 2007b; Todaka *et al.*, 2009).

Previously, we demonstrated that *C. formosanus*-derived endogenous endo-β-1,4-glucanase, expressed in *Escherichia coli*, can digest filter-paper cellulose releasing predominantly cellotriose and cellobiose (Zhang *et al.*, 2009). This suggests that this salivary gland-abundant cellulase (Nakashima *et al.*, 2002) could be involved in initial cleavage of cellulosic materials ingested by the termite. The function of this cellulase also implies that cellulose could be converted to glucose when an additional β-glucosidase is present, without the aid of a 1,4-β-D-glucan cellobiohydrolase (cellobiohydrolase, exoglucanase), which is thought necessary for effective biological hydrolysis of cellulose to glucose in fungal and bacterial cellulolytic systems (Béguin & Aubert, 1994).

Correspondence: Dunhua Zhang, Formosan Subterranean Termite Research Unit, Southern Regional Research Center, ARS, USDA, 1100 Robert E. Lee Boulevard, New Orleans, LA 70124, USA. Tel: (504) 286 4382; email: dunhua.zhang@ars.usda.gov

A β-glucosidase is known to be present in the salivary gland and midgut of *C. formosanus* (Itakura *et al.*, 1997; Zhu *et al.*, 2005). However, the gene sequence of the putative β-glucosidase has not been analyzed and its cellulolytic activity is unknown.

In this study, the endogenous β-glucosidase gene of *C. formosanus* was cloned from a cDNA library of worker termites and heterologously expressed in *E. coli*. The enzymatic activity of the recombinant β-glucosidase was determined using cellodextrins and enzymatic end products of filter-paper cellulose catalyzed by a recombinant endogenous endo-β-1,4-glucanase.

Materials and methods

Cloning of β-1,4-glucanase and β-glucosidase genes

The β-1,4-glucanase gene (accession No. EU853671) was cloned as described previously (Zhang *et al.*, 2009). The primers used for cloning of β-glucosidase gene were designed based on the sequence derived from a *C. formosanus* expression sequence tag (EST) library (which was made from a pool of normalized mRNA, including different developmental casts; the EST data is under annotation). The full-length cDNA of β-glucosidase was cloned from the termite worker rapid amplification of cDNA ends (RACE)-ready cDNA using the gene-specific primers and the SMART RACE cDNA Amplification kit (Clontech, Mountainview, CA, USA). Resulting cDNA clones were sequenced and analyzed using basic local alignment search tool (BLAST) search and Vector NTI program (Invitrogen, Carlsbad, CA, USA).

Production of recombinant proteins in E. coli

The coding sequences for the mature peptides of endo-β-1,4-glucanase (Zhang *et al.*, 2009) and β-glucosidase (predicted by on-line program SignalP 3.0 Server: www.cbs.dtu.dk/services/SignalP) were polymerase chain reaction (PCR)-amplified using the following primer pairs: (i) 5′-CTAGCCATG GCT TAC GAC TAC AAG ACA GTA CTG AAG A-3′ (forward) and 5′-CAGACTCGAG CAC GGC TGC CTT GAG GAG ACC-3′ (reverse) for endo-β-1,4-glucanase; (ii) 5′- CTAGCCATG GAT GAC GTC GAT AAC GAC ACC CTT G -3′ (forward) and 5′-CAGACTCGAG GTC TCG GAA GCG CTC TGG AAT CTG-3′ (reverse) for β-glucosidase. Both forward primers flanked an *Nco* I restriction site at the 5′ end and reverse ones had an *Xho* I site at the 5′ end (underlined). Amplicons were gel-purified and subjected to double digestion with *Nco* I and *Xho* I (New England BioLabs, Ipswich, MA, USA). Following further purification, both amplicons were cloned into pET28a vector plasmids (Novagen, Madison, WI, USA) at corresponding restriction sites. Recombinant plasmids were propagated in *E. coli* NovaBlue cells (Novagen) and purified for sequence analysis.

Competent cells of *E. coli* Rosetta 2 (DE3) (Novagen) were transformed with recombinant plasmids carrying an endo-β-1,4-glucanase gene or β-glucosidase gene, respectively, and selected on Luria-Bertani (LB) agar supplemented with antibiotics (50 μg/mL kanamycin and 34 μg/mL chloramphenicol). Resulting colonies were propagated in LB broth containing the antibiotics for glycerol stocks and recombinant protein production.

