

Comparative Lignocellulase Activity and Distribution Among Selected Termite (Isoptera) Genera¹

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Abstract Lignocellulase activity and distribution within the alimentary canal were compared among 4 termite (Isoptera) genera representing 2 taxonomic families: *Coptotermes* and *Reticulitermes* in Rhinotermitidae; *Macrotermes* and *Odontotermes* in Termitidae. Activity of β -glucosidase and xylanase was higher in *Macrotermes barneyi* Light than observed in 5 other termite species examined; thus, indicating that *M. barneyi* might be a prospective organism for discovery of novel lignocellulases. Xylanase activity was primarily restricted to the hindgut of *Coptotermes formosanus* Shiraki, *Reticulitermes guangzhouensis* Ping, and *R. dichrous* Ping (Family Rhinotermitidae); whereas, xylanase activity occurred in both the midgut and hindgut of *M. barneyi*, *Odontotermes formosanus* (Shiraki), and *O. hainanensis* (Light) (Family Termitidae). β -glucosidase activity was primarily detected in the salivary gland and foregut of *O. formosanus* and *O. hainensis*, but β -glucosidase activity was equally distributed in salivary glands, foregut, and midgut of *M. barneyi*.

Key Words enzymatic activity, β -glucosidase, lignocellulase, xylanase, Rhinotermitidae, Termitidae, *Macrotermes barneyi*, *Coptotermes formosanus*, *Reticulitermes guangzhouensis*, *Reticulitermes dichrous*, *Odontotermes formosanus*, *Odontotermes hainanensis*

Lignocelluloses are the most abundant and renewable biomasses on earth and are mainly composed of celluloses and hemicelluloses (xylan being the dominant component) (Tokuda et al. 2009, Ohkuma 2003). Termites (Isoptera) digest an estimated 74 - 99% of the cellulose in the world and are considered to be the most important lignocellulose-decomposing animal (Arakawa et al. 2009, Tokuda and Watanabe 2007, Tayasu et al. 2000).

The lignocellulases of termites are comprised of the following enzymes: endo- β -1, 4-glucanase (EC; EG. 3.2.1.4), exo- β -1, 4-cellobiohydrolase (CBH; EC. 3.2.1.91) and β -glucosidase (BG; EC. 3.2.1.21) for hydrolysis of cellulose, and; endo- β -D-xylanase or xylanase (EC. 3.2.1.8) for hydrolysis of hemicellulose (Willis et al. 2010, Arakawa et al. 2009, Clarke et al. 1997). Endogenous cellulases are secreted by the salivary glands (Ohkuma 2008), and cellulases and hemicellulases are produced by endo-symbiotic flagellates in the hindgut (Tokuda et al. 2009, Warnecke et al. 2007, Tokuda et al. 1997) of phylogenetically lower termite groups (Matsui et al. 2009). Phylogenetically

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higher groups generally lack symbiotic protozoa in the hindgut, except for those belonging to the subfamily Marotermitinae. These higher groups of termites reportedly have endosymbiotic cellulases produced by bacteria in the hindgut and also secrete endogenous cellulase in the midgut rather than from the salivary gland (Matsui et al. 2009, Arakawa et al. 2009, Tokuda et al. 2005, Tokuda et al. 2004).

Gene expression and enzyme distribution of the lignocellulases have been studied in the phylogenetically-lower termites, including *Coptotermes* spp. and *Reticulitermes* spp. (Zhang et al. 2010, Cho et al. 2010, Arakawa et al. 2009, Tokuda et al. 2009, Ohkuma 2008, Brune and Stingl 2006). However, studies of the lignocellulolytic systems in higher termite groups are limited and primarily focused on the *Nasutitermes* spp. (Todaka et al. 2010a, Fujita et al. 2008, Tokuda et al. 2007, Tokuda and Watanabe 2007, Tokuda et al. 2005). Our objectives in the study reported herein were to (1) identify lignocellulolytic enzyme activity in 3 major regions of the alimentary canal of 6 species of termites – *Coptotermes formosanus* Shiraki (Rhinotermitidae), *Reticulitermes guangzhouensis* Ping (Rhinotermitidae), *Reticulitermes dichrous* Ping (Rhinotermitidae), *Odontotermes formosanus* (Shiraki) (Termitidae), *Odontotermes hainanensis* (Light) (Termitidae), *Macrotermes barneyi* Light (Termitidae); and (2) compare the source and distribution of enzymatic activity between the phylogenetically-lower Rhinotermitidae and the higher Termitidae.

