THE TERMITE CELLULASE SYSTEM AS A NOVEL TARGET SITE FOR TERMITE CONTROL

By

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To my family and to Andrew Magis
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Termites are among the few insects capable of efficiently digesting cellulose, the major component in wood. This singular ability enables termites to play important ecological roles and makes them major structural pests worldwide. All termite species require the presence of specialized enzymes called cellulases for the digestion of cellulosic materials. In many termite pest species, cellulases are both symbiotic and endogenous in origin. This collaborative cellulase system is central to termite nutrition and survival, and consequently considered a potential target site for novel termite control agents, including cellulase inhibitors. Thus, the overall objective of this research project was to identify novel compounds as potential inhibitors of termite cellulases. Specifically, three prototype cellulase inhibitors were tested against workers of the Eastern subterranean termite, *Reticulitermes flavipes*, including the disaccharide-based cellbioimidazole (CBI) and fluoro-methyl celllobiose (FMCB) and the monosaccharide-based fluoro-methyl glucose (FMG). These compounds were tested *in vitro* through cellulase activity assays, as well as *in vivo* in 24-day bioassays. In addition, this research developed a technique to monitor cellulolytic protozoan populations in termites, as an effort to clarify whether decreases in cellulase activity are the result of protozoan death or enzymatic inhibition. This study provides novel data indicating that the disaccharide-based inhibitor CBI is a strong inhibitor of termite
cellulases and caused moderate termite mortality. This research also suggests that quantitative real time -PCR is a viable method to monitor shifts in cellulytic protozoa populations.
CHAPTER 1
LITERATURE REVIEW

Introduction

Despite the number and diversity of insects, few are able to exploit the nutritive value of cellulose, the most abundant polymer on earth. Unlike glycogen and starch that have a helical structure and α -1,4- glucosidic bonds, cellulose has β -1,4- bonds which gives cellulose a highly rigid structure. Specifically, cellulose consists of composite forms of highly crystallized microfibrils among amorphous matrices. For this reason, cellulose digestion is a complex process that requires the presence of specialized enzymes called cellulases. These enzymes are capable of hydrolyzing glucosidic linkages in cellulose and degrading it into the universal energy source, glucose.

Termites are the most efficient cellulose-digesters, with assimilation efficiencies often approaching 99% (Breznak and Brune 1994). They thrive in a variety of ecosystems worldwide and play an important role in the biorecycling of plant material (Ohkuma 2003). Phylogenetically, termites are classified into two major groups, a more primitive group known as the lower termites and a more advanced group known as the higher termites. Three-fourths of all termite species are higher termites and belong to the family Termitidae. Members of the families Mastotermitidae, Kalotermitidae, Termopsidiae, Rhinotermitidae, Serritermitidae and Hodotermitidae all belong to the lower termite group (Krishna and Weesner 1969).

Lower termite species, particularly subterranean species, have been the focus of many studies because several pest species belong to this group (Su and Scheffrahn 1990). In addition, lower termites have long been known to rely on their hindgut protozoa to produce cellulases (Cleveland 1924, Hungate 1939). The relationship between lower termite species and protozoa is considered a textbook case of insect symbiosis, in which termites require the cellulolytic action
of protozoa for survival and in return they provide a habitat and nutrition to the protozoa. Recent studies have shown that lower termites also possess endogenous cellulases and that both endogenous and symbiotic cellulases are important for cellulose digestion (Watanabe et al. 1998). Higher termites do not harbor cellulolytic flagellates and instead acquire cellulases by cultivating fungi (suggested for Macrotermiteinae only) or rely on endogenous cellulases (Breznak and Brune, 1994).

**Colony Structure**

Termites and Hymenoptera (ants, wasps, and bees) are also noteworthy because they are among the few insect orders that have evolved eusociality. Their colonies exhibit a division in reproductive labor, cooperative brood care, and overlapping adult generations (Wilson 1971). However, unlike Hymenoptera which exhibits haploidiploidy, both males and females within the termite colony are diploid. Termite colonies are polyphenic and most termite colonies can be divided into a reproductive caste, a defensive or soldier caste, a worker caste, and an immature brood. However, unlike the general portrayal of termite societies where the queen and king live with a group of sterile workers, in many termite species individuals retain developmental and reproductive options (Thorne 1997).

Caste differentiation, in termites, is not a genetically predetermined process but rather a postembryonic process that is induced by hormonal and environmental signals. There are two developmental pathways involved in differentiation, a sexual line also called the imaginal or nymphal line that exhibits wing buds and developing eyes and an apterous line that leads to wingless and eyeless individuals that function as workers. Those that differentiate into the sexual line comprise the reproductive caste. This caste is divided into alates and non-alate derived reproductives. Alates are fully pigmented, winged adults with compound eyes (Krishna and Weesner 1969). They are produced seasonally, disperse and found new colonies. Colony
establishment was traditionally thought to occur through “royal pairs” (single male and female alate); however the occurrence of primary polyandry in laboratory and field colonies has also been documented (Grube and Forschler 2004), as well as colony establishment by non-alate reproductives. There are two types of non-alate reproductives that can differentiate to replace or supplement the founding king and queen. Neotenic reproductives differentiate as a result of the death or senescence of reproductives. Those that differentiate to aid the reproductive potential of a colony are called supplementary reproductives (Lainé and Wright 2003, Thorne et al. 2003). All of these reproductive forms are physiologically specialized to produce large numbers of offspring. The abdomen of both the queen and female neotenics becomes distended through a process called physogastry.

In the majority of lower termite species, soldiers have a morphologically distinct head region with long mandibles and usually comprise a low percentage of the termite colony (1-3 % for R. flavipes) (Howard and Haverty 1981). They are traditionally considered the defensive caste but may also participate in food scouting and foraging. Additionally, termite soldiers are thought to play a role in caste differentiation, primarily by regulating JH titers in nestmates. In recent years, significant progress has been made delineating the proximate mechanisms in the soldier differentiation process, including the role of juvenile hormone and hexamerin proteins in the induction and suppression of Reticulitermes flavipes Kollar worker to soldier differentiation (Zhou et al. 2006).