Actively growing colonies on LB agar (37°C, overnight) were inoculated in LB broth with antibiotics and shake-cultured at 250 r/p at 37°C for approximately 6 h. Bacterial cells were pelleted by centrifugation and were further propagated in 40 mL fresh LB broth with antibiotics by shaking at 37°C until reaching optical density (OD_{600}) of approximately 0.4–0.5. Protein overexpression was induced by addition of IPTG ((isopropyl-β-D-thiogalactopyranoside), at a final concentration of 0.5 mmol/L, to the bacterial cultures with continuous shaking at 23°C for 16 h. The induced bacterial cells were then aliquoted and pelleted by centrifugation. Total cell proteins were extracted using 1× CelLytic B Cell Lysis Reagent (Sigma, St Louis, MO, USA), which was diluted with a buffer containing 20 mmol/L Tris, 0.5 mol/L NaCl, pH 7.9. The cell lysates were clarified by centrifugation. Target proteins were purified from the resulting supernatants using His•Mag Agrose Beads (Novagen) in accordance with the manufacturer's instruction. Protein concentration of each preparation was estimated with Bradford Reagent (Sigma) using bovine serum albumin (BSA) as the protein standard. Protein profiles of crude extracts and purity of purified proteins were determined by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) using NuPAGE 4%–12% Bis-Tris pre-cast gel and MOPS/SDS running buffer (Invitrogen).

Digestion of cellodextrins/filter paper with the recombinant proteins

Cellodextrins used included cellobiose (99% purity, Sigma), cellotriose (98% purity, Sigma), cellotetraose (90% purity, Sigma) and cellopentaose (80% purity, Sigma). Each was dissolved in 50 mmol/L sodium acetate (pH 5.0) at concentrations of 10 mg/mL. An aliquot of 100 μL of the cellodextrin was individually mixed with

5 μL (100 ng) of purified β-glucosidase. The mixture was incubated at 37°C for 1 h. An aliquot of 1 μL of the reaction was analyzed using thin layer chromatography (TLC) described below.

Filter paper digestion was conducted by mixing 15 mg of pre-cut filter paper (~1–2 × 1–2 mm, Whatman #4) with 200 μL 50 mmol/L sodium acetate buffer (pH 5.0) containing 200 ng of purified endo-β-1,4-glucanase. The mixture was incubated at 37°C for 24–48 h. An aliquot of 5 μL was analyzed using TLC. Additionally, an aliquot of 50 μL of the reaction was mixed with 2.5 μL (50 ng) of purified β-glucosidase and incubated at 37°C for 1 h. An aliquot of 5 μL of the second reaction was also analyzed using TLC.

Thin layer chromatography conditions were performed as described previously (Zhang *et al.*, 2009). Briefly, after spotting the samples on an TLC plate (Silica Gel 60, EMD Chemicals Inc, Gibbstown, NJ, US), chromatography was developed in a solvent of n-butanol/glacial acetic acid/water (2 : 1 : 1) for 1.5 h. The plate, after drying with a stream of warm air, was stained by spraying with *p*-anisaldehyde reagent (ethanol : glacial acetic acid : sulfuric acid : *p*-anisaldehyde = 9.0 : 0.1 : 0.75 : 0.75) and heated in an oven at 110°C for 5–10 min. Reference sugar standard, containing 10 μg each of glucose, cellobiose, cellotriose, cellotetraose and cellopentaose, was included in each TLC run.

β-glucosidase activity on cellobiose at different pH and temperature

The optimal ranges of pH and temperature for β-glucosidase activity on cellobiose were determined by the following settings. Buffers with pH range from 3.8 to 7.8 were prepared using 100 mmol/L acetic acid per 100 mmol/L sodium acetate (pH 3.8–5.6) and 100 mmol/L NaH_2PO_4 per 100 mmol/L Na_2HPO_4 (pH 6.2–7.8). Aliquots of 100 μL cellobiose solution at concentration of 5 μg/μL with different pH units, containing 200 ng of β-glucosidase, were incubated at 37°C for 75 min. Following incubation, an aliquot of 2 μL for each reaction was analyzed by the TLC method described above.

Five temperature assays were conducted between 29°C and 60°C. Aliquots of 100 μL cellobiose solution at 5 μg/μL in 100 mmol/L sodium acetate buffer (pH 5.0), containing 200 ng of β-glucosidase, were incubated at different temperatures. At a 15-min interval an aliquot of 10 μL reaction was taken from individual reactions and placed on ice until TLC analysis, the same method as described above.

Results

β-glucosidase gene structure

The complete cDNA of *C. formosanus* β-glucosidase, as shown in Fig. 1, was the first of its kind cloned from this insect (GenBank accession no.: GQ911585). The full-length sequence consists of 1 745 bp excluding the poly-A tail. The single open reading frame indicates that the translation starts at nucleotide 106 and ends at thenucleotide 1 593, encoding 495 amino acids. There is a predicted 17-amino acid signal peptide. The 478-amino acid mature peptide has a calculated molecular weight of 54.58 kDa with isoelectric point (pI) of 4.77. Protein BLAST search shows that the protein is similar to β-glucosidases in glycosyl hydrolase (GH) family 1 and possesses corresponding amino acid residues for catalysis and substrate binding (Marana *et al.*, 2001).

Expression of recombinant proteins

Production of recombinant endo-β-1,4-glucanase and β-glucosidase in *E. coli* was achieved using IPTG induction. Apparently homogeneous recombinant proteins were recovered from the crude extracts following affinity purification (Fig. 2). The endo-β-1,4-glucanase shows a distinctive band with molecular weight of ~48 kDa while the distinctive band for β-glucosidase has a molecular weight of ~ 56 Daltons on SDS-PAGE. From a 10 mL culture, approximately 150–200 μg purified proteins were obtained for both endo-β-1,4-glucanase and β-glucosidase.

