Changes in *Reticulitermes flavipes* (Kollar) Gut Cellulolytic Activities in Response to Hardwood, Softwood and Cellulose Diets[1]

Joseph A. Smith, Michael E. Scharf,[2] Roberto M. Pereira, and Philip G. Koehler[2]

Department of Entomology and Nematology, University of Florida, Gainesville, Florida 32611 USA

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Abstract Three cellulase activities (endoglucanase, exoglucanase and β-glucosidase) were assayed for each gut region of field-fed *Reticulitermes flavipes* (Kollar) workers, in comparison with population subsamples kept in the laboratory on 3 diets: red oak (*Quercus* spp.), pine (*Pinus* spp.) and filter paper. The 3 cellulase activities were generally higher in the field-fed termites compared with the 3 subpopulations that were fed on laboratory diets, especially the oak and pine diets. Endoglucanase activity was evenly distributed in the foregut and hindgut with ~36 - 50% of the activity associated with the hindgut. This finding provides good supporting evidence that endoglucanases are produced both endogenously and also by symbionts that enhance cellulose digestion in the hindgut. Exoglucanase activities were mainly located in the hindgut (>87 - 95% of total), suggesting that partially digested cellulose travels from the foregut and midgut to the hindgut where it is exposed to symbiont-derived exoglucanases. β-glucosidase activity was found distributed through all 3 gut regions with ~37 - 56% of the activity found in the hindgut. Cellulolytic activities in *R. flavipes* can thus change to accommodate dietary composition. One hypothesis is that this change is accomplished via changing hindgut protozoan community composition. However, another equally plausible hypothesis is that endogenous cellulase activities can be altered via differential gene expression in response to changing diet. This flexibility apparently allows *R. flavipes* to efficiently use a variety of wood and wood-derived materials that contain a variety of lignocellulose compositions.

Key Words cellulase, endoglucanase, exoglucanase, β-glucosidase

Subterranean termites, such as *Reticulitermes flavipes* (Kollar), subsist largely on a diet of wood and other materials primarily made of cellulose (Noirot and Noirot-Timothee 1969). Wood is not a substance that most animals are capable of digesting to any significant degree, and termites are well known for the ability to digest cellulose with the aid of microbial hindgut symbionts. However, the degree to which termites and their symbionts collaborate, and the specific roles of individual enzyme families in the termite gut still remain largely undefined (Scharf and Tartar 2008).

In wood, cellulose chains are typically arranged in parallel bundles known as microfibers which are embedded in a matrix of lignin and hemicelluloses (Astley et al. 1997, Timell 1964). Cellulose occurs in either a tightly aligned crystalline matrix or a more randomly arranged amorphous form. Three major types of cellulases work together to digest cellulose (Breznak and Brune 1994). Exoglucanases (EC 3.2.1.91) cleave the cellulose chain from the ends, typically producing cellobiose, and are most

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[2]Address inquiries email: (mescharf@ufl.edu; pgk@ufl.edu).

active against crystalline cellulose. Endoglucanases (EC 3.2.1.4) cleave the cellulose chain randomly along its length and are most active against amorphous cellulose. β-glucosidases (EC 3.2.1.21) cleave cellobiose and other small cellulose fragments, hydrolyzing them to glucose.

To effectively digest wood, termites like *R. flavipes* have evolved with their symbionts to produce an array of enzymes. Endogenous cellulases in the Rhinotermitidae have been characterized and are produced naturally by the termite (Watanabe et al. 1998). Those of symbiotic origin are produced by flagellate symbionts within the hindgut (Nakashima et al. 2002a, Watanabe et al. 2002, Ohtoko et al. 2000). Zhou et al. (2007) and Smith et al. (2009) demonstrated that exoglucanase activity is largely localized to the hindgut of *R. flavipes*, suggesting resident flagellates as the potential agents of crystalline cellulose digestion. Inoue et al. (1997) showed that the protozoan composition of the *Reticulitermes speratus* (Kolbe) hindgut significantly changes when the termites are fed pure cellulose or pure xylan, as opposed to wood. Smith and Koehler (2007) demonstrated that xylanolytic enzyme activities change in response to various diets so it is probable that the levels of different cellulase activities in the gut would also change in response to dietary cellulose composition. Our objectives here were to (1) determine cellulase baseline activities in each gut region of field-fed termites, (2) determine changes in the cellulase activities for each gut region in response to laboratory-fed wood or paper diets, and (3) compare cellulase activities for field-fed termites to those fed the 3 laboratory diets.