The vast majority of a termite colony is made up of workers. In R. flavipes workers can comprise up to 90% of the colony (Howard and Haverty 1981). A true worker refers to a nonreproductive, nonsoldier individual that has reached at least its third instar. Unlike larvae, workers have sclerotized mandibles and a darkened abdomen. This true worker caste is found in
almost all lower termites and it is found among termite species that forage away from their nest (Thorne 1997). Many morphologically and developmentally distinct groups such as nymphs, later instar “larvae”, and pseudergates can perform worker tasks. Pseudergates are non-reproductive individuals that have regressed from the imaginal line at a late larval instar (Snyder 1926, Miller 1969, Lainé and Wright 2003). However, the majority of tasks including tunneling, foraging, alarm giving, and broodcare are performed by the worker caste (Crosland et al. 1997).

**Termite Digestion**

The digestive physiology of termite species is governed by cellulose degradation process and by a continuous exchange of nutrients between diverse castes (Krishna and Weesner 1969). The worker caste is the primary wood consumer and provides nourishment to all other castes by means of trophallaxis, or liquid food transfer. In lower termites, proctodeal trophallaxis, which involves anal-oral food transfer, is probably of greater importance than oral to oral trophallaxis; because symbiotic protozoa are transferred from one nestmate to another by proctodeal trophallaxis (Thorne 1997). Recent research has shown that the highest number of cellulase gene transcripts are found in the worker caste (Scharf et al. 2003a). More specifically, endogenous cellulases were found in workers, nymphs, alates and supplementary reproductives, while symbiotic cellulases were found in all forms except supplementary reproductives (Scharf et al. 2005); thus, suggesting a correlation between digestive physiology and the role each caste plays in colony digestion and nutrition.

In termites, several important metabolic activities are aided by gut microbiota including cellulose hydrolysis, fermentation of depolymerized products and intestinal nitrogen cycling and nitrogen fixation (Brune and Friedrich 2000). In lower termites, cellulose and hemicellulose digestion is aided by anaerobic protozoa. Protozoa occupy a substantial portion of the hindgut in lower termites and are known to phagocytize wood particles and ferment polysaccharides to
acetate, CO₂ and H₂ (Cleveland 1924, Hungate 1939, Yamin 1981). Recent studies have shown that termites also depolymerize cellulose through the action of endogenous cellulases (Watanabe et al. 1998, Nakashima et al. 2002, Zhou et al. 2007). Unlike symbiont-produced cellulases which are localized in the hindgut, endogenous cellulases are found in the salivary glands/foregut or the midgut, depending on whether the termite species belongs to the lower or higher termite group, respectively (Tokuda et al. 2004). Thus, in higher termites which do not harbor cellulolytic protozoa, endogenous cellulases can at least partially account for their cellulolytic capabilities.

More specifically, cellulose digestion is thought to be achieved through the collaborative action of three different types of cellulases namely, endoglucanases, exoglucanases and β-glucosidases. Endoglucanases internally cleave 1,4 β-D-glucosidic linkages and release glucose, cellobiose and cellotriose units while, exoglucanases terminally cleave cellulose units from the ends of cellulose chains. β-glucosidases release glucose by hydrolyzing β-D-glucose residues from cellobiose and cellotriose units (Martin 1991, Breznak and Brune 1994). Similarly hemicelluloses are degraded by the action of specialized enzymes called xylanases which cleave 1,4 β-D xylosidic linkages to release xylose (Breznak and Brune 1994). In lower termite R. speratus, xylanase activity was mainly restricted to the hindgut, suggesting that in this species xylanases are primarily produced by gut symbionts (Inoue et al. 1997).

Endogenous termite cellulases have been classified as endoglucanases for R. flavipes, R. speratus, and Coptotermes formosanus Shiraki and have been structurally classified as members of the glucoside-hydrolase family 9 (GHF9) (Watanabe et al. 1998, Nakashima et al. 2002, Zhou et al. 2007). However, symbiotic cellulases for these species have been classified into GHF7 and GHF45 and are thought to have both endoglucanase and exoglucanase activity (Ohtoko et al. 2004).
The synergistic action of both endoglucanases and exoglucanases in lower termite hindguts is thought to make the symbiotic cellulase system more efficient than endogenous cellulases which primarily have endoglucanase activity (Tokuda et al. 2005). Prokaryotes in the termite hindgut have been hypothesized to play a role in cellulose digestion, particularly in the flagellate-free higher termites. However, there is currently insufficient research that supports this hypothesis, and consequently the contribution of bacteria to the cellulose digestion is thought to be negligible (Breznak and Brune 1994). Instead, bacteria are thought to play major roles in acetogenesis, methanogenesis and nitrogen fixation and form highly compartmentalized communities. In *R. flavipes*, bacterial communities have been found to range from aerotolerant lactic-acid bacteria, facultative anaerobic enterobacteria and strictly aerobic bacteria (Brune 1998).

**Statement of Purpose**

The overall objective of this research project is to investigate the potential of cellulase inhibition as novel termite control method. More specifically, this project investigates the potential of monosaccharide and disaccharide-based compounds as cellulase inhibitors of *R. flavipes* workers. The central hypothesis is that if cellulase inhibition occurs it will impact termite feeding and cause termite mortality. The specific research objectives that tested the central hypothesis are: i) to assess whether cellulase inhibition can be observed *in vitro* by conducting termite gut dissections and cellulase activity assays, ii) to examine the impact of cellulase inhibitors on termite feeding and mortality and iii) to develop a method to monitor shifts in cellulolytic protozoan populations in order to assess whether decreases in cellulase activity are due to cellulase inhibition or death of cellulolytic protozoa. This project provides...
important information regarding the cellulase system of *R. flavipes* and the viability of the
termite cellulase system as a target site for novel termite control agents.
CHAPTER 2

IN VITRO INHIBITION OF TERMITE CELLULASES BY SUGAR-BASED INHIBITORS

Introduction

Termites are efficient cellulose-digesters and thrive in a variety of ecosystems worldwide. Although they play an important ecological role in the biorecycling of plant material, they are notorious for their status as major structural pests, causing an estimated 20 billion dollars in structural damage each year (worldwide) (Su 2002). Based on the presence or absence of symbiotic protozoa, termites are divided into two major phylogenetic groups: a more primitive group known as the lower termites and a more derived group known as the higher termites. From early studies done by Cleveland (1924), lower termites have been known to rely on cellulolytic protozoa for the digestion of cellulosic materials. Specifically, protozoan symbionts contribute specialized enzymes called cellulases. Recent research has shown that termites also produce their own cellulases and that endogenous cellulases are important to both lower and higher termite cellulose digestion (Watanabe et al. 1998, Nakashima et al. 2002, Tokuda et al. 2005, Zhou et al. 2007).