Materials and Methods

Worker termites were collected from field colonies of the 6 species of termites from the Lianhua Mountain area in Guangzhou, the Maofeng Mountain area in Guangzhou, and the Luofu Mountain in Huizhou, all in the subtropical region of China. Collected termites were immediately placed in liquid nitrogen, returned to the laboratory, and stored at -80°C until used in the study. Classification of each termite species is indicated in Table 1. Individuals were taxonomically identification to species using Maiti (2006) and Huang et al. (2000).

The enzymes targeted for activity and distribution within the termite guts were the composite cellulases (abbreviated FPase because of the use of filter paper as the substrate for the activity test), xylanase, and β -glucosidase. Fifteen termites for each species were individually dissected to remove and isolate the salivary glands/foreguts, midguts, and hindguts. Individual sections were placed in separate microtubes, homogenized with ice-cold 0.1 M sodium acetate buffer (SAB, pH 5.6), and centrifuged at 12,000 g for 15 min at 4°C. The supernatants were used in the assays for FPase, endo- β -D-xylanase and β -glucosidase activity. One unit (U) of enzyme activity was defined as the amount of enzyme capable of releasing 1 μ mol reducing sugar per min under the defined reaction conditions. Specific activity was expressed as units per mg of protein (U/mg protein). Each assay for each species and gut section was performed in triplicate.

Using bovine serum as a standard, the protein content of each sample was determined spectrophotometrically at 660 nm using the Coomassie Brilliant Blue G-250 method (Fujita et al. 2008, Lott et al. 1983). FPase, xylanase and β -glucosidase activity were assayed by measuring the release of deducing sugars from filter paper, xylan and salicin, for the respective enzymes, using the dinitrosalicylic acid (DNS) method (Eveleigh et al. 2009, Cai et al. 2008, Lott et al. 1983). Glucose and xylose production was detected colorimetrically at 540 nm (Miller 1959) using glucose and xylose as separate standards.

Table 1. Mean (\pm SD) Units/mg protein of enzymatic activity of FPase, xylanase and β -glucosidase in termite workers of *C. formosanus*, *R. guangzhouensis*, *R. dichrous*, *M. barneyi*, *O. formosanus*, and *O. hainanensis* collected from different locales in China.

Species	Family	Locale**	Filter paper activity	Xylanase activity	β -glucosidase activity
<i>C. formosanus</i>	Rhinotermitidae	LH	0.257 \pm 0.011	7.037 \pm 0.067	0.416 \pm 0.027
<i>C. formosanus</i>	Rhinotermitidae	MF	0.116 \pm 0.002	6.731 \pm 0.081	0.303 \pm 0.010
<i>C. formosanus</i>	Rhinotermitidae	LF	0.303 \pm 0.055	6.508 \pm 0.044	0.538 \pm 0.089
<i>R. guangzhouensis</i>	Rhinotermitidae	LH	0.124 \pm 0.016	10.812 \pm 0.070	0.234 \pm 0.005
<i>R. guangzhouensis</i>	Rhinotermitidae	MF	0.141 \pm 0.033	16.126 \pm 0.037	0.110 \pm 0.000
<i>R. dichrous</i>	Rhinotermitidae	LF	0.195 \pm 0.009	14.89 \pm 0.033	0.124 \pm 0.002
<i>M. barney</i>	Termitidae	LH	0.126 \pm 0.015	20.185 \pm 0.050	2.644 \pm 0.198
<i>M. barney</i>	Termitidae	MF	0.231 \pm 0.018	19.064 \pm 0.018	5.111 \pm 0.030
<i>M. barney</i>	Termitidae	LF	0.179 \pm 0.109	17.397 \pm 0.027	2.937 \pm 0.085
<i>O. formosanus</i>	Termitidae	LH	0.125 \pm 0.009	0.785 \pm 0.030	2.103 \pm 0.029
<i>O. formosanus</i>	Termitidae	MF	0.078 \pm 0.007	0.787 \pm 0.013	2.026 \pm 0.088
<i>O. hainanensis</i>	Termitidae	LF	0.163 \pm 0.009	0.415 \pm 0.027	3.099 \pm 0.085

