Cellulase Activity in Higher and Lower Wood-Feeding Termites

by

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ABSTRACT

Wood-feeding termites have evolved efficient cellulose-decomposing systems. The cellulase activity and distribution of workers were studied in three wood-feeding termites from phylogenetically different lineages; the lower termites, *Crypototermes domesticus* (Haviland) (Isoptera: Kalotermitidae) and *Coptotermes formosanus* Skiraki (Rhinotermitidae), and the higher termite, *Ahmaditermes sichuanensis* Xia *et al*. (Termitidae). The results showed that the total cellulase activity of the higher termite *A. sichuanensis* was markedly higher than that of the lower termites. *Co. formosanus* had the highest activity of endo-β-1,4-glucanase and cellobiohydrolase. The activity of β-glucosidase was not significantly different among the three species. The proportion of cellobiohydrolase was higher in the higher termite. In terms of distributions of cellulolytic activity in the gut, the primary site of endo-β-1,4-glucanase activity was presented in the hindgut of both the lower termites but in the midgut of the higher termite, and the primary site of β-glucosidase activity was restricted to the midgut in the lower termites and the head/foregut in the higher termite. The functions of the gut segments were apparently differentiated between the lower and higher termites, with the role of the midgut becoming more important in the higher termite. For the endogenous cellulases, the main site of endo-β-1,4-glucanase activity was concentrated in the midgut in both the lower termite and the higher termite, but the main site of β-glucosidase activity was in the head/foregut in the higher termite. The results suggest that characters of cellulase activity could reflect the phylogeny of wood-feeding termites to a certain extent.

Key words: *Crypototermes domesticus*, *Coptotermes formosanus*, *Ahmaditermes sichuanensis*, endo-β-1,4-glucanase, exoglucanase, β-glucosidase, cellobiohydrolase

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INTRODUCTION

Cellulose is the most abundant, widespread and renewable biomass on the earth. Biotic fuels generated from cellulose could replace petroleum, but economic production of cellulosic biotic fuels is dependent on the growth of more efficient and cheaper cellulases that can be applied into both pretreatment and cellulolytic technologies (Mosier *et al*. 2005; Emsley 2008). In nature, xylophagous insects have evolved strategies to derive their energy needs from celluloses with cellulases, of which termites are the most efficient decomposers of celluloses (Sugimoto *et al*. 2000; Watanabe & Tokuda 2010). Termites are important decomposers in tropical ecosystems (Noble *et al*. 2009), and cellulase enzymes and cellulase genes in the digestive systems of termites may have potential for cellulosic ethanol production by biological processes (Ni 2007; Warnecke *et al*. 2007; Li *et al*. 2009).

Termites have specialized cellulose-digesting systems (Nakashima *et al*. 2002; Tokuda *et al*. 2007; Zhou *et al*. 2007). Various cellulases are involved in degradation of celluloses in termites and their symbionts. The related investigation of cellulase activity in termites is an important research field. The three main types of cellulases are endo-β-1,4-glucanases (EGs; EC 3.2.1.4), cellobiohydrolases (CBHs; EC 3.2.1.91), and β-glucosidases (BGs; EC 3.2.1.21), and cellulose degradation requires the synergistic action of the three types of glycoside hydrolases (Lo *et al*. 2011). Patterns and characters of cellulases in termites and their symbionts have been described extensively (Watanabe and Tokuda 2010; Willis *et al*. 2010), and cellulase activity and their distributions in the digestive systems were different among diverse termites. Recently, distributions of diverse cellulase activity in each gut segments of termites were mostly studied (Willis *et al*. 2010), and Tokuda *et al*. (2004, 2005) found that the expression of the endogenous cellulase genes has shifted from the salivary glands of lower termites to the midgut of higher termites. In addition, differences in total cellulase activity were compared among lower termites (Mo *et al*. 2004; Lu *et al*. 2010), and EG expression among different castes of *Hodotermopsis sjostedti* (family Termopsidae) and *Nasutitermes takasagoensis* (family Termitidae) have been reported (Fujita *et al*. 2008).

Recent research suggests that the distributions of cellulase activity in termites are related to their evolutional levels (Tokuda *et al*. 2004, 2005).