Unlike higher termites which have a broader feeding guild, lower termite feeding habits are mainly restricted to wood at varying stages of decay (Waller and La Fage 1987). Consequently, the majority of pest species belong to this group and the lower termite cellulase system is considered a potential target for innovative termite control agents, such as cellulase inhibitors (Zhu et al. 2005). In general, the cellulase system is composed of three different functional types of cellulases, namely endoglucanases, exoglucanases and β-glucosidases. These enzymes collaboratively degrade cellulose chains into simple sugars including glucose.

Inhibition of any of these three types of cellulases would lessen the efficiency of cellulose digestion and potentially cause termite mortality. To date, however, only a single study has
tested different compounds as potential inhibitors of β-glucosidases for the species *Coptotermes formosanus* Shiraki (Zhu et al. 2005). Therefore, the present study is an effort to continue to identify novel compounds that serve as termite cellulase inhibitors. Specifically, the objectives of this investigation were to (1) survey the distribution of endoglucanase, exoglucanase and β-glucosidase enzymes across the gut of *R. flavipes* workers and (2) test the efficacy of cellulase inhibitors fluoro-methyl glucose (FMG), cellobioimidazole (CBI) and fluoro-methyl cellobiose (FMCB) *in vitro*. Both objectives used homogenates of dissected guts as an enzyme source and were tested using cellulase activity assays. This study suggests the disaccharides CBI and FMCB are strong and moderate inhibitors (respectively) of exoglucanase and β-glucosidase enzymes.

**Materials and Methods**

**Termites**

The *R. flavipes* colony was collected from the University of Florida campus and was identified as *R. flavipes* using a PCR-RFLP identification key (Szalanski et al. 2003) and soldier head morphology. The colony was held in a plastic container supplied with moist brown paper towels and pine shims as food. Termites were kept in an environmental chamber in darkness at approximately 22°C and 70 % RH for 6 months before including them in this study.

**Chemicals**

The sugar-based compounds cellubioimidazole (CBI), fluoro-methyl cellobiose (FMCB), and fluoro-methyl glucose (FMG) were synthesized by Carbohydrate Synthesis Ltd (Oxford, UK) (Fig. 2-1). Inhibitor concentrations were diluted using reagent-grade methanol as a solvent. The concentration range tested for *in vitro* cellulase enzyme assays was $10^{-3}$ to $10^{-9}$ M. The model substrates used were carboxy-methyl cellulose (CMC), p-nitrophenol cellobioside (pNPC), and p-nitrophenol glucopyranoside (pNPG). All three substrates were diluted in homogenization buffer (0.1 M sodium acetate, pH of 5.8).
Optimization of Cellulase Activity Assays

Optimization of cellulase activity assays was carried out using whole-body homogenates as an enzyme source. Homogenates were prepared by homogenizing worker termites using a Teflon-glass tissue homogenizer and ice-cold homogenization buffer. Following homogenization, whole-body extracts were centrifuged at 14,000 rpm at 4°C for 15 min and then passed through a glass wool filter to remove excess lipids. Protein concentration was determined using a commercially available bicinchoninic acid assay (Pierce; Rockford, IL) with bovine serum albumin as a standard.

The CMC assay is an endpoint assay, which allowed for the optimization of more conditions than pNPC and pNPG assays, which are kinetic assays. Assay conditions examined for CMC assays included substrate concentration (0.0625 - 2%), protein concentration (0.3125 - 40 termite / ml; 0.0331 - 3.2513 mg protein / ml), assay time (10-70 min), assay temperature (22 - 57°C), and homogenization buffer pH (3.4 - 6.6). The impact of residual glucose present in the termite gut was assessed by comparing CMC hydrolysis activity with and without denatured protein. The conditions tested for the kinetic pNPC and pNPG assays were substrate concentration (0.125 - 16 mM and 0.25 - 32 mM, respectively), protein concentration (0.3125 - 40 termite / ml; 0.0331 - 3.2513 mg protein / ml), assay time (10 - 70 min), and homogenization buffer pH (3.4 - 6.6).

Enzyme Preparation for Cellulase Distribution and in vitro Inhibition Assays

Gut dissections were carried out for cellulase distribution studies and for in vitro inhibition assays. In both of these studies, the gut was dissected by decapitating each worker termite, opening the abdomen, and removing the gut intact. In the cellulase distribution study, the gut was divided into three major parts: salivary glands/foregut, midgut and hindgut. For in vitro
inhibition assays, the gut was divided into two regions in which the hindgut remained separate from the salivary glands/foregut and the midgut.

In both experiments, 25 gut regions were placed in a 2 ml Tenbroeck tissue grinder and homogenized in 1 ml of ice-cold homogenization buffer (0.1 M sodium acetate, pH of 5.8). Gut homogenates were then centrifuged (14,000 rpm at 4°C for 15 min); and the resulting supernatant was used directly as an enzyme source for the cellulase distribution study and \textit{in vitro} inhibition assays. Protein concentration was determined using the same protein assay described in the optimization study.

**Cellulase Activity Assays**

Cellulase activity assays were modified from Han et al. (1995) and optimized for running in 96-well microtiter plates and reading with a microplate spectrophotometer. A substrate concentration of 0.5 % CMC was used for CMC-based endoglucanases assays and a 4mM substrate concentration was used for the pNPC-based exoglucanase and pNPG-\(\beta\)-glucosidase assays. Endpoint CMC assays were incubated at 32°C, for 30 min and the reaction terminated by the addition of 100 \(\mu\)l of 1% 3, 5-dinitrosalicylic acid (DNSA), 30% sodium potassium tartrate, and 0.4 M NaOH to each sample well and fixed by placement of the microtiter plate on a 100°C water bath for 10 minutes. To achieve color formation microtiter plates were placed on ice for 15 min and then read at 520 nm. The spectrophotometer absorbance readings were analyzed relative to a glucose standard curve.