Enzymatic hydrolysis of cellodextrins and filter-paper cellulose

The recombinant endo-β-glucosidase could hydrolyze cellobiose, cellotriose, cellotetraose and cellopentaose and generated only glucose as the end product as shown in Fig. 3. Filter paper was not hydrolyzed by the enzyme even in a prolonged incubation (at 37°C for 4 days; lane 5 of Fig. 4). On the other hand, the recombinant endo-β-1,4-glucanase could hydrolyze filter-paper cellulose and produced mainly cellobiose and cellotriose in a 24-h incubation at 37°C (Lane 2, Fig. 4). When the reaction was kept at 37°C for another 24 h or longer, the end products of the hydrolysis were mainly cellobiose and glucose, and no cellotriose was detected (Lane 3, Fig. 4). By addition of recombinant β-glucosidase to the hydrolytic end products of filter paper catalyzed by endo-β-1,4-glucanase (24 h, 37°C), only glucose was

```
1     TGGCCGGGGACACACACACATATATAATATATAAAAAGAAAGGCTTTCTCTGTTACTCTGGTACTCTGACACTCCTACGGCAAGGGCGCT
                                                                                              25
                        M  R  F  Q  T  L  C  L  V  V  F  V  T  T  V  F  G  D  D  V  D  N  D  T  L
91    TCACGGAGCAACGAGATGAGGTTCCAAACGCTTTGCCTCGTCGTCTTTGTGACGACAGTATTCGGAGATGACGTCGATAACGACACCCTT
                                                                                              55
       V  T  F  P  D  D  F  K  L  G  A  A  T  A  S  Y  Q  I  E  G  G  W  D  A  D  G  K  G  P  N
181   GTTACGTTTCCTGACGATTTTAAGTTAGGTGCCGCTACAGCTTCATACCAGATTGAAGGAGGATGGGATGCGGATGGAAAGGGTCCCAAC
                                                                                              85
       I  W  D  T  L  T  H  E  R  P  H  L  V  V  D  R  S  T  G  D  V  A  D  D  S  Y  H  L  Y  L
271   ATCTGGGACACTCTGACACACGAGCGCCCACACTTAGTGGTTGATCGTTCAACAGGTGACGTAGCGGATGATTCGTATCACTTGTACCTG
                                                                                             115
       E  D  V  R  L  L  K  D  M  G  A  E  V  Y  R  F  S  I  S  W  A  R  I  L  P  E  G  H  D  N
361   GAAGACGTTCGACTTCTGAAGGACATGGGGGCAGAAGTTTATCGCTTCTCCATATCTTGGGCTCGCATCCTCTACGGAATGGAATAGAGC
                                                                                             145
       N  V  N  E  A  G  I  E  Y  Y  N  K  L  I  D  A  L  L  R  N  G  I  E  P  M  V  T  M  Y  A
451   TGCCTGAAGGACACGATAACAACGTGAACGAAGCGGGTATTGAGTACTATAACAAACTCATAGACGCACTCGATGGTTACTATGTATCAC
                                                                                             175
       Y  H  W  D  L  P  Q  K  L  Q  D  L  G  G  W  P  N  R  I  L  A  K  E  N  Y  A  R  V  L  F
541   TGGGATCTACCCCAGAAACTCCAAGACCTAGGAGGATGGCCAAATAGGATATTGGCCAAATACGCCGAGAATTACGCCCGCGTTTTGTTT
                                                                                             205
       S  N  F  G  D  R  V  K  Q  W  L  T  F  N  E  P  L  T  F  M  D  A  Y  A  S  D  T  G  M  A
631   AGTAACTTTGGTGACAGGGTCAAACAGTGGCTCACCTTCAACGAACCTCTGACATTCATGGATGCATACGCATCTGACACAGGAATGGCT
                                                                                             235
       P  S  V  D  T  P  G  I  G  D  Y  L  T  A  H  T  V  I  L  A  H  A  N  I  Y  R  L  Y  E  R
721   CCATCAGTCGACACACCCGGTATCGGTGACTACCTCACGGCACATACTGTAATCCTTGCCCATGCCAATATCTACCGTTTGTATGAGAGG
                                                                                             265
       E  F  R  E  E  Q  Q  G  Q  V  G  I  A  L  N  I  H  W  C  E  P  E  T  G  S  P  K  D  V  E
811   GAATTCAGAGAGGAACAGCAAGGACAGGTTGGTATCGCACTCAATATCCACTGGTGTGAGCCGGAGACTGGTTCGCCAAAAGACGTTGAG
                                                                                             295
       A  C  E  R  Y  Q  Q  F  N  L  G  I  Y  A  H  P  I  F  S  E  N  G  D  Y  P  S  V  L  K  A
901   GCTTGTGAAAGGTACCAACAGTTCAATCTGGGAATATACGCTCATCCCATCTTCAGCGAAAACGGCGACTACCCCAGTGTTTTGAAAGCG
                                                                                             325
       R  V  D  A  N  S  A  S  E  G  Y  T  T  S  R  L  P  K  F  T  P  E  E  V  A  F  V  N  G  T
991   AGGGTGGACGCCAACAGCGCATCGGAAGGTTACACAACATCGCGGCTACCAAAGTTCACTCCAGAGGAAGTAGCTTTCGTCAATGGAACA
                                                                                             355
       Y  D  F  L  G  L  N  F  Y  T  A  V  V  G  R  D  G  V  E  G  E  P  P  S  R  Y  R  D  M  G
1081  TATGATTTCCTGGGTCTGAATTTCTACACTGCTGTAGTCGGAAGAGATGGAGTTGAAGGGGAACCGCCTTCACGGTACAGAGACATGGGC
                                                                                             385
       T  I  T  S  Q  D  P  E  W  P  E  S  A  S  S  W  L  R  V  V  P  W  G  F  R  K  E  L  N  W
1171  ACGATAACATCACAGGATCCGGAGTGGCCCGAGTCTGCTTCTTCATGGCTCAGAGTTGTACCGTGGGGATTCCGCAAGGAACTCAACTGG
                                                                                             415
       I  A  N  E  Y  G  N  P  P  I  F  I  T  E  N  G  F  S  D  Y  G  G  V  N  D  T  N  R  V  L
1261  ATCGCCAACGAATATGGTAACCCCCCTATATTCATCACTGAAAACGGCTTCTCAGACTACGGTGGAGTCAATGACACTAACAGAGTACTT
                                                                                             445
       Y  Y  T  E  H  L  K  E  M  L  K  A  I  H  I  D  G  V  N  V  I  G  Y  T  A  W  S  L  I  D
1351  TACTACACTGAACATTTAAAGGAGATGCTGAAGGCAATTCACATAGACGGAGTTAACGTAATCGGATACACAGCTTGGAGCCTCATAGAC
                                                                                             475
       N  F  E  W  L  R  G  Y  T  E  R  F  G  I  H  A  V  N  F  I  D  P  S  R  P  R  T  P  K  E
1441  AACTTCGAATGGCTGCGAGGATATACTGAGAGGTTCGGTATACATGCAGTGAATTTCATCGACCCAAGTCGCCCACGAACTCCGAAGGAG
                                                                                             495
       S  A  R  V  L  T  E  I  F  K  T  R  Q  I  P  E  R  F  R  D  *
1531  TCGGCCAGGGTACTCACAGAGATCTTCAAAACAAGACAGATTCCAGAGCGCTTCCGAGACTAACTTCATATTCAAGGCGCTACGACTTAT

1621  ACAAAAAAAAAAAAATTCAAATACGGCATACTGCTTCTATGAGTTCCCTTGAAAACAGCAGTAAGGTCATGGAAAACAGTTGTAATTAAAT
1711  AAACATATACACAAAAAAAAAAAAAAAAATCCTTCGAAAAAAAAAAAAAAAAAA
```

