Symbiotic Fungus and Enzymatic Digestion in the Gut of the Termite, *Macrotermes barneyi* (Light) (Isoptera: Termitidae)[1]

M. W. J. Crosland, L. K. Chan and J. A. Buswell

Department of Biology, The Chinese University of Hong Kong
Shatin, N.T., Hong Kong

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ABSTRACT Gut polysaccharidase activity was examined in the fungus-growing termite, *Macrotermes barneyi* (Light). Rates of enzymatic digestion of 7 polysaccharides and 5 synthetic heterosides were compared among major and minor worker midguts and hindguts and mycotêtes of the *Termitomyces* fungus. Major workers showed substantially more hindgut polysaccharidase activity than minor workers, though for both worker types midgut activity was substantially higher than in the hindgut. For 10 out of the 12 enzymes tested (including cellulases), activities were significantly higher in the midguts of major workers than in the fungus mycotêtes. Acquired polysaccharidases from the mycotêtes have been suggested to play a role in digestion in the termite midgut in some other *Macrotermes* species. However, in *M. barneyi*, our results indicate that for 11 of the 12 enzymes we tested acquired fungal polysaccharidases are probably of relatively little importance.

KEY WORDS Insecta, Isoptera, termite, *Macrotermes, Termitomyces,* cellulose digestion

Fungus-growing termites (sub-family Macrotermitinae) live in a remarkable and complex symbiotic relationship with fungi of the genus *Termitomyces*. These termites cultivate large fungal gardens of virtually pure cultures of *Termitomyces*, which they construct on collected plant material (Wood and Thomas 1989). Termite workers then eat the fungus comb, which includes *Termitomyces* mycelium and mycotetes (round asexual structures).

The role of the fungus garden remains incompletely understood (Wood and Thomas 1989). Enzymes from *Termitomyces* mycotêtes are ingested by termite workers, and some authors claim these provide essential "missing enzymes" that are active in the termite gut and essential for the "completion of cellulose digestion" in termites (Martin and Martin 1978, Martin 1987, 1991, 1992). However, this view has become controversial (Slaytor 1992, Bignell et al. 1994), and we have now investigated the potential role of enzymes acquired from *Termitomyces* in a broader context. Following the approach of Rouland et al. (1991), we have examined the digestion of not only cellulose but also other plant polysaccharides expected to comprise part of the Macrotermitinae diet. Moreover, whereas previous studies have focused on African species, in this study, we have examined an Asian termite species, *Macrotermes barneyi* (Light).

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Materials and Methods

Samples of major and minor termite workers and fungus comb were collected during summer 1993 from the campus of The Chinese University of Hong Kong. Samples were taken from within a mature (alate-producing) nest of *M. barneyi* containing *T. microcarpus* (Heim). Within 5 hours of collection, mycotêtes were removed and termite mid- and hindguts were dissected. Both gut tissue and contents were dissected and assayed together (following Martin and Martin 1978, though we also found more activity in the lumen than in the tissue). Individual samples each consisted of at least 30 midguts or hindguts from each worker type and 20 mycotêtes. Samples were initially frozen in 8 to 10 ml of liquid nitrogen, vigorously ground for 20 s using a mortar and pestle, and mixed with 3 ml of 50 mM potassium phosphate buffer, pH 6.2. Homogenates were centrifuged for 5 to 10 mins at 16,000 × g and the supernatants retained and assayed for enzyme activities. Amylase, cellulase (exo- and endoglucanase), xylanase, pectinase, laminarinase, and pullulanase were determined by measuring the amount of reducing sugar released from starch, avicel, carboxymethylcellulose, oat-spelt xylan, arabinogalactan, laminarin, and pullulan, respectively using the Somogyi-Nelson procedure (Somogyi 1952). Reaction mixtures containing 0.8 ml of 1% w/v substrate solution, 1.7 ml 50 mM phosphate buffer, pH 6.2, and 0.5 ml sample were incubated at 50°C for 30 min. α-Glucosidase, β-glucosidase, β-galactosidase, β-xylosidase, and chitinase were determined by release of p-nitrophenol from the corresponding p-nitrophenol derivatives (Cai et al. 1994). Values were corrected for the presence of end-product in both test material and substrates. This was done by including controls containing buffer and either enzyme extract of test material or substrate alone. Protein was determined by the Lowry method, following Veivers et al. (1991).

One unit (U) of enzyme activity is defined as the amount of enzyme required to produce 1 μ-mole of end-product under the assay conditions. Each measurement was replicated 3 times, each time using workers and fungus freshly collected on a different day. The activity of each enzyme was separately compared among the 5 sources (mycotêtes, midguts of majors, midguts of minors, hindguts of majors and hindguts of minors), using a 1-way ANOVA followed by the Tukey test (Zar 1984).

Results

Enzymatic activity was clearly shown in the midguts of major workers on all polysaccharides and synthetic heterosides tested (Table 1). The same was found for minor workers except for amylase and pectinase activity (the only two cases not significantly different [$P > 0.05$, t-test] to controls [=zero]). Especially high levels of laminarinase, xylanase, and β-glucosidase activity occurred in midguts of both worker types.