In pNPC and pNPG assays, enzyme and substrate reacted for 20 minutes at 32°C before being read kinetically at 420 nm at room temperature. PNPG assays were read for at total of 1 h, while pNPC assays were read for 1.5 h. An absorbance reading for each sample was measured every 2 min and mean velocity was used as a measure of activity.
Cellulase Distribution across *R. flavipes* Gut

The distribution of endoglucanase, exoglucanase, and β-glucosidase activities across the *R. flavipes* gut was assessed by dissecting the gut into three regions: salivary glands/foregut, midgut, and hindgut and by estimating the enzyme activity in each region through CMC, pNPC and pNPG assays. Homogenates were prepared using worker termites from a single termite colony. Three separate preparations, as well as three sub-samples were included for each gut region. Reactions consisted of 10 μl of enzyme and 90 μl of substrate. The specific activity and % total activity were calculated for each gut region. Statistical analyses were carried out using SAS software and data were analyzed using a one-way analysis of variance (ANOVA) on raw data. Means were separated using the Tukey-Kramer HSD method (SAS Institute).

**In vitro Inhibition**

The efficacy of the three prototype cellulase inhibitors FMG, FMCB and CBI was tested against two different gut enzyme sources that included endogenous termite cellulases from salivary gland, foregut and midgut, and symbiotic cellulases from the hindgut. Assays were initiated by addition of 5 μl inhibitor (in methanol) in a 95-μl reaction that contained 10-μl enzyme preparation and 85 μl substrate solution. For each inhibitor, a concentration range of $10^{-3}$ - $10^{-9}$ M (serial dilution) was tested and appropriate concentrations were identified. Percent inhibition was calculated relative to methanol controls. Using a range of inhibitor concentrations, inhibition curves were generated and used to determine 50% inhibition (*i.e.*, $I_{50}$) by linear regression and extrapolation. Each inhibition curve was derived from three independent preparations with three determinations for each concentration.
Results

Optimization of Cellulase Activity Assays

Optimization of CMC, pNPC and pNPG assays were carried out to ensure accurate measurement of termite cellulase activity. The results indicate that a protein concentration between 0.5 to 1.5 mg/ml and a homogenization buffer pH of 5.8 was optimal for all three assay types (Fig. 2-2 and 2-3). Results also indicate that optimal conditions for CMC assays include a substrate concentration of 0.5 % CMC and an assay time and temperature of 30 min and 32°C. In addition, a comparison of active and denatured protein suggests residual glucose does not affect CMC assay results (Fig. 2-2). Optimal conditions for pNPC and pNPG included a substrate concentration of 4mM pNPC/G; and showed cellulase activity to be stable after 20 min of assay time (Fig. 2-3). The conditions used in cellulase activity assays were all within the linear activity range.

Cellulase Distribution across R. flavipes Gut

Baseline cellulase distribution data indicates the amount of total protein is higher in the hindgut than in the salivary gland/foregut and midgut tissues. Based on specific activity results endoglucanase, exoglucanase and β-glucosidase activity were highest in the salivary gland/foregut region. However, when the data was corrected for total gut protein concentration and represented as a “% of total activity”, endoglucanase and exoglucanase activity are highest in the hindgut followed by salivary glands/foregut and midgut tissues. In contrast, when correcting for % total activity, β-glucosidase activity was higher in the salivary glands/foregut and had a similar activity level in midgut and hindgut regions (Table 2-1).

In vitro Inhibition

The I₅₀ determinations for each inhibitor are shown in Figure 2-4. Cellulase activity assays indicate the monosaccharide FMG did not strongly inhibit cellulase activity. The disaccharides
CBI and FMCB, however, inhibited both exoglucanase and β-glucosidase activity in both gut regions. Cellbio-imidazole (I50s in nM - μM range) had a greater inhibitory effect than FMCB (I50s in mM range) and was more active against cellulase activities located in the salivary gland/foregut/midgut extracts. None of the three prototype inhibitors had a strong inhibitory effect against endoglucanase activity.

Discussion

This study assessed three sugar-based compounds and their potential as cellulase inhibitors for the subterranean termite, *R. flavipes*. In addition, baseline distribution of endoglucanase, exoglucanase and β-glucosidase activity was determined for salivary gland/foregut, midgut and hindgut regions. The conditions for conducting cellulase activity assays were optimized, and % total activity results indicate the majority of endoglucanase and exoglucanase activities are localized in the hindgut. This finding is in agreement with previous research and supports the notion that endoglucanase and exoglucanase enzymes are produced by hindgut protozoa in lower termite species and that a considerable portion of endoglucanase activity is endogenous and located in the salivary gland/foregut region (Breznak and Brune 1994, Watanabe et al. 1998, Zhou et al. 2007). β-glucosidase assays indicate this activity is both endogenous and symbiotic in origin since significant levels of β-glucosidase total activity were comprised in the salivary gland/foregut and hindgut regions and because specific activities within these two regions were significantly different from each other. Similar endogenous β-glucosidase distribution results have been reported for several termite species, including the lower termite *Neotermes koshunensis* Shiraki and the higher termite *Nasutitermes takasagoensis* Shiraki (Tokuda et al. 1997, Tokuda et al. 2002). Significant β-glucosidase activity has also been found in the hindgut of *R. speratus* Kolbe (Inoue et al. 1997).
The main goal of this investigation was to test three sugar-based compounds against *R. flavipes* workers and to assess whether they inhibit cellulases, *in vitro*. To our knowledge, the monosaccharide FMG and disaccharide FMCB have not been previously identified as cellulase inhibitors. Conversely, CBI is already known to non-competitively inhibit cellobiohydrolases (exoglucanases) in the cellulolytic fungi *Trichoderma reesei* and *Phanerochaete chrysosporium* (Vonhoff et al. 1999, Ubhayasekera et al. 2005). Results from the present study revealed CBI is also a strong inhibitor (nM-μM range) of termite cellulases and is particularly active against exoglucanase and β-glucosidase enzymes. Similarly, FMCB inhibited exo- and β-glucosidase activities, but was moderate (mM range) in comparison with CBI results. CBI and FMCB inhibited both endogenous (salivary gland/foregut/midgut) and symbiotic (hindgut) cellulases. However, FMCB did not show differences between the two gut tissues, while CBI had a more pronounced effect on endogenous exoglucanases than on symbiotic exoglucanases. Differences in inhibition efficiencies were previously reported and discussed for cellobiohydrolases of *T. reesei* and *P. chrysosporium* and were shown to be the result of structural differences within the sub-sites for both fungal cellulase enzymes (Ubbayasekera et al. 2005). Thus, the different inhibition efficiencies observed in this study may reflect structural differences between endogenous and symbiotic cellulases; however further research is needed to clarify this contention.