Fig. 1 The complete cDNA and translated amino acid sequences of *Coptotermes formosanus* β-glucosidase. The 5′ and 3′ untranslated sequences are in italic; the poly-A signal is underlined. The 17-amino acid signal peptide is highlighted in bold. Amino acid residues, involved in catalysis and substrate binding, are underlined.

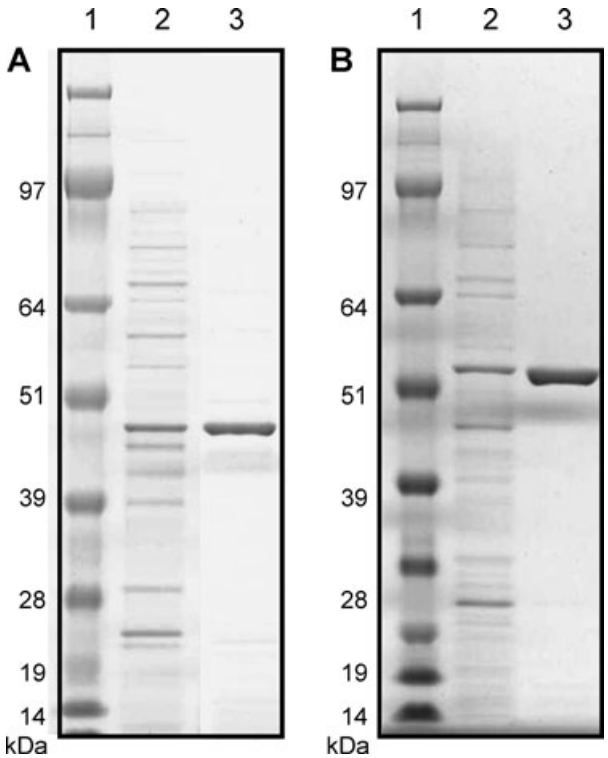

Fig. 2 Sodium dodecyl sulfate – polyacrylamide gel electrophoresis of recombinant endo-β-1,4-glucanase (A) and β-glucosidase (B). A: Lane 1: molecular marker (SeeBlue® Plus 2, Invitrogen, Carlsbad, CA, US); Lane 2: crude extract of recombinant endo-β-1,4-glucanase; Lane 3: purified endo-β-1,4-glucanase. B: Lane 1: molecular marker (Invitrogen); Lane 2: crude extract of recombinant β-glucosidase; Lane 3: purified β-glucosidase. The gels were stained with SimpleBlue SafeStain (Invitrogen).

detected in the reaction after 1-h incubation at 37°C (Lane 4, Fig. 4).