* Units = amount of enzyme capable of releasing 1 μ mol of reducing sugar/min under defined reaction conditions.

** Collection locales: LH, Lianhua Mountain area; MF, Maofeng Mountain area; LF, Loufu Mountain area.

Results

Total enzymatic activity of FPase, endo- β -D-xylanase, and β -glucosidase when combined for all regions of the alimentary canal for each of the 6 termite species are shown in Table 1. Cellulase (FPase) activity was relatively similar across all species and collection sites, ranging from a mean (\pm SD) of 0.078 ± 0.007 - 0.257 ± 0.011 U/mg protein. Xylanase activity varied among the species studied with *M. barneyi* demonstrating the highest activity (17 - 20 U/mg protein) and *O. formosanus* demonstrating the lowest activity (0.415 - 0.787 U/mg protein) of the 6 species examined. β -glucosidase activity was 5X to 10X lower in the rhinotermitid species when compared with the activity observed in the termitid species examined in this study.

Some interesting trends were observed in the distribution of the activity of these enzymes through the alimentary canals of the termite species (Fig. 1). First, the relative proportions of cellulase (FPase) activity within each of the 3 regions of the alimentary canal remained relatively constant across all species with 27% of the total activity observed in the salivary gland/foregut, 35% in the midgut, and 38% in hindgut (Fig. 1-A). The obvious exception was for the *C. formosanus* specimens collected from the Lianhua Mountain area in which 19% of the total activity was observed in the salivary gland/foregut, 55% in the midgut, and 26% in the hindgut. Second, xylanase activity predominantly occurred in the hindguts of *R. guangzhouensis* and *R. dichrous* (> 90% of the total xylanase activity observed) (Fig. 1-B). Similar trends were observed for *C. formosanus*, with the exception being the specimens collected from the Maofeng Mountain area (13% of total activity observed in the salivary gland/foregut, 50% in the midgut, 37% in the hindgut). Xylanase activity, in general, was equally distributed between the midgut (44% of total activity) and hindgut regions (42% of total activity) of the termitid species with *Odontotermes* spp. having comparatively low levels of xylanase activity overall. Third, β -glucosidase activity was higher in the termitid species than in the rhinotermitid species (Fig. 1-C). The salivary gland/foregut region yielded 89 - 90% of the total β -glucosidase activity *Odontotermes* spp. examined, whereas the proportion of total activity observed in *M. barneyi* were approximately equal in the salivary gland/foregut (34 - 41%) and in the midgut (36 - 45%).

Discussion

Previous studies report that *C. formosanus* digests cellulose with cellulase activity sourced from salivary glands in the foregut region and from symbiotic flagellates in the hindgut regions (Arakawa et al. 2009, Nakashima et al. 2002a). Our results with FPase activity demonstrated that, in addition to the cellulase activity in the salivary gland/foregut and in the hindgut, that cellulose is also digested in the midgut of *C. formosanus* and the other 5 species of termites examined herein.