For all enzymes statistically compared (Table 1), midgut activities were significantly higher than hindgut activities in major workers. Minor workers showed the same trend with midgut activities either not different from (4 out of the 10 cases statistically tested) or significantly higher than hindgut activities (6 out of 10 cases).

Table 1. Polysaccharidase activities in major and minor workers of *Macrotermes barneyi* and fungal mycotêtes.

	Amylase	Laminarinase	Pectinase	Pullulanase	Endoglucanase	Exoglucanase
Mycotêtes	**0.48** ± 0.03 (1) a	**1.75** ± 0.05 (1) NT§	**0.31** ± 0.01 (1) a	**0.49** ± 0.01 (1) a	**1.44** ± 0.03 (1) a	**0.12** ± 0.01 (1) a
	* ** † ‡					
Midgut (Major)	**0.26** ± 0.06 (0.5) b	**10.15** ± 0.49 (5.8) a	**0.36** ± 0.06 (1.2) a	**0.73** ± 0.03 (1.5) b	**2.71** ± 0.06 (1.9) b	**0.72** ± 0.08 (6.0) b
Midgut (Minor)	**0.03** ± 0.03 (0.06) cd	**11.88** ± 0.30 (6.8) b	**0.02** ± 0.02 (0.06) b	**0.60** ± 0.02 (1.2) b	**2.87** ± 0.03 (2.0) b	**0.23** ± 0.02 (1.9) a
Hindgut (Major)	**0.05** ± 0.01 d	**1.46** ± 0.15 c	**0.20** ± 0.14 NT £	**0.29** ± 0.04 c	**0.71** ± 0.13 NT	**0.08** ± 0.04 a
Hindgut (Minor)	**0.00** ± 0.00 c	**0.11** ± 0.09 c	**0.04** ± 0.03 b	**0.12** ± 0.08 NT	**0.45** ± 0.22 NT	**0.07** ± 0.07 a

	Xylanase	β-galactosidase	Chitinase	β-glucosidase	α-glucosidase	β-xylosidase
Mycotêtes	**1.57** ± 0.12 (1) a	**0.53** ± 0.02 (1) a	**0.12** ± 0.01 (1) a	**2.90** ± 0.39 (1) NT §	**0.07** ± 0.01 (1) a	**0.17** ± 0.00 (1) ab
Midgut (Major)	**10.49** ± 0.30 (6.7) b	**1.73** ± 0.03 (3.3) b	**0.75** ± 0.01 (6.3) b	**13.54** ± 0.26 (4.7) a	**1.84** ± 0.01 (26.3) b	**1.86** ± 0.07 (10.9) c
Midgut (Minor)	**12.11** ± 0.55 (7.7) c	**0.08** ± 0.01 (0.2) c	**1.58** ± 0.06 (13.1) c	**11.55** ± 0.31 (4.0) b	**0.53** ± 0.03 (7.5) c	**0.65** ± 0.04 (3.8) d
Hindgut (Major)	**3.99** ± 0.63 d	**0.35** ± 0.03 d	**0.45** ± 0.04 d	**1.51** ± 0.08 c	**0.36** ± 0.02 d	**0.22** ± 0.02 b
Hindgut (Minor)	**0.69** ± 0.37 a	**0.01** ± 0.02 c	**0.00** ± 0.00 a	**0.17** ± 0.08 d	**0.03** ± 0.03 a	**0.03** ± 0.03 a

* Mean enzyme activity (U/mg protein) of 3 replicates (see Methods). (Bold type)

** Standard error. (±)

† Ratio of activity in midgut to activity in mycotêtes. For midgut major amylase 0.26/0.48 = (0.5). (Brackets)

‡ Different letters (a, b, c, d) indicate a significant difference between enzyme activities between enzyme sources (e.g., mycotêtes, midguts and hindguts) for that enzyme ($P < 0.05$ Tukey test). (Letters for one enzyme cannot be compared with letters of another enzyme. For example, letter a of amylase is completely different to letter of a laminarinase.)

£ NT = Not Tested with Tukey test whether or not significant difference. Not tested because if included homoscedasticity of that ANOVA would be violated. (NT is the value with the most widely different standard error.)

§ Mycotête activity significantly different from both major and minor midgut activity (*t*-test, $P < 0.01$).

Comparing major and minor workers, the hindgut of the major workers had more activity than the hindgut of minor workers (7 out of the 9 cases statistically compared, with no significant difference in the remaining 2 cases). Furthermore, in the midgut, where most of the digestion occurs, 7 of the 12 enzymes had significantly higher activity in major workers. For three of these enzymes (amylase, pectinase and β-galactosidase) activities were 8 to 21 times higher. For midgut activity of minor workers of the three enzymes that were slightly (but significantly) higher, only one enzyme had over twice the activity of major workers (chitinase, 2.1 times).