β-glucosidase activity at different pH and temperature

With cellobiose as substrate, enzymatic conversion to glucose by β-glucosidase was almost completed in 75 min at pH 5.0–6.6. Beyond this pH range the enzymatic activity decreased gradually (Fig. 5). The optimal temperature range for the β-glucosidase activity is shown in Fig. 6. For the complete conversion it took ~75 min at 29–37°C, 60 min at 42°C, 45 min at 48–54°C, and 60 min at 60°C. While the optimal temperature for the enzymatic activity ranged from 42°C to 54°C, the enzyme was apparently unstable when the temperature reached to 60°C.

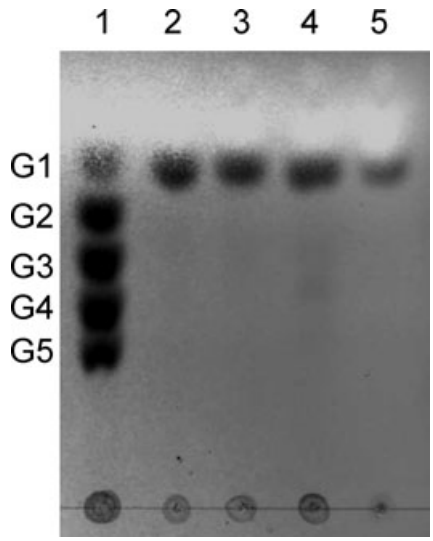

Fig. 3 Thin layer chromatography of cellodextrin hydrolytic products by the recombinant β-glucosidase. In Lane 1, cellodexrins, G1–G5, indicate glucose, cellobiose, cellotriose, cellotetraose and cellopentaose, respectively; each loaded at approximately 10 μg. Lanes 2–5: hydrolytic products (at 37°C for 1 h) of G2, G3, G4 and G5, respectively (approximately 10 μg of substrate equivalent were spotted).

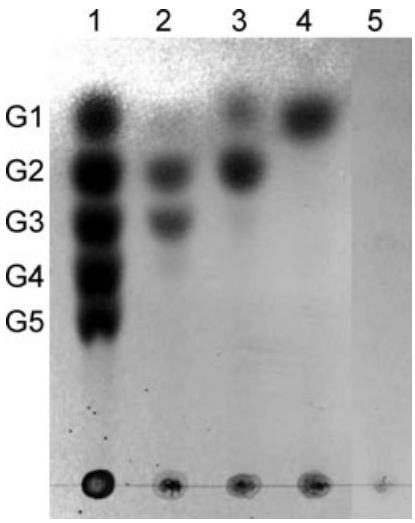

Fig. 4 Thin layer chromatography of hydrolytic products of filter paper. Lane 1: cellodextrin standards, the same as depicted in Figure 3. Lane 2: recombinant endo-β-1,4-glucanase with filter paper (at 37°C for 24 h); Lane 3: the same reaction as Lane 2 but incubated at 37°C for 48 h; Lane 4: the end products of Lane 2 with addition of recombinant β-glucosidase and incubation at 37°C for 1 h; Lane 5: recombinant β-glucosidase with filter paper (at 37°C for 4 days).

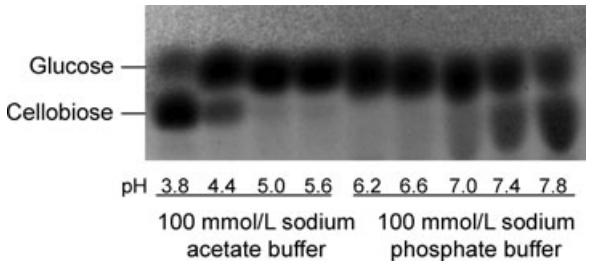

Fig. 5 Thin layer chromatography of cellobiose hydrolytic product by the recombinant β-glucosidase at different pH solutions. Cellobiose was dissolved in 100 mmol/L sodium acetate buffer (pH 3.8–5.6) or 100 mmol/L sodium phosphate buffer (pH 6.2–7.8) at concentration of 5 μg/μL and incubated at 37°C for 75 min. An aliquot of 2 μL was applied to the analysis.