Tokuda et al. (2004) postulated that cellulase activity was relatively low in the phylogenetically-higher termites than in lower termites. However, our data show that xylanase and β -glucosidase activity was higher in *M. barneyi* than in the phylogenetically-lower rhinotermitids. Furthermore, a novel polysaccharidase was recently purified from the major soldier of *M. subhyalinus* (Rambur) and was identified as an endo- β -xylanase with EG activities (Blei et al. 2010). Thus, *Macrotermes* spp. appear to be excellent prospects for discovery of novel lignocellulases.

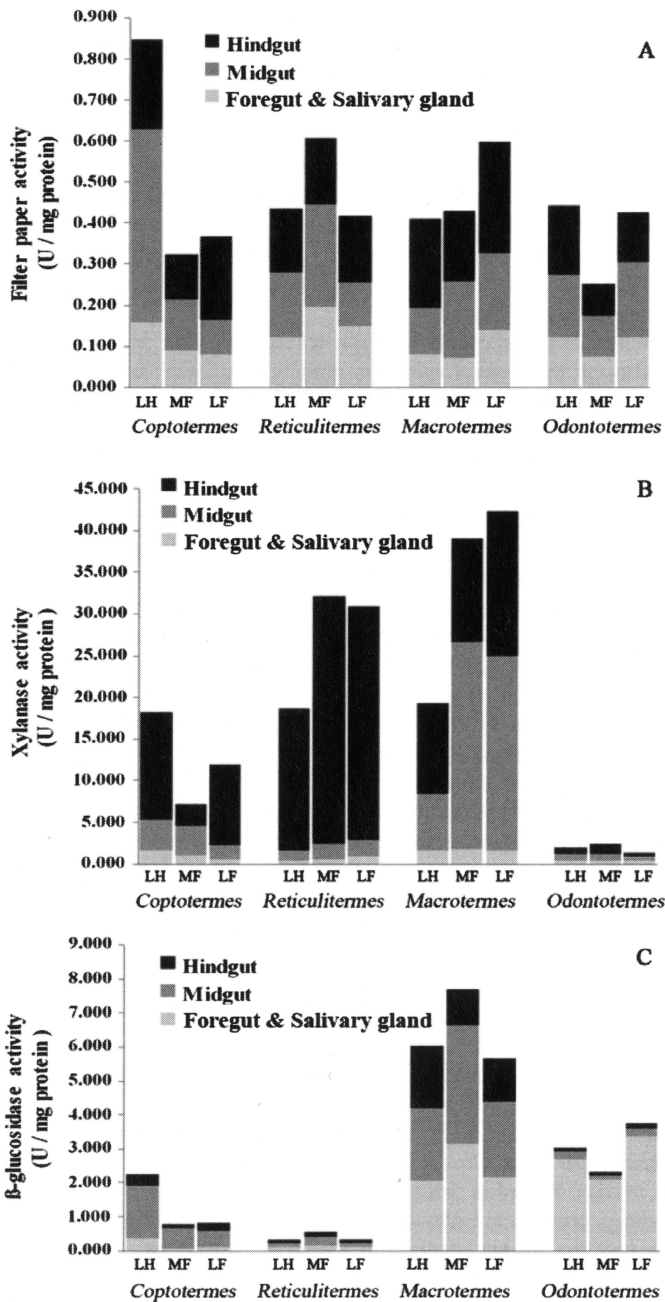


Fig. 1. Distribution of PFase (A), xylanase (B) and β -glucosidase (C) activity within the alimentary canal of worker termites of *Coptotermes*, *Reticulitermes*, *Macrotermes*, and *Odontotermes* collected from different locales in China.