At present, there are approximately 2,600 described termite species around the world (Kambhampati & Eggleton 2000), and comprehensive studies of phylogenetic and taxonomic relationships among termite groups have been published (Donovan *et al*. 2000; Legendre *et al*. 2008). However, the cellulase activities of only around 16 termite species have been reported (Lu *et al*. 2010; Lo *et al*. 2011). Additional research can expand the knowledge of the cellulose-digesting mechanisms of termites, and provide a reference basis for the development of cellulosic ethanol (Rubin 2008) and environmentally-friendly biotermicides (Zhang *et al*. 2011).

Termites feed on dead plant material at all stages of decomposition. Besides wood, they feed on soil, leaf litter, fungi, grass, or lichen (Donovan *et al*. 2000). On the whole, cellulase activity levels in wood-feeding termites are far higher than those found in the fungus-growers and the soil-feeders (Tokuda *et al*. 2004; Lo *et al*. 2011). To provide further information about the relationship between cellulolytic activity and evolutionary status in wood-feeding termites from phylogenetically different lineages, the cellulase activity and distribution of workers were studied in three wood-feeding termites from phylogenetically different lineages; the lower termites, *Crypototermes domesticus* (Haviland) (Isoptera: Kalotermitidae) and *Coptotermes formosanus* Skiraki (Rhinotermitidae), and the higher termite, *Ahmaditermes sichuanensis* Xia *et al*. (Termitidae).

MATERIALS AND METHODS

Termites

Cr. domesticus were collected from two laboratory-maintained colonies. Three *Co. formosanus* colonies and two *A. sichuanensis* colonies were collected on Luofu mountain, Guangdong Province in China. The caste of termites used for experiments were healthy adult workers, but pseudoworkers for *Cr. domesticus*. The workers or pseudoworkers were directly put into liquid nitrogen before enzyme extraction.

Preparation of crude enzyme

To prepare enzyme extracts, the workers or pseudoworkers were washed with precooling 0.09% normal saline. Fifteen sets per termite colony of heads (including salivary glands) and whole guts were dissected from termites, and

each set was divided into head/foregut, midgut, and hindgut. The three sections and other five bodies of worker per colony were collected in tubes and homogenized in 500 μL of 0.1 M sodium acetate buffer (SAB) (pH 5.6) on ice. The tubes were centrifuged at 12,000 rpm for 15 min at 4 °C, and the supernatants were brought to volume of 500 μL by adding 0.1 M SAB and used as the enzyme extract. The same volume of 0.1 M SAB was used as the control.

Assay of cellulase activity

Filter paper degrading activity

Circular filter paper (3.5 mg per piece) after high temperature sterilization was put into the microtubes with 120μL SAB (pH 5.6), and the crude enzyme (12 μL) was incubated with the filter paper at 37 °C for 60 min. Based on dinitrosalicylic acid method (Eveleigh *et al.* 2009), 120 μL dinitrosalicylic acid solution was added and boiled for 5min. Then, the tubes were cooled to room temperature rapidly. The glucose production was detected colorimetrically with Victor 3 Multi-label Microplate Reader (Perkin Elmer, US) at 540nm, using glucose as a standard. The protein content of the sample was determined spectrophotomerically at 660nm according to the Coomassie Brilliant Blue G-250 method (Lott *et al*. 1983), using bovine serum as a standard. One unit (U) of enzyme activity was defined as the amount of enzyme capable of releasing one μmol reducing sugar per minute. Specific activity was expressed as units per mg protein. The enzyme assays were repeated three times.

Endo-β-1,4-glucanase and β-glucosidase activity

The activity of both EG and BG were determined using 120 μL of 1% sodium carboxymethylcellulose and 120 μL of 1% salicin as the substrates, respectively. Other steps and definitions were the same as the assay of filter paper degrading activity mentioned above.

Cellobiohydrolase activity

Cellobiohydrolase was assayed using *p*-nitrophenyl-β-D-cellobioside (pNPC) as the substrate. 120 μL of 0.1 mM pNPC was mixed with 12 μL crude enzyme at 37 °C for 60 min. The incubation was stopped by adding 120 μL of 1 M sodium carbonate, and diluted with SAB to 500 μL. The amount of *p*-nitrophenyl was measured at 405 nm.

Data analysis

The data were analyzed by one-way analysis of variance with least significant difference post-hoc tests (LSD) with SPSS 16.0 for Windows Software.

RESULTS

Differences in cellulase activity among termites

The data (Table 1) of cellulase activity of whole bodies of termite workers (or pseudoworkers) showed that the BG activities were not significantly different among the three termites, but activity of both EG and CBH was high in *Co. formosanus*. Regarding the proportion of different cellulose types within species, EG activity was the principal component in all three termites. However, CBH activity of *A. sichuanensis* was not significantly different from EG or BG.