Discussion

Lignocellulosic biomass stores energy in the form of plant cell wall polymers (cellulose, hemicellulose and lignin) and offers a renewable source of sugars that can be converted to ethanol and other liquid fuels (Rubin, 2008). Termites are considered as an extremely successful group of wood-degrading organisms (Sugimoto *et al.*, 2000). Extensive research efforts have been made to determine the lignocellulose-degrading/energy-conversion biocatalysts residing in termites and their symbiotic/mutualistic microbes, such as *Nasutitermes* sp. hindgut microbiota (Warnecke *et al.*, 2007), protists of *Reticulitermes speratus* (Todaka *et al.*, 2007), an endosymbiont of *C. formosanus* protist (Hongoh *et al.*, 2008), and *Reticulitermes flavipes* host and symbionts (Scharf & Tartar, 2008).

To identify and characterize the unique molecular-processing mechanisms of *C. formosanus*, which enable the termite to undergo caste differentiation and metabolize wood lignocellulose, we have sequenced approximately 75 000 clones from a normalized *C. formosanus* EST library (the annotation is ongoing). Preliminary assessments show that genes encoding multiple families of glycosyl hydrolases were present in the *C. formosanus* transcriptome as seen from other termite species mentioned above, though sequence (codon) variations were observed. Most of the endogenous glycosyl hydrolases are expressed predominantly in *C. formosanus* salivary glands as revealed by quantitative reverse transcription-PCR (D. Zhang & A.R. Lax, manuscript in preparation), suggesting that they may be directly involved in wood lignocellulose digestion.

The demonstrative experiments conducted in this study provide evidence that the recombinant

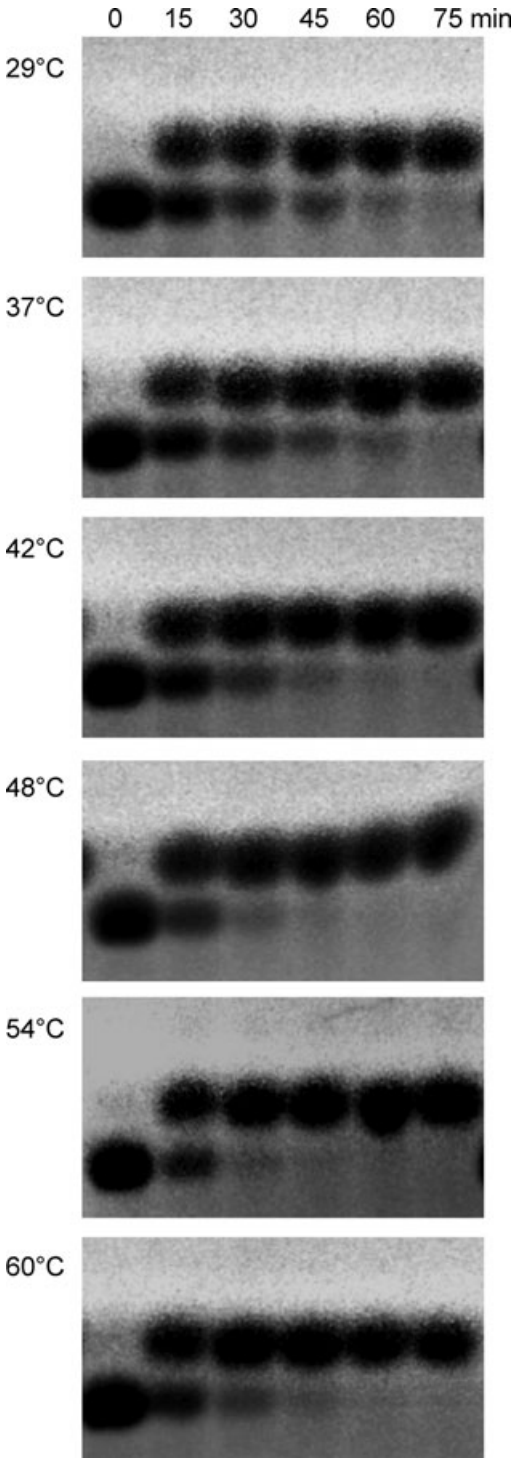

Fig. 6 Thin layer chromatography of cellobiose hydrolytic product by the recombinant β-glucosidase at time course from 0 to 75 min at different temperatures. Cellobiose was dissolved in 100 mmol/L sodium acetate buffer (pH 5.0) at concentration of 5 μg/μL and incubated at corresponding temperature settings. An aliquot of 2 μL was applied to the analysis.

endo-β-1,4-glucanase and β-glucosidase derived from *C. formosanus* and produced in *E. coli* are active in hydrolyzing cellulose to glucose. The endo-β-1,4-glucanase produced mainly cellobiose and cellotriose from the cellulose and could convert cellotriose into cellobiose and glucose in a prolonged reaction. Although endoglucanases are generally thought to hydrolyze internal β-1,4-glycosidic bonds, the insect-origin endo-β-1,4-glucanases seem to possess characteristic properties of cellobiohydrolase, an exoglucanase, as observed in others extracted/purified from a higher termite (*Nasutitermes walkeri*; Schulz *et al.*, 1986), a lower termite (*Reticulitermes speratus*; Watanabe *et al.*, 1997) and a cockroach (*Panesthia cribrata*; Scrivener & Slaytor, 1994). Recently, an endoglucanase (GH family 7), derived from an *R. speratum* symbiont and expressed in *Aspergillus oryzae*, was shown to have similar properties (Todaka *et al.*, 2009). The molecular mechanism underlying this property is currently unknown. The *C. formosanus* β-glucosidase, as first reported here in recombinant form, hydrolyzed 2–5 units of cellodextrin polymers but not cellulose. This property resembles that of a recombinant β-glucosidase of *Neotermes koshunensis* (Ni *et al.*, 2007b), although differential enzymatic activities were observed (D. Zhang & A.R. Lax, manuscript in preparation).