Noticeable differences occurred in the activities of xylanase and β -glucosidase between *M. barneyi* and the *Odontotermes* spp. whereas their FPase activity was almost the same. It is generally believed that the FPase activity assay is the best measure of the true activities of EG and CBH in an organism, both of which are considered to work synergistically to degrade the substrate (Willis et al. 2010, Eveleigh et al. 2009, Tokuda et al. 2005). In our results, FPase activities among different termites genus were similar; namely, termites with higher β -glucosidase and xylanase activities did not always possess correspondingly higher filter paper digestion abilities. Thus, it is possible that xylanase belonging to the hemicellulase system would not directly affect the cellulose digestion system and that β -glucosidase is not the "short plank" in termite cellulose digestion.

Lower termites are commonly thought to digest xylan in hindgut; however, we found that, although the overall xylanase activity was lower in *C. formosanus* than in either *R. guangzhouensis* or *R. dichrous*, xylanase activity was higher in midgut than in the hindgut of *C. formosanus*. We postulate that these results might be due to the release of xylanase in the midgut of *C. formosanus* when the enzyme is in short supply in the hindgut.

Hydrolysis of lignocelluloses by members of the subfamily Macrotermitinae is, to a great extent, due to endosymbiosis and exosymbiosis with fungi (Binate et al. 2008). For example, the wood-feeding higher termite *Nasutitermes* produces β -glucosidase and endo- β -1, 4-glucanase primarily in the midgut (Tokuda et al. 2009, Slaytor 2000), whereas metagenomic studies have identified various bacteria genes in hindgut relating to cellulose and xylan hydrolysis in that region (Burnum et al. 2011, Cantarel et al. 2009, Tokuda et al. 2009, Warnecke et al. 2007). We did not observe a similar trend with *M. barneyi* in that we detected high xylanase activity in midgut and the hindgut, and xylanase genes expressed in the termite midgut have not yet been reported. Therefore, whether xylanase activity exhibited in the midgut of *M. barneyi* is endogenous remains uncertain. Furthermore, β -glucosidase and xylanase activity in the termitids examined herein demonstrated diverse distributions and activity levels in the alimentary canal.

Perhaps diet might explain some observed discrepancies. Other studies have reported significant differences in the gut distribution of cellulase activity among different fungus-growing termites (Burnum et al. 2011, Tokuda and Watanabe 2007, Tokuda et al. 2005). In our study, the fungus-growing higher termite *O. formosanus* showed only a trace of crystalline cellulose hydrolysis throughout the gut. Yet, the midgut and hindgut of wood-feeding higher termite *N. takasagonensis* (Shiraki) primarily contributed in crystalline cellulose degradation (Todaka et al. 2010a). Furthermore, earthworms, grasshoppers and beetles reportedly have the capacity to produce endogenous cellulases in their foreguts and midguts, and the levels of cellulase found in grasshopper were similar to those in termites and beetles (Cho et al. 2010, Todaka et al. 2010b, Wei et al. 2006).

These findings lead us to believe that there is a link between lignocellulose digestion systems and the insect phylogeny. Recently, molecular investigations with the cellulases have provided insight into the phylogeny of termites. Multiple gene sequences indicate that termites evolved from wood-feeding cockroaches, and that chromosomal rearrangement events may have occurred in the course of evolution from wood roaches to termites (Todaka et al. 2010a). The endogenous cellulases GHF5 (glycosyl hydrolase families) and GHF45 have been discovered in various animals, including beetles, molluscs and nematodes. The GHF45 of termites has been

cloned from the hindgut protists (Ni et al. 2010, Nakashima et al. 2002b); the GHF5, shared by the symbiotic system of many lower termites and the wood-feeding cockroach *Cryptocercus punctulatus* Scudder was transferred from bacteria to the protistan symbiotic system (Todaka et al. 2010a). Accordingly, gene interactions may happen between fungus-growing higher termites and fungi as well as diverse organisms in various evolutionary contexts.

Overall, our results suggest that lignocellulase activity and distribution in single groups of termites might have broader application to different species. More specifically, these results can be applied to future studies of termite phylogeny and for discovery of a broad range of novel biocatalysts for lignocellulose biomass conversion.

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