Filter paper assay

Filter paper degradation is directly related to digestibility of naturally occurring cellulose. According to the data of filter paper degrading activity (Table 2), the most evolved species, *A. sichuanensis,* had the highest activity of filter paper degrading activity in the whole body of three wood-feeding

Table 1 Complete cellulase activity of whole bodies in the termite workers tested.

Species	Whole body activity		
	endo-β-1,4-glucanase	β-glucosidase	cellobiohydrolase
Crypototermes domesticus	0.347±0.028 bA	0.116±0.007 aB	0.016±0.000 bC
Coptotermes formosanus	0.780±0.047 aA	0.426±0.057 aB	0.243±0.079 aB
Ahmaditermes sichuanensis	0.525±0.122 bA	0.747±0.470 aA	0.015±0.000 bA

Mean±S.E. with different small letter means significant difference in the same column, while different capital letter means significant difference in the same line at the 0.05 probability level. The same as below.

Table 2 Filter paper degrading activity of different segments and whole body in the termite workers.

Species	Filter paper degrading activity			
	Whole Body	Head/Foregut	Midgut	Hindgut
Crypototermes domesticus	0.167±0.036 b	0.139±0.055aA	0.130±0.057aA	0.155±0.072aA
Coptotermes formosanus	0.241±0.031ab	0.131±0.027aB	0.107±0.017aB	0.229±0.022aA
Ahmaditermes sichuanensis	0.417±0.102a	0.213±0.087aC	0.326±0.157aA	0.288±0.051aB

Table 3 Endo-β-1,4-glucanase and β-glucosidase activity of different segments in the termite workers.

Cellulases	Speices	Cellulase acitivity in each gut segment		
		Head/Foregut	Midgut	Hindgut
endo-β-1,4-glucanase	*Crypototermes domesticus*	0.152±0.016bB	0.185±0.073aB	0.499±0.020bA
	Coptotermes formosanus	0.363±0.016aB	0.295±0.120aB	0.818±0.126aA
	Ahmaditermes sichuanensis	0.214±0.075bC	0.399±0.085aA	0.289±0.079bB
β-glucosidase	*Crypototermes domesticus*	0.080±0.005bC	0.160±0.006aA	0.116±0.007aB
	Coptotermes formosanus	0.153±0.068bA	0.314±0.107aA	0.200±0.033aA
	Ahmaditermes sichuanensis	0.363±0.033aA	0.305±0.162aB	0.289±0.103aC

termites, but there were no significant differences among filter paper degrading activity of the three termites in each gut segment. Regarding distributions of filter paper degrading activity in guts of the termites, *A. sichuanensis* showed significantly different filter paper degrading activity among three gut segments, and the midgut was the main segment of filter paper degrading activity in *A. sichuanensis*.

Distribution of EG and BG activities in the gut

According to the data from the hindgut (Table 3), it is suggested that flagellate-harbouring termites possess a higher percentage of intestinal microbial EG and BG than *A. sichuanensis*. In addition, activities of both EG and BG were not significantly different in the midgut among the three wood-feeding termites, and neither was BG activity in the hindgut. *Co. formosanus* had the highest activity of EG in head/foregut and hindgut, and *A. sichuanensis* had the highest activity of BG in head/foregut.

For endogenous endoglucanase and β-glucosidase, EG activities were not significantly different between the head/foregut and midgut of *Cr. domesticus*, or *Co. formosanus*. However, the higher termite *A. sichuanensis* had higher EG activity in the midgut. In contrast, the higher BG activity in *A. sichuanensis* was in the head/foregut.

DISCUSSION

Termites play an important role in degradation of cellulosic materials in nature, and special attention is paid to the activity and expression of termite cellulases (Watanabe & Tokuda 2010). Efficient cellulose digestion in termites requires both endogenous and intestinal microbial cellulases (Tokuda 2007;

zhou 2007). The present study showed that the hindgut was the primary site of cellulose digestion in lower termites, which was consistent with the report of Tokuda *et al*. (2005). As for endogenous termite cellulases, Tokuda *et al*. (2004) proposed that the expression of the endogenous cellulase genes has shifted from the salivary glands of lower termites to the midgut of higher termites.