In conclusion, this study represented an important first step in identifying potential inhibitors that target the *R. flavipes* cellulase system. Specifically, this study showed that CBI is a strong inhibitor of termite and symbiont-produced cellulases, while FMCB and FMG are moderate to weak cellulase inhibitors, respectively. Moreover, this study supports previous research and further validates that *R. flavipes* cellulases are both endogenous and symbiotic in
origin (Zhou et al. 2007). Further research has investigated the effects of CBI, FMCB and FMG on termite feeding and mortality (See Chapter 3). Feeding bioassays characterized the biological effect of CBI, FMCB and FMG and tested for cellulase inhibition after bioassays were completed.
Figure 2-1. Chemical structure of fluoro-methyl glucose (FMG), fluoro-methyl cellobiose (FMCB) and cellobioimidazole (CBI).
Figure 2-2. Optimization conditions for CMC assays including (A) substrate concentration, (B) protein concentration, (C) assay time, (D) homogenization buffer, (E) assay temperature and (F) residual glucose. Arrows indicate the conditions used throughout CMC assays.
Figure 2-3. Optimization condition for pNPC and pNPG assays including (A) substrate concentration, (B) protein concentration, (C) assay time, (D) homogenization buffer. Arrows indicate the specific conditions used in pNPC and pNPG activity assays.
Table 2-1. Distribution of cellulase activity across the R. flavipes gut. Data points within row with the same letter are not significantly different by the LSD t-test (n = 3; df = 2; p<0.05). All ANOVAs were significant at p<0.05.

<table>
<thead>
<tr>
<th>Activity</th>
<th>Assay</th>
<th>Data Type</th>
<th>Foregut</th>
<th>Midgut</th>
<th>Hindgut</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>Pierce</td>
<td>mg / termite $^1$</td>
<td>9.9 ± 1.0</td>
<td>8.4 ± 0.6</td>
<td>20.4 ± 0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total Activity</td>
<td>1.2 b</td>
<td>1.0 b</td>
<td>2.4 a</td>
</tr>
<tr>
<td>Endoglucanase</td>
<td>CMC</td>
<td>nmol / min / mg $^2$</td>
<td>44.6 ± 6.4</td>
<td>13.0 ± 5.6</td>
<td>13.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ratio</td>
<td>3.4 a</td>
<td>1.0 b</td>
<td>1.0 b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>% of Total $^3$</td>
<td>39.8 ± 2.8 b</td>
<td>8.5 ± 3.6 c</td>
<td>51.7 ± 1.4 a</td>
</tr>
<tr>
<td>Exoglucanase</td>
<td>pNPC</td>
<td>mmol / min / mg $^2$</td>
<td>0.97 ± 0.24</td>
<td>0.30 ± 0.01</td>
<td>0.78 ± 0.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ratio</td>
<td>3.2 a</td>
<td>1.0 b</td>
<td>2.6 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>% of Total $^3$</td>
<td>21.3 ± 5.0 b</td>
<td>4.9 ± 0.4 c</td>
<td>73.8 ± 4.7 a</td>
</tr>
<tr>
<td>β-glucosidase</td>
<td>pNPG</td>
<td>mmol / min / mg $^2$</td>
<td>6.2 ± 0.7</td>
<td>2.8 ± 0.2</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ratio</td>
<td>9.8 a</td>
<td>4.4 b</td>
<td>1.0 c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>% of Total $^3$</td>
<td>56.4 ± 3.9 a</td>
<td>18.9 ± 1.9 b</td>
<td>24.8 ± 2.0 b</td>
</tr>
</tbody>
</table>

1. Total μg of protein per termite per gut region.
2. Specific activity for each substrate by gut region.
3. Specific activity corrected for total gut protein by gut region and then converted to a percentage.
Figure 2-4. I50 determination for each inhibitor tested on termite cellulases.
CHAPTER 3
EFFECTS OF THREE SUGAR-BASED CELLULASE INHIBITORS ON FEEDING AND MORTALITY OF Reticulitermes flavipes WORKERS

Introduction

Termites are important structural pests, causing an estimated global impact of $20 billion annually (Su 2002). Many pest species, including the widely-distributed Coptotermes and Reticulitermes spp., belong to the lower termite group. Lower termites rely on the production of both endogenous cellulases and symbiotic cellulases to digest cellulose, a major component in wood (Cleveland 1924, Watanabe et al. 1998). In lower termites, endogenous cellulases are localized in the salivary/foregut or midgut regions, while symbiotic cellulases are produced by anaerobic protozoa and restricted to the hindgut (Tokuda et al. 1999, Nakashima et al. 2002, Zhou et al. 2007). Through the action of cellulases, specifically endoglucanases, exoglucanases and β-glucosidases, termites are able to degrade cellulose chains into glucose. The collaboration of symbiotic and endogenous cellulases enables lower termites to efficiently digest cellulose and is the main reason why termites are considered important economic pests.