Materials and Methods

Termite collection. *Reticuliterme flavipes* termites were field collected on the University of Florida campus in Gainesville, FL, as described in Smith and Koehler (2007). Briefly, a PVC bucket with holes to allow termite access was placed in the ground covered with a PVC lid. Two to 3 rolls of single-faced corrugated cardboard were placed into the bucket as a food source. Termites were separated from the cardboard, and either placed in feeding bioassays or immediately frozen and kept at –80°C until dissection. Collections were restricted to a single colony.

Termite diets and feeding. Three laboratory diets were prepared for the termites identical to Smith and Koehler (2007). These diets were red oak (*Quercus* sp.), pine (*Pinus* sp.), or filter paper. Wood diets were generated by drilling into craft wood boards (0.6 × 5.1 × 61.0 cm) with a 2.54 cm spade drill bit. Sawdust was collected and weighed. Filter paper diet consisted of crumpled 100% cellulose filter paper disks (42.5 mm diam, Whatman, grade 4).

Each laboratory diet (20 g of sawdust or paper) was added to a sterilized and loosely capped 250-mL glass bottle (Pyrex) with 5 mL of deionized water. Termites were added (~300 workers and 3 soldiers per bottle) and kept in the dark at 21°C for 6 wk, with deionized water (2 mL) added every 2 wk. After 6 wk, live laboratory fed termites were collected, frozen and kept at –80°C until dissection.

Termite dissection and enzyme extraction. Termites were processed and enzymes were extracted as described by Smith et al. (2009). Briefly, each termite's gut was removed in either sodium acetate buffer (0.1 M, pH 5.5 for endogluconase assay) or sodium phosphate buffer (0.1 M pH 6.5 for exoglucanase and β-glucosidase assay) and separated into foregut (and salivary glands), midgut, and hindgut. An enzyme extract was prepared for each feeding treatment/gut region from 50 termites for the endoglucanase assay or 35 termites for exogluconase and β-glucosidase assays

using a single biological replication and using an experimental design similar to previous termite carbohydrolase experiments (Hogan et al. 1988, Inoue et al. 1997, Nakashima et al. 2002b, Smith and Koehler 2007, Smith et al. 2009) with statistical analysis performed on technical replicates. The 3 gut regions were placed into separate 1.5-mL microcentrifuge tubes (Eppendorf) containing the appropriate buffer, and kept on ice. Final concentrations were equivalent to 50 termite gut regions per mL.

Enzymes were extracted using the method of Smith et al. (2009). The contents of each microcentrifuge tube were placed in a 2-mL Tenbroeck glass tissue grinder (Pyrex) and manually homogenized on ice. The homogenates were centrifuged at 20,800 g at 4°C for 15 min. The supernatants were collected, frozen, and kept at -80°C until use in the enzyme assays. All assays were conducted in clear 96-well microplates using methods adapted from Han et al. (1995), Smith and Koehler (2007), and Smith et al. (2009).

For the endoglucanase assays, 10 µL of tissue extract was combined with 90 µL of 2% carboxymethylcellulose solution (CMC, Sigma-Aldrich, in 0.1 M sodium acetate buffer, pH 5.5) in each well and allowed to react for 70 min at 23°C. A 100-µL volume of 1% 3,5-dinitrosalicylic acid (DNSA), 0.4M sodium hydroxide and 30% sodium potassium tartrate was added to each well. The microplate was immediately placed in boiling water for 10 min and then on ice for 15 min. Each cooled microplate was read at 540 nm using a µQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, VT). Control plates were allowed to react for 10 min to allow for passive mixing of solutions before boiling with DNSA solution (Zhou et al. 2007, 2008a, b); standards used dilutions of glucose. For all replicates, control plates were used to adjust for 540 nm absorbance in gut extracts and had the same number of replicates as the assay plates using one microplate well per technical replicate.

For the exoglucanase and β-glucosidase assays, 10 µL of tissue extract was combined in each well with 90 µL of pNPC (4 mM p-nitrophenyl-β-D-cellobioside) or pNPG (4 mM p-nitrophenyl-β-D-glucopyranoside) prepared in sodium phosphate buffer (0.1 M, pH 6.5), respectively. After 10 min, the plates were read in the microplate spectrophotometer (described above) at 420 nm every 2 min for 30 min at 23°C. Mean velocities (mOd/s) were recorded. Standard curves used dilutions of p-nitrophenol.