In terms of the dynamic shift in termites, our results showed that activity of EG and filter paper degrading activity were most highly concentrated on the midgut of the higher termite studied than that of the lower termites, which supported the previous studies on EG (Mo *et al*. 2004; Tokuda *et al*. 2004, 2005; Fujita *et al*. 2008; Tokuda *et al*. 2009; Lo *et al*. 2011), but the dynamic change of BG was contrary to the related report of Fujita *et al*. (2008) and Tokuda *et al*. (2009). They suggested that the main position of BG activity tended to the head/foregut with evolution of wood-feeding termites, and the evolved wood-feeding termites such as the termites of the Rhinotermitidae and Termitidae may have higher BG activity in the head/foregut.

As for the comparison of cellulase activities in whole bodies of termites, it was suggested that the degrading activity of filter paper and the percentage value of CBH in complete cellulases were increased with a rise in evolutionary status. The higher termite *A. sichuanensis* had the highest degrading activity of filter paper and the highest percentage value in complete cellulases. However, a previous study showed that activity in the higher termite *Nasutitermes takasagoensis* was markedly lower using microcrystalline cellulose as substrate than that of flagellate-harbouring termites (Tokuda *et al*. 2005).Because of this, Tokuda *et al*. (2005) considered the cellulase activity of *N. takasagoensis* was likely to be similar to its requirement for energy metabolism. In addition, filter paper, microcrystalline cellulose and cotton have been used as cellulase substrates to determine the existence of complete cellulases (Willis *et al*. 2010), but there is evidence for the degradation of microcrystalline cellulose in insects lacking CBH activity (Scrivener and Slaytor 1994).

Co. formosanus is one of the most destructive and invasive pests around the world, and its cellulase activity has been intensively studied on different substrates (Willis *et al*. 2010). *Co. formosanus* had the highest activity of EG and BG among termites (Tokuda *et al*. 2005; Lu *et al*. 2010). The present study showed that *Co. formosanus* had the highest degrading activity of not

only EG but also CBH in the three termites studied, but its BG activity was not significantly different from species of the family Kalotermitidae which was consistent with the report of Mo *et al*. (2004). In addition, In terms of the synthetic cellulase activity in wood-feeding termites, *A. sichuanensis* and *Co. formosanus* showed higher activities of EG and CBH. Use of diverse cellulase substrates can help differentiate the specific contribution of specific insect gut areas to the digestion of plant material (Tokuda *et al*. 2005).

For cellulase activity assays, the data are often difficult to compare among studies. Multinomial factors can influence the cellulase activity, such as temperature, substrate, detection method and so on (Tokuda *et al*. 2005; Willis *et al*. 2010). Furthermore, phylogenetic analysis of cellulase genes from the termite lineages has received special attention (Todaka *et al*. 2010). The relative expression levels of cellulase genes do not correspond with their activity, which could be affected by different regulators (Fujita *et al*. 2008). In addition, for the workers of higher termites, the cellulose-digesting division of labor might indirectly influence the determination results (Fujita *et al*. 2008).

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REFERENCES

Donovan S., D. Jones, W. Sands, P. Eggleton 2000. Morphological phylogenetics of termites (Isoptera). Biological Journal of the Linnean Society 70(3): 467-513.

Emsley A.M. 2008. Cellulosic ethanol reignites the fire of cellulose degradation. Cellulose 15(2): 187-192.

Eveleigh D.E., M. Mandels, R. Andreotti, C. Roche 2009. Measurement of saccharifying cellulase. Biotechnology for Biofuels 2(1): 1-8.

Fujita A., T. Miura, T. Matsumoto 2008. Differences in cellulose digestive systems among castes in two termite lineages. Physiological Entomology 33(1): 73-82.

Gusakov A.V., T.N. Salanovich, A.I. Antonov, B.B. Ustinov, O.N. Okunev, R. Burlingame, M. Emalfarb, M. Baez, A.P. Sinitsyn 2007. Design of highly efficient cellulase mixtures for enzymatic hydrolysis of cellulose. Biotechnology and bioengineering 97(5): 1028-1038.

Kambharnpati S. & P. Eggleton 2000. Taxonomy and phylogeny of termites. In: Abe T, Bignell DE, Higashi M (eds) Termites: evolution, sociality, symbioses, ecology, pp.1-23. Kluwer academic publishers, Dordrecht.