The functions of most sequencing-derived cellulolytic genes are predicted, and their enzymatic activities, substrates and eraction end products have yet to be determined. Actually, some cellulases may have altered activities or even gained new functions (Davison & Blaxter, 2005). A β-glucosidase (GH family 1) in *Cryptotermes secundus* has apparently evolved from an ancestral role of wood digestion to pheromonal communications (Korb *et al.*, 2009) and a β-1,3-glucanases (GH family 16) in *Nasutitermes corniger* (Bulmer *et al.*, 2009) and in *Helicoverpa armigera* (Pauchet *et al.*, 2009) was found to serve as an antimicrobial effector. Copper oxidase (laccase) was reported to be responsible for the cuticle sclerotization and tanining in *Bombyx mori* (see Yatsu & Asano, 2009); however, in wood-feeding termites the enzyme may be related to lignin oxidation or depolymerization (Scharf & Tartar, 2008). Therefore, functional analysis of candidate lignocellulytic proteins in recombinant form in large quantities, as demonstrated in this study, could reveal potential applications in the biofuel conversion industry.

Further understanding of the efficient wood-degrading/energy-conversion enzyme system of termites, especially the molecular mechanism of lignin/crystalline microfibril depolymerization, could lead to development of better enzyme cocktails with higher efficiency in bacterial or yeast expression systems for green energy production.

References

Archicentre (2003) Australian termites $780 million smorgasbord. *Archicentre News Release*, 18 January 2003, Sydney, Australia.

Béguin, P. and Aubert, J.-P. (1994) The biological degradation of cellulose. *FEMS Microbiology Reviews*, 13, 25–58.

Bulmer, M.S., Bachelet, I., Raman, R., Rosengaus, R.B. and Sasisekharan, R. (2009) Targeting an antimicrobial effector function in insect immunity as a pest control strategy. *Proceedings of the National Academy of Sciences of the United States of America*, 106, 12652–12657.

Davison, A. and Blaxter, M. (2005) Ancient origin of glycosyl hydrolase family 9 cellulase genes. *Molecular Biology and Evolution*, 22, 1273–1284.

Hongoh, Y., Sharma, V.K., Prakash, T., Noda, S., Toh, H., Taylor, T.D., Kudo, T., Sakaki, Y., Toyoda, A., Hattori, M. and Ohkuma, M. (2008) Genome of an endosymbiont coupling N2 fixation to cellulolysis within protist cells in termite gut. *Science*, 322, 1108–1109.

Itakura, S., Tanaka, H. and Enoki, A. (1997) Distribution of cellulases, glucose and related substances in the body of *Coptotermes formosanus*. *Material und Organismen*, 31, 17–29.

Korb, J., Weil, T., Hoffmann, K., Foster, K.R., Rehli, M. (2009) A gene necessary for reproductive suppression in termites, *Science*, 324, 758.

Lax, A.R. and Osbrink, W.L.A. (2003) United States Department of Agriculture – Agriculture Research Service research on targeted management of the Formosan subterranean termite *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae). *Pest Management Science*, 59, 788–800.

Marana, S.R., Jacobs-Lorena, M., Terra, W.R. and Ferreira, C. (2001) Amino acid residues involved in substrate binding and catalysis in an insect digestive β-glycosidase. *Biochimica et Biophysica Acta*, 1545, 41–52.

NaKashima, K., Watanabe, H., Saitoh, H., Tokuda, G. and Azuma, J.-I. (2002) Dual cellulose-digesting system of the wood-feeding termite, *Coptotermes formosanus* Shiraki. *Insect Biochemistry and Molecular Biology*, 32, 777–784.

Ni, J., Takehara, M., Miyazawa, M. and Watanabe, H. (2007a) Random exchanges of non-conserved amino acid residues among four parental termite cellulases by family shuffling improved thermostability. *Protein Engineering, Design and Selection*, 20, 535–542.

Ni, J., Takehara, M. and Watanabe, H. (2005) Heterologous overexpression of a mutant termite cellulase gene in *Escherichia*

coli by DNA shuffling of four orthologous parental cDNAs. *Bioscience, Biotechnology and Biochemistry*, 69, 1711–1720.

Ni, J., Tokuda, G., Takehara, M. and Watanabe, H. (2007b) Heterologous expression and enzymatic characterization of β-glucosidase from the drywood-eating termite, *Neotermes koshunensis*. *Applied Entomology and Zoology*, 42, 457–463.