Data analysis. Experiments were set up as one-factor designs with a single homogenization for each gut region/diet/substrate combination. Thus, the experiments only had a single biological replication, a previously accepted method (Hogan et al. 1988, Inoue et al. 1997, Nakashima et al. 2002b), with technical replicates being used for statistical analysis. The endoglucanase assays had 4 technical replicates for each gut region, and the exoglucanase and β-glucosidase assays had 3 technical replicates for each gut region. Enzymatic activities were calculated using the formulae presented in Smith et al. (2009) and analyzed by one-way analysis of variance for each gut region. Tukey's Studentized Range Test ($\alpha = 0.05$) was used to separate the mean activities on each diet within each gut region (SAS Institute 2001).

Results

The color of the dissected termite guts reflected the color of the diets fed to the termites, similar to observations by Smith and Koehler (2007). Termites fed on red oak had brownish-orange gut contents; those fed pine had pale yellow gut contents; and those fed on paper had white gut contents. Field-fed termites had dark brown gut contents.

Most of the endoglucanase activity for field-fed termites was located in the foregut and the hindgut, with little in the midgut (Fig. 1A). About 50% of the activity was in the foregut and was probably endogenous in origin. Among the laboratory-fed diets, foregut endoglucanase activity was highest on paper and significantly lower on oak. The 100% cellulose paper diet resulted in ~62% of activity in the foregut; whereas, the pine and oak diets resulted in activity being evenly distributed between the foregut and hindgut. Hindgut endoglucanase activity in the field-fed termites was significantly higher than the hindgut activity seen on all 3 laboratory-fed diets. Among the diets, hindgut activity was highest on oak and lowest on paper, with only 37% of the activity in the hindgut. Among the laboratory-fed diets, endoglucanase activities were lower on paper than on oak and pine.

Exoglucanase activity in field-fed termites was mainly associated with the hindgut which accounted for ~90% of activity (Fig. 1B). Foregut exoglucanase activity in field-fed termites was significantly higher than the foregut activity seen in all 3 laboratory-fed diets. Among the diets, foregut exoglucanase activity was highest on paper with lower activity on pine and oak diets. Midgut exoglucanase activity in the field-fed termites was intermediate between the midgut activities seen on oak and pine diets. Among the laboratory-fed diets, midgut activity was highest on paper and lowest on oak. Hindgut exoglucanase activity in the field-fed termites was significantly higher than the hindgut activity seen on the 3 laboratory-fed diets. Among the diets, hindgut activity was lowest on paper and highest on oak, with activity on pine diet nearly as high as oak.

Activity of β-glucosidase in field-fed termites was distributed among all 3 gut regions with about 18% of activity in the foregut, 32% in the midgut, and 49% in the hindgut (Fig. 1C). Foregut β-glucosidase activity was higher for the paper-fed termites than field-fed; however, the wood-fed termites had significantly lower activity, with the lowest activity for the oak diet. Midgut β-glucosidase activity was about one-third of the activity for both the field-fed and the laboratory-fed diets. Midgut activity in the paper-fed was similar to field-fed termites; however, activity in wood-fed termites was significantly lower, with oak being the lowest. Hindgut β-glucosidase activity in the field-fed termites was 49% of the activity and similar to those fed wood (pine and oak) diets. However, those fed paper had significantly less hindgut activity which only accounted for 37% of the activity. β-glucosidase activity was highest in field-fed termites and lowest in wood-fed termites.

Discussion

The appearance of the termite guts upon dissection was consistent with that described previously for *R. flavipes* (Smith and Koehler 2007, Smith et al. 2009, Zhou et al. 2007) and indicated that the termites had fed on their respective diets. Based on the relatively dark gut contents of the field-fed termites, as well as the presence of dark oval objects in some cases, it is possible that these termites were feeding on cardboard partially digested by wood decay organisms. Regardless of diet, cellulose digestion likely begins in the foregut due to endogenous endoglucanase production, as demonstrated in the current study and through previous research (Smith et al. 2009). Even though the proportion of cellulose in the oak and pine diets should be equivalent, only the oak diet significantly reduced endoglucanase activity in the foregut perhaps due to the higher levels of xylan known to exist in oak wood.