Current control options for subterranean termites include soil termiticides and baiting systems (Su and Scheffrahn 2000). Soil termiticides are liquid insecticides that are applied in large volumes along foundation perimeters. Liquid insecticides are often neurotoxins which elicit acute toxicity in insects. However, the environmental persistence of liquid termiticides has raised public health and environmental concerns. Bait systems are considered more environmentally sound and provide colony elimination through the action of chitin synthesis inhibitors or metabolic inhibitors; however, bait systems provide complete colony elimination more slowly than soil termiticides. In this respect, a faster and more environmentally friendly termite control method is needed.
Several studies have investigated the potential of sugar-based compounds as termite feeding stimulants and bait additives (Waller and Curtis 2003, Swoboda et al. 2004). Feeding stimulants could increase consumption of termiticides and provide faster colony elimination. However, compounds that act as both feeding stimulants and termiticides remain largely unidentified. Recently, Zhu et al. (2005) researched the potential of cellulase inhibitors as a novel termite control method. Cellulase inhibitors would decrease digestion efficiencies and could potentially elicit a compensatory feeding response and cause termite mortality. Thus, the goal of this study was to evaluate three prototype cellulase inhibitors against *R. flavipes* and assess their potential as novel termite control agents, as well as potential termite feeding stimulants. The inhibitors used were the disaccharides cellobioimidazole (CBI) and fluoro-methyl cellobiose (FMCB) and the monosaccharide fluoro-methyl glucose (FMG). The specific objectives for this study were to 1) conduct cellulase inhibitor bioassays that assess the impact of cellulase inhibitors on the feeding and survivorship of *R. flavipes* workers, 2) conduct mono- and disaccharide bioassays to further verify the feeding and mortality results from inhibitor bioassays and 3) verify cellulase inhibition via cellulase activity assays. This study provides novel data indicating after 24-day feeding bioassays the disaccharide-based inhibitor CBI inhibits exoglucanase and β-glucosidase activity and causes moderate termite mortality.

**Material and Methods**

**Termites**

Termite colonies were collected from nearby field sites at the University of Florida campus. Colonies were identified as *Reticulitermes flavipes* using a PCR-RFLP identification key (Szalanski et al. 2003), and were held in plastic containers provisioned with moist, brown paper towels and pine shims. Termite colonies were held in darkness at approximately 22°C and
70% RH. Colonies were allowed to acclimate to laboratory conditions for at least one month before including them in these experiments.

**Cellulase Inhibitor Bioassays**

Cellulase inhibitor bioassays were modeled after previous caste differentiation assays described by (Scharf et al. 2003b, Zhou et al. 2007), and consisted of sets of 15 worker termites in plastic petri dishes (10 x 15 mm, Nunc Inc., Naperville, IL) with treated paper disks (Georgia-Pacific). Worker termites were used because of their status as primary wood consumers and because cellulase gene and protein expression are highest in the worker caste (Scharf et al. 2003a, Scharf et al. 2005). One termite colony was used to test the effects of CBI and FMG\(^1\) and a different colony was used to repeat the FMG assay and to test the effects of FMCB. The CBI and FMG bioassays were conducted in May of 2006, while the FMG and FMCB bioassays were carried out in July of 2006.

High-purity CBI, FMCB and FMG were synthesized by Carbohydrate Synthesis Ltd (Oxford, UK). Different concentrations for each inhibitor were prepared using reagent-grade methanol as a solvent. The concentrations prepared were 75, 50, 25, 10, 5, 1, 0.5, and 0.1 mM (approximately 3, 2, 1, 0.4, 0.2, 0.04, 0.02 and 0.004 % wt/wt). Paper disks were pre-weighed and then treated with 50 μl of a given inhibitor concentration. Controls consisted of filter paper disks treated with 50 μl of methanol. Treated paper disks were placed in a fume hood for approximately 30 m before including them in bioassays. Treatments and controls were held in complete darkness at approximately 27°C and 70% RH. For each inhibitor, three replicate dishes of 15 termites each were assayed per concentration.

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\(^1\) The colony replicate for CBI and FMG was done by Dr. M.E. Scharf and Dr. X. Zhou.
All assays were carried out for a total of 24 days. Termite mortality and filter paper moisture were monitored every 4th day. Every 8th day, paper disks were replaced with a new, treated disk. To estimate paper consumption, paper disks were dried and re-weighed. After 24-day feeding bioassays surviving termites were frozen at -20°C for post-inhibition studies.

**Validative Bioassays with Mono- and Disaccharides**

Mono- and disaccharide bioassays were conducted to further validate the results from feeding bioassays. Three different sugars were chosen based on structural similarity to the cellulase inhibitors used in feeding bioassays including glucose (Fisher Scientific), D(+) -maltose monohydrate (90%) and D(+) -cellobiose (98%) (Acros Organics). Glucose is a monosaccharide similar in structure to FMG, while maltose and cellobiose are alpha and beta-linked disaccharides, respectively, similar to both CBI and FMCB. Mono- and disaccharide bioassays were completed in a similar manner as feeding assays; however due to differences in solubility, mono-and disaccharides sugars were dissolved in dH₂O instead of methanol. Sugar bioassays consisted of three replicate dishes per concentration and were replicated across two different termite colonies. The first colony replicate was carried out in August of 2006 and the second in October of 2006.

**Post-feeding Inhibition**

Cellulase activity assays tested for cellulase inhibition in termites surviving the 24-day feeding bioassays. Cellulase activity assays were adapted from Han et al. (1995) and optimized for a 96-well microplate format. Whole-body homogenates were used as an enzyme source and were prepared using a motorized Teflon-glass tissue homogenizer. Homogenization was carried out by using 10 termites/ml of homogenization buffer. After homogenization, preparations were centrifuged at 14,000 rpm at 4°C for 15 min and then filtered with glass wool to remove excess
lipids. The protein concentration in each sample was determined using a commercially available bicinchoninic acid assay (Pierce; Rockford, IL) with bovine serum albumin as a standard.

The model substrates used to estimate endoglucanases, exoglucanases and β-glucosidase activity were carboxy-methyl cellulose (CMC), p-nitrophenol cellobioside (pNPC), and p-nitrophenol glucopyranoside (pNPG), respectively. All three substrates were diluted in homogenization buffer (0.1 M sodium acetate, pH of 5.8). The substrate concentration for CMC assays was 0.5%. A 4 mM substrate concentration was used for pNPC and pNPG assays.

CMC assays were carried out as endpoint assays and were incubated at 32°C, for 30 minutes. After incubation, the reaction terminated with the addition of 100 μl of 1% 3, 5-dinitrosalicylic acid (DNSA), 30% sodium potassium tartrate, and 0.4 M NaOH to each sample. The reaction was then fixed by placing the microtiter plate in a 100°C water bath for 10 minutes. To achieve color formation microtiter plates were placed on ice for 15 minutes. Absorbance was measured at 520 nm and compared to a glucose standard curve.