Pauchet, Y., Freitak, D., Heidel-Fischer, H.M., Heckel, D.G. and Vogel, H. (2009) Immunity or digestion: Glucanase activity in a glucan-binding protein family from Lepidoptera. *Journal of Biological Chemistry*, 284, 2214–2224.

Rubin, E.M. (2008) Genomics of cellulosic biofuels. *Nature*, 454, 841–845.

Scharf, M.E. and Tartar, A. (2008) Review: Termite digestomes as sources for novel lignocellulases. *Biofuels, Bioproducts and Biorefining*, 2, 540–552.

Schulz, M.W., Slaytor, M., Hogan, M. and O'Brien, R.W. (1986) Components of cellulase from the higher termite *Nasutitermes walkeri*. *Insect Biochemistry*, 16, 929–932.

Scrivener, A.M. and Slaytor, M. (1994) Properties of endogenous cellulase from *Panesthia cribrata* and purification of major endo-β-1,4-glucanase components. *Insect Biochemistry and Molecular Biolology*, 24, 223–231.

Su, N. and Scheffrahn, R.H. (2000) Termites as pests of buildings. *Termites: Evolution*, *Sociality*, *Symbiosis*, *Ecology* (eds. T. Abe, D.E. Bignell & M. Higashi), pp. 437–454. Dordrecht, Kluwer Academic Publishers.

Sugimoto, A., Bignell, D.E. and MacDonald, J.A. (2000) Global impact of termites on the carbon cycle and atmospheric trace gases. *Termites: Evolution*, *Sociality*, *Symbiosis*, *Ecology* (eds. T. Abe, D.E. Bignell & M. Higashi), pp. 409–435. Kluwer Academic, Dordrecht.

Todaka, N., Moriya, S., Saita, K., Hondo, T., Kiuchi, I., Takasu, H., Ohkuma, M., Piero, C., Hayashizaki, Y. and Kudo, T. (2007) Environmental cDNA analysis of the genes involved in lignocellulose digestion in the symbiotic protist community of *Reticulitermes speratus*. *FEMS Microbiology Ecology*, 59, 592–599.

Todaka, N., Lopez, C.M., Inoue, T., Saita, K., Maruyama, J.-i., Arioka, M., Kitamoto, K., Kudo, T. and Moriya, S. (2009) Heterologous expression and characterization of an endoglucanase from a symbiotic protist of the lower termite, *Reticulitermes speratus*. *Applied Biochemistry and Biotechnology*. DOI 10.1007/s12010-009-8626-8 (in press).

Tsunoda, K. (2003) Economic importance of Formosan termite and control practices in Japan (Isoptera: Rhinotermitidae). *Sociobiology*, 41(1 A), pp. 27–36.

Warnecke, F., Luginbühl, P., Ivanova, N., Ghassemian, M., Richardson, T.H., Stege, J.T., Cayouette, M., McHardy, A.C., Djordjevic, G., Aboushadi, N., Sorek, R., Tringe, S.G., Podar, M., Martin, H.G., Kunin, V., Dalevi, D., Madejska, J., Kirton, E., Platt, D., Szeto, E., Salamov, A., Barry, K., Mikhailova, N., Kyrpides, N.C., Matson, E.G., Ottesen, E.A., Zhang, X., Hernández, M., Murillo, C., Acosta, L.G., Rigoutsos, I., Tamayo, G., Green, B.D., Chang, C., Rubin, E.M., Mathur, E.J., Robertson, D.E., Hugenholtz, P. and Leadbetter, J.R. (2007) Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. *Nature*, 450, 560–565.

Watanabe, H., Nakamura, M., Tokuda, G., Yamaoka, I., Scrivener, A.M. and Noda, H. (1997) Site of secretion and properties of endogenous endo-β-1,4-glucanase components from *Reticulitermes speratus* (Kolbe), a Japanese subterranean termite. *Insect Biochemistry and Molecular Biology*, 27, 305–313.

Yatsu, J. and Asano, T. (2009) Cuticle laccase of the silkworm, *Bombyx mori*: Purification, gene identification and presence of its inactive precursor in the cuticle. *Insect Biochemistry and Molecular Biology*, 39, 254–262.

Zhang, D., Lax, A.R., Raina, A.K. and Bland, J.M. (2009) Differential cellulolytic activity of native-form and C-terminal tagged-form cellulase derived from Coptotermes formosanus and expressed in *E. coli*. *Insect Biochemistry and Molecular Biology*, 39, 516–522.

Zhou, X., Wheeler, M.M., Oi, F.M. and Scharf, M.E. (2008) RNA interference in the termite *Reticulitermes flavipes* through ingestion of double-stranded RNA. *Insect Biochemistry and Molecular Biolology*, 38, 805–815.

Zhu, B.C.R., Henderson, G., Laine, R.A. (2005) Screening methods for inhibitors against Formosan subterranean termite β-glucosidase in vivo. *Journal of Economic Entomology*, 98, 41–46.

Accepted December 3, 2009