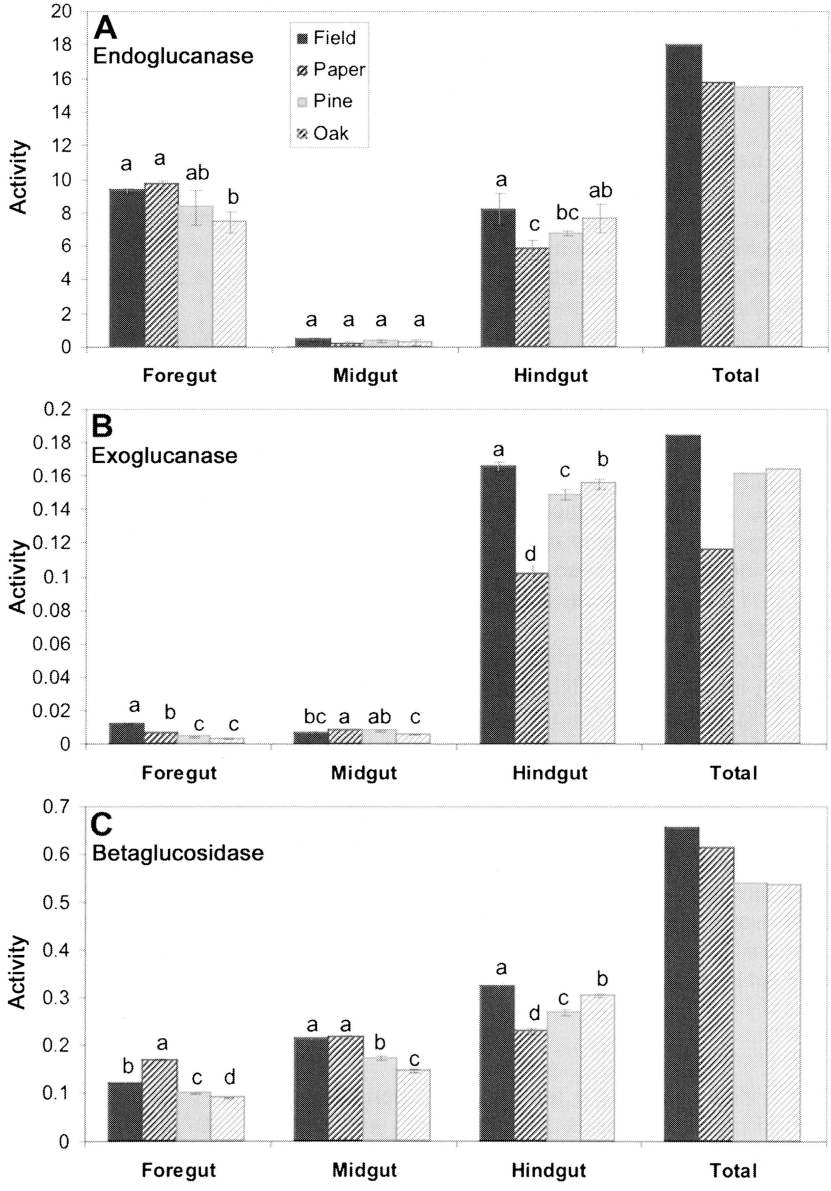

Fig. 1. Endoglucansase (A), exoglucanase (B), β-glucosidase (C) and total activities in each gut region. Means within gut region or for the total insect with the same letter are not significantly different (Tukey's Studentized Range Test, α = 0.05 [SAS Institute, 2001]). There were *n* = 4 technical replicates for endoglucanase expressed as nmol reducing sugar per termite equivalent per min; *n* = 3 for exoglucanase and β-glucosidase expressed as nmol p-nitrophenol per termite equivalent per min.

The lowest observed endoglucanase activity was in termites that were provided paper diets. The acid degradation of cellulose during paper production may result in better digestion by endoglucanase and less need for enzyme induction. Foregut digestion of paper perhaps resulted in less available cellulose in the hindgut, ultimately impacting symbiont populations. It appears that the amount of amorphous cellulose in the diet may significantly affect the location and level of endoglucanase activity in both the foregut and the hindgut.

The cellulose in field-fed termites was probably partially predigested by microorganisms (e.g., fungi), and thus, the termites likely adjusted production of endoglucanase to exploit the simpler arrangement of the cellulose form and fibers. Similarly, the reduction in endoglucanase activity with both pine and oak diets may have been due to the increased quantities of hemicelluloses present in these diets.

Exoglucanase activity was almost entirely associated with the hindgut, supporting that this activity is symbiotic in origin, as suggested previously (Watanabe et al. 2002, Nakashima et al. 2002a, Zhou et al. 2008a, Smith et al. 2009). Significantly higher exoglucanase activity in the foregut of field-fed termites may be a result of greater trophallaxis rates/levels in field termites than those held in smaller groups in the laboratory.