Legendre F., M.F. Whiting, C. Bordereau, E.M. Cancello, T.A. Evans, P. Grandcolas 2008. The phylogeny of termites (Dictyoptera: Isoptera) based on mitochondrial and nuclear markers: implications for the evolution of the worker and pseudergate castes, and foraging behaviors. Molecular phylogenetics and evolution 48(2): 615-627.

Li X., H. Yang, B. Roy, D. Wang, W. Yue, L. Jiang, E.Y. Park, Y. Miao 2009. The most stirring technology in future: Cellulase enzyme and biomass utilization. African Journal of Biotechnology 8(11): 2418-2422.

Lo N., G. Tokuda, H. Watanabe 2011. Evolution and function of endogenous termite cellulases. In: D.E. Bignell et al. (eds.), Biology of Termites: a modern synthesis, pp.51-67. Springer Science+Business Media, London New York.

Lott J., V.A. Stephan, Jr K. Pritchard 1983. Evaluation of the Coomassie Brilliant Blue G-250 method for urinary protein. Clinical chemistry, 29(11): 1946-1950.

Lu J., T. Deng, J. Li, J. Mo 2010. Activities of some lignocelluloses-degrading enzymes in workers of five common termites (Isoptera). Sociobiology 55(3): 749-762.

Mo J., T. Yang, X. Song, J. Cheng 2004. Cellulase activity in five species of important termites in China. Applied Entomology and Zoology, 39, 635–641.

Mosier N., C. Wyman, B. Dale, R. Elander, Y.Y. Lee, M. Holtzapple, M. Ladisch 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresource technology 96(6): 673-686.

Nakashima K., H. Watanabe, H. Saitoh, G. Tokuda, J.I. Azuma 2002. Dual cellulose-digesting system of the wood-feeding termite, Coptotermes formosanus Shiraki. Insect biochemistry and molecular biology 32(7): 777-784.

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

Noble J.C., W.J. Miller, W.G. Whitford, G.H. Pfitzner 2009. The significance of termites as decomposers in contrasting grassland communities of semi-arid eastern Australia. Journal of arid environments 73(1): 113-119.

Rubin E.M. 2008. Genomics of cellulosic biofuels. Nature 454(7206): 841-845.

Scrivener A. & M. Slaytor 1994. Properties of the endogenous cellulase from Panesthia cribrata saussure and purification of major endo-β-1, 4-glucanase components. Insect biochemistry and molecular biology 24(3): 223-231.

Sugimoto A., D.E. Bignell, J.A. MacDonald 2000. Global impact of termites on the carbon cycle and atmospheric trace gases. In: Abe T, Bignell DE, Higashi M. (eds) Termites: evolution, sociality, symbioses, ecology, pp.409–435. Kluwer Academic Publishers, Dordrecht.

Todaka N., T. Inoue, K. Saita, M. Ohkuma, C.A.Nalepa, M. Lenz, T. Kudo, S. Moriya 2010. Phylogenetic analysis of cellulolytic enzyme genes from representative lineages of termites and a related cockroach. PLoS One 5(1): e8636.

Tokuda G., N. Lo, H. Watanabe 2005. Marked variations in patterns of cellulase activity against crystalline- vs. carboxymethyl-cellulose in the digestive systems of diverse, wood-feeding termites. Physiological Entomology 30(4): 372-380.

Tokuda G., Lo N., Watanabe H., Arakawa G., Matsumoto T., Noda H. 2004. Major alteration of the expression site of endogenous cellulases in members of an apical termite lineage. Molecular Ecology 13(10): 3219-3228.

Warnecke F., P. Luginbühl, N. Ivanova, M. Ghassemian, T.H. Richardson, J.T. Stege, M. Cayouette, A.C. McHardy, G. Djordjevic, N. Aboushadi 2007. Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. Nature 450(7169): 560-565.

Watanabe H. & G. Tokuda 2001. Animal cellulases. Cell. Mol. Life Sci. 58(9): 1167-1178.

Watanabe H. & G. Tokuda 2010. Cellulolytic systems in insects. Annual review of entomology 55: 609-632.

Willis J.D., C. Oppert, J.L. Jurat-Fuentes 2010. Methods for discovery and characterization of cellulolytic enzymes from insects. Insect Science 17(3): 184-198.

Zhang D., A.R Lax., J.M. Bland, A.B. Allen 2011. Characterization of a new endogenous endo-β-1,4-glucanase of Formosan subterranean termite (Coptotermes formosanus). Insect biochemistry and molecular biology 41: 211-218.