In pNPC and pNPG assays, enzyme and substrate reacted for 20 minutes at 32°C, before being read kinetically at 420 nm at room temperature. pNPG assays were read for a total of 1 h, while pNPC assays were read for 1 h and 30 m. An absorbance reading for each sample was read every 2 m and mean velocity results were used as activity data.

**Data Analysis**

At the end of each assay, the data were summarized as cumulative feeding and cumulative termite mortality. The cumulative feeding and mortality for each concentration were converted into a percentage of feeding relative to that of untreated (MeOH) controls. The FMG data for both termite colonies was compiled and averaged into a single data set, as well as analyzed separately to observe colony variation. Data for cellulase inhibitor bioassays, post-feeding inhibition and mono/disaccharide assays were analyzed using SAS (SAS Institute; Cary, NC).
(p<0.05). A one-way analysis of variance (ANOVA) was used to analyze cellulase inhibitor bioassays and post-feeding inhibition data. The exoglucanase (pNPC) and β-glucosidase (pNPG) assays were analyzed on ranked data because untransformed data did not meet the assumptions of ANOVA. A two-way ANOVA was used for mono/disaccharide data. Mean separation was carried out using Tukey-Kramer HSD and Student-Newman-Keuls (SNK) methods.

Results

Cellulase Inhibitor Bioassays

This research investigated the effect three potential cellulase inhibitors had on termite filter paper consumption and termite mortality. The monosaccharide FMG did not stimulate filter paper consumption at any concentration and had significant inhibitory effects throughout the 25-75 mM concentration range \((F= 7.14, \ df= 8, 51, P< 0.0001)\) (Fig.3-1). However, the disaccharide CBI had both inhibitory and stimulatory feeding effects \((F= 6.48, \ df =8, 24, P= 0.0002)\). The inhibitory effect occurred at the lowest and the highest concentrations, with significant inhibition at the 75 mM concentration. A stimulatory effect for CBI occurred at midrange inhibitor concentrations and was significant at the 5 mM concentration. A similar inhibitory and stimulatory feeding effect occurred in FMCB bioassays; however the results were not significant at any inhibitor concentration.

Overall, FMCB caused greater cumulative termite mortality than CBI and FMG (Fig. 3-1). The highest FMCB-induced mortality was associated with the highest concentrations, with 75 mM causing a mortality rate greater than 40%. Similarly, the mortality observed in FMG replicates was concentration-dependent. However, the highest FMG concentration reached a maximum of approximately 20% mortality (combined colony results) and was not significantly different from methanol-treated controls. Although not significant, CBI-induced mortality was
the highest throughout the same midrange concentrations that were associated with feeding induction.

**Validative Bioassays with Mono- and Disaccharides**

Mono- and disaccharide bioassays were carried out to further define the effects of cellulase inhibitor bioassays on termite feeding and mortality. No significant feeding stimulation was observed in any of the sugar bioassays (Fig. 3-2). However, a significant inhibitory effect occurred in the 10 mM treatment in the maltose bioassay \( (F = 3.64, \text{df}=8, 41, P = 0.0028) \). No significant termite mortality was observed in glucose \( (F = 1.54, \text{df}=8, 44, P = 0.1711) \), cellobiose \( (F = 1.41, \text{df}=8, 43, P = 0.2206) \) or maltose \( (F = 1.06, \text{df}=8, 41, P = 0.4120) \) bioassays.

**Post-feeding Inhibition**

The most pronounced inhibition occurred for exoglucanase and β-glucosidase activities after exposure to CBI \( (F = 18.64, \text{df}=8, 30, P < .0001) \) (Fig. 3-3). Inhibition by CBI occurred in a dose-dependent manner with the highest concentrations causing the greatest inhibition (70-90%). By comparison, FMG and FMCB did not strongly inhibit cellulase activity after feeding. FMG showed the greatest impact on termites treated with 10 mM concentration. At this concentration, only the endoglucanase activity was significantly affected. FMCB showed a significant β-glucosidase inhibition at the 0.1 and 75 mM concentrations.

**Discussion**

The eastern subterranean termite, *R. flavipes*, is a major pest species within the United States (Su and Scheffrahn 1990). The *R. flavipes* cellulase system is comprised of both endogenous and symbiotic cellulases (Zhou et al. 2007). This system is considered a potential target site for novel termite control agents including cellulase inhibitors (Zhu et al. 2005). The main objective of this study was to test the effects of three prototype cellulase inhibitors against *R. flavipes* workers. The results from cellulase inhibitor bioassays indicate that CBI, and to a
lesser extent FMCB, caused both stimulatory and inhibitory feeding effects, while FMG largely elicited an inhibitory effect. Feeding stimulation in the inhibitor bioassays may be a compensatory feeding response, where termites increase feeding to compensate for a decrease in cellulose-digesting efficiency and subsequent nutritional deprivation. This contention was supported by disaccharide bioassays and post-feeding inhibition assays (for CBI only). Although many sugars are considered termite phagostimulants (Waller and Curtis 2003), validative mono/disaccharide bioassays suggest that stimulatory feeding is not a generalized response to disaccharides and is instead a specific response induced by CBI and FMCB. Swoboda et al. (2004) described a similar result, where sugars including several disaccharides did not elicit a stimulatory response throughout non-choice feeding bioassays.

Post-feeding bioassays showed that for CBI, cellulase inhibition occurred throughout concentrations that caused stimulatory feeding. However, there was no correlation between concentrations that elicited feeding stimulation and those which had the highest inhibition percentage. Therefore the highest CBI concentrations, may act as feeding deterrents, while at midrange concentrations of CBI may act as a feeding stimulants, indicating an interaction between inhibitor palatability and concentration. Additionally, CBI inhibitor bioassays indicate that termite mortality was highest at midrange concentrations suggesting a correlation between feeding stimulation and termite mortality.