Mycotêtes had detectable enzymatic activity significantly greater than controls (=zero) against all 12 substrates ($P < 0.05$, t-tests). However, comparing midgut activity with activity in the mycotêtes, activities were significantly higher in midguts of major workers for 10 out of the 12 enzymes. For many of these enzymes, midgut activities were many fold higher than activities in the mycotêtes. Mycotête activities of pectinase and β-galactosidase were higher than in the midgut of minors, though not majors. However, for only one enzyme (amylase) was activity in the mycotêtes higher than activities in the midgut of both major and minor workers.

Discussion

Our results support Rouland et al. (1991) by showing many poly- and disaccharidases present in the *Termitomyces* mycotêtes. However, we find that the enzyme activity in the *T. microcarpus* mycotêtes is relatively low compared with the midgut of *M. barneyi*. In only one of the 12 enzymes tested (i.e., amylase) was activity in the mycotêtes substantially higher than in the termite midgut. Rouland et al. (1991) divided fungus-growing termites into species where the fungus exhibited relatively higher enzymatic production (*Macrotermes bellicosus*, *Odontotermes* near *pauperans* and *Pseudacanthotermes militaris*) and species where one can question the role of the fungus as they exhibited very low enzymatic activities (*Ancistrotermes cavithorax* and *Microtermes toumodiensis*). Our results indicate that *M. barneyi* falls into the second category (i.e., not the category of its congener *Macrotermes bellicosus*). Previous researchers also used this same method of comparing enzymatic activity in the midgut with the mycotêtes (Martin and Martin 1978, Martin 1987, Rouland et al. 1991, Veivers et al. 1991). For the mycotêtes to actively supply enzymes for cellulose digestion in the termite gut, we would expect the specific activities of enzymes in the mycotêtes to be several times higher than in the midgut (where they would be "diluted" by the ingested food). Very high activity in the mycotêtes was indeed found in *Macrotermes natalensis* (Martin and Martin 1978, Martin 1987), though not in *M. barneyi* (Table 1).

If fungal enzymes could be concentrated in the gut (though this was not part of Martin and Martin's [1978] original suggestion), widely differing proportions of enzymes between the mycotêtes and midgut would not be expected. For example, the midgut of major workers can have between 0.5 and 26.3 times the concentrations of different enzymes in the mycotêtes (Table 1). Furthermore, sometimes similar and sometimes widely differing concentration ratios between

major and minor midguts (e.g., laminarinase 5.8 and 6.8, whereas 1.2 and 0.06 for pectinase) also would not be expected.

In contrast to the situation in *M. bellicosus* (Rouland et al. 1991), our data show that enzymes acting on xylan and xylooligomers were produced in lower amounts in the *Termitomyces* mycotêtes compared to the *M. barneyi* midgut. High laminarinase activity found in the termite midgut (e.g., *M. barneyi* and *M. bellicosus*) indicates ability to cleave β-1,3-glucans, a substrate commonly found in fungal cell walls (Rouland et al. 1991).

In the case of *M. natalensis* it has been strongly argued that while the termite can digest non-crystalline cellulose (e.g., carboxymethylcellulose) largely unaided, enzymes acquired from the fungal mycotêtes are necessary to digest crystalline cellulose (e.g., avicel) and cellobiose (Martin and Martin 1978, Martin 1987, Martin 1992, but see Slaytor 1992). Our results show that for *M. barneyi*, most cellulolytic activity (exoglucanase, endoglucanase, and β-glucosidase) is located in the midgut of the workers and not in mycotêtes. Thus, in *M. barneyi* the mycotêtes are probably not important in digestion of cellulose. Intracellular gut symbionts have been found in no termite species (Breznak and Brune 1994) and specific microflora are confined to the anaerobic hindgut of fungus-growing termites (Rouland et al. 1991). Therefore, the possibility is being increasingly approached that at least some termites may be able to produce many or all of the cellulases themselves (Slaytor 1992, Bignell et al. 1994, Breznak and Brune 1994). Little cellulase activity occurs in the foregut compared with the midgut (Veivers et al. 1991). Our results confirm those of Rouland et al. (1991) that indeed the midgut is more important than the hindgut for cellulase activity in *Macrotermes*. This contrasts with some other termites (Slaytor 1992).

The substantially higher activity of major worker midguts and hindguts (compared with those of minor workers) might be correlated with the role of major workers as the principal foraging caste in *M. barneyi* (unpublished observations) (i.e., the first caste to ingest newly-collected polysaccharides [Wood and Thomas 1989, Veivers et al. 1991]). Minor workers carry out more activities in the center of the nest (e.g., around the chitin-containing fungus comb, unpublished observations).

Rouland et al. (1991) found that different genera of fungus-growing termites showed considerable variation in the enzymatic role of the *Termitomyces* fungus. The present study (when compared with earlier studies of Martin [1987], and Rouland et al. [1991]) highlights that considerable variation can occur in the enzymatic role of the fungus even within a single termite genus (*Macrotermes*).

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