Exoglucanases are thought to be oriented toward digestion of crystalline cellulose and are apparently mostly symbiotic in origin. However, because wood can be composed of up to 70% crystalline cellulose and 25% xylan by dry weight (Biermann 1996), it was surprising that wood diets of both pine and oak eaten by termites in the laboratory significantly reduced exoglucanase activity. With wood diets, the production of xylanase by hindgut symbionts may come at the expense of exoglucanase production. High exoglucanase activity in field-fed termites suggests a robust symbiont community in the hindgut of these insects; whereas, the lowest exoglucanase activity in paper-fed termites reflects the reduced need for symbionts and their digestion of cellulose.

Regardless of diet, significant β-glucosidase activity was observed in all 3 gut regions and increased progressively along the gut as observed previously (Smith et al. 2009); however, diet significantly affected levels of activity. β-glucosidase digests small molecules produced during cellulose digestion; therefore, β-glucosidase activity in the foregut of paper-fed termites probably contributed to the final digestion of products derived from the amorphous cellulose/endoglucanase reaction or partially digested materials obtained via proctodeal trophallaxis. Because wood diets were probably more complex, β-glucosidase activity in the foregut of wood-fed termites was significantly lower, and more of the digestion of small molecules occurred further in the digestive tract.

β-glucosidase was the only cellulase activity associated with endogenous (i.e., nonsymbiotic) production in the midgut. Unlike xylanase activity (Smith and Koehler 2007), cellulase activity was highest in field-fed termites probably due to consumption of at least partially decayed and, consequently, partially digested wood. Because cellulolytic activity in field-fed termites increased only for exoglucanase, and not endoglucanase or β-glucosidase, it is unlikely that endoglucanase was ingested with the decayed wood. A loss of symbiont fauna in the laboratory in response to a nondecayed wood diet is perhaps a more plausible explanation for the proportionally lower cellulolytic activity of the laboratory-fed termites. Whether the termites changed endogenous enzyme output in response to fluctuations in symbiont enzymes, or the symbiont population changed in response to the levels of endogenous termite enzymes, was not determined. Previous studies have suggested that xylan content of a

given diet may affect symbiont populations (Zhou et al. 2007, Smith and Koehler 2007), and therefore, xylan content may affect the activity levels of the enzymes produced by these symbionts. Studies on *R. speratus* (Azuma et al. 1993, Inoue et al. 1997), *R. virginicus* (Banks) (Cook and Gold 2000) and *Coptotermes formosanus* (Shiraki) (Mannesmann 1972, Waller and La Fage 1987) also have demonstrated significant changes in the hindgut protozoan communities in response to different diets, including different wood species, pure cellulose and pure xylan.

The overall cellulase activity pattern found in this study was consistent with that described for *R. flavipes* (Zhou et al. 2007, 2008a) and *R. speratus* (Inoue et al. 1997); although activity levels in *R. speratus* were roughly 10- to 100-fold higher than in our study. Higher activity in *R. speratus* may be due to the assay used (DSNA [our study] versus tetrazolium blue [Inoue et al. 1997]), assay temperature (23°C versus 25°C) or termite population/species. More precise reaction times and lower variation can be obtained with the DSNA assay format used in the present study (Smith et al. 2009).

Based on the observed locations of the activities, our findings strongly suggest that both endoglucanases and β-glucosidases are produced by both the termite and its symbionts. β-glucosidase activities were seen throughout the termite gut, with endogenous β-glucosidases being produced in the foregut and midgut, and symbiont β-glucosidases likely being produced in the hindgut. Exoglucanases appear to be produced almost entirely by hindgut symbionts.

In summary, findings presented here suggest that the quality of the cellulose content in the diet likely affects the termite's ability to digest it without the aid of symbionts. Because the termites produce endogenous endoglucanases and β-glucosidases, they may be capable of degrading amorphous cellulose without the need of symbionts, but likely they rely heavily upon symbiont exoglucanases to digest crystalline cellulose, or predigested cellulose. It is apparent that *R. flavipes* workers are capable of digesting cellulose, following the same pattern reported in previous literature (Zhou et al. 2007). Crystalline cellulose is mainly digested by hindgut symbionts whereas amorphous cellulose and cellodextrins are digested by both the termites and its symbionts. In addition, the balance of endogenous versus symbiont cellulolytic activities appears to change in response to their diet, most likely by changes in the hindgut protozoan communities as well as the termite enzyme expression. This flexibility allows the termites to efficiently use a variety of wood species and wood-derived materials which have different qualities of cellulose. This capacity for adaptation and partial balancing between the termite and its symbionts may also make termite control by means of cellulase inhibition more difficult.

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