Overall the highest percentage of termite mortality was observed for FMCB. However, post-feeding inhibition assays and previous in vitro studies (Chapter 2) show CBI as being the most potent cellulase inhibitor. This discrepancy is likely the result of colony variation. FMCB and CBI were tested using two different termite colonies: a field colony was collected and acclimated to laboratory conditions for 1 month and a laboratory colony which had acclimated to
laboratory condition for > 1 year (respectively). Consequently, FMCB and CBI mortality results may not be directly comparable, since termite colonies are known to vary in their physiological status and thus may respond differently to treatment. This occurrence was noted by Zhu et al. (2005), and in the present study was supported by FMG mortality results, in which the field colony showed significantly higher mortality than the laboratory colony (Fig. 3-4). Therefore, CBI may have shown mortality comparable to that of FMCB if assayed using the same field colony. Unfortunately, the cost and limited availability of each inhibitor played a major role in bioassay succession and colony replication.

In summary, this study shows that the disaccharide-based inhibitor CBI caused significant stimulatory feeding in termites at midrange inhibitor concentrations. This feeding stimulation resulted in greater inhibitor intake and caused moderate termite mortality (~20%). Bioassays involving mono- and disaccharides confirmed that stimulatory feeding is a specific response elicited by CBI. These observations suggest that stimulatory feeding may be the result of a decrease in cellulose digestion efficiency. However, cellulase “inhibition” in termites may occur because of enzyme inhibition, in which there is a direct interaction between inhibitor and enzyme, or due to cellulolytic symbiont death. Thus, future studies should also clarify how enzyme inhibition is achieved: through either symbiont death or enzymatic inhibition. In addition, subsequent bioassays that focus on midrange CBI and FMCB concentrations are necessary to confirm feeding and mortality results, as well as an assessment of colony to colony variation.
Figure 3-1. Effects on feeding (TOP) and mortality (BOTTOM) by three prototype cellulase inhibitors FMG (A, B), CBI (C, D) and FMCB (E, F). Results are shown as percentage relative to methanol-treated controls, (*) indicates a significant difference between treatments and controls.
Figure 3-2. Results from mono- and disaccharide bioassays. (A) Shows cumulative feeding results after 24 days for glucose, maltose and cellobiose, while (B) shows cumulative mortality for the three sugars. Cumulative feeding and mortality results are shown as a percentage of water-treated controls, (*) indicates a significant difference between treatments and water-treated controls.
Figure 3-3. Post-feeding inhibition results for FMCB, FMG and CBI. (*) indicates a significant difference between treatment and controls. See Appendix A for averaged results.
Figure 3-4. Comparison of field and laboratory colonies used to test FMG. The lab colony was kept under laboratory conditions for $> 1$ year and the field colony was held under laboratory conditions for $< 1$ month.
CHAPTER 4
MOLECULAR AND BIOCHEMICAL MARKERS FOR MONITORING DYNAMIC SHIFTS OF CELLULOLYTIC PROTOZOA IN Reticulitermes flavipes

Introduction

The mutualism between lower termite species and a variety of symbiotic protozoa has been of interest to termite researchers since early studies done by Cleveland (1924). Symbiotic protozoa have long been known to be important to the process of termite cellulose digestion. Namely, protozoan species produce cellulases, which are enzymes capable of cleaving the 1,4 β-D-glucosidic linkages in cellulose (a major component of wood). In recent years, additional advances have been made in characterizing the cellulase system of many lower termite species, including Reticulitermes flavipes Kollar, R. speratus Kolbe and Coptotermes formosanus Shiraki (Inoue et al. 1997, Nakashima et al. 2002, Zhou et al. 2007).

The cellulase system in lower termites is known to involve both endogenous and symbiotic cellulases (Watanabe et al. 1998, Nakashima et al. 2002, Tokuda et al. 2005, Zhou et al. 2007). Unlike symbiotic cellulases which are confined to the hindgut, endogenous cellulases are produced and found in the salivary glands or the midgut, depending on termite family (Tokuda et al. 1999, Zhou et al. 2007). This study investigated the cellulase system of the lower termite R. flavipes, which is one of the most destructive termite species in the United States. The R. flavipes cellulase system, like that of all other lower termite species, is known to be comprised of three main types of enzymes including endoglucanases, exoglucanases and β-glucosidases. These enzymes degrade cellulose by cleaving different linkages along the cellulose chain. Endoglucanases internally hydrolyze glucosidic linkages while exoglucanases terminally cleave cellulose units from the ends of cellulose chains. β-glucosidases release glucose by hydrolyzing β-D-glucose residues from cellobiose and cellotriose units (Breznak and Brune 1994).
Thus far, the full-length protein coding regions of four cellulase genes have been isolated from R. flavipes, including an endogenous (Cell-1) and three symbiotic cellulases (Cell-2, Cell-3 and Cell-4). The endogenous Cell-1 and the symbiotic Cell-2 are classified as endoglucanases, while Cell-3 and Cell-4 are exoglucanases. In R. flavipes, endogenous endoglucanase activity is mainly localized in the salivary glands and symbiotic cellulase activity is localized in the hindgut (Zhou et al. 2007).

The process of cellulose degradation can sustain nearly 100% of lower termite metabolism (Breznak and Brune 1994). Thus, given the dependence of lower termites on wood, the lower termite cellulase system is considered a potential target for termite control agents (Zhu et al. 2005, Zhou et al. 2007). However despite the major role of protozoa in the lower termite cellulase system, to date there are few techniques beyond actual protozoan counts available to monitor the effects that novel termite control agents could have on cellulolytic protozoan populations. Although protozoan counts provide a quantitative and direct measure of protozoan populations, they are also time-consuming and labor intensive. Therefore, the main goal of this investigation was to develop an effective, alternative method to monitor fluctuations of cellulolytic termite protozoa. Specifically, the objectives of this study were to (1) use UV irradiation to remove hindgut protozoa from R. flavipes workers and to test whether (2) quantitative real time-PCR (qRT-PCR) and/or cellulase enzyme assays can be used to monitor changes in termite cellulolytic protozoan populations. In addition, an estimate of endoglucanase, exoglucanase and β-glucosidase stability in vitro was also included. Our results suggest that qRT-PCR is a viable method to monitor shifts in cellulolytic protozoa populations and verify specifically that the R. flavipes genes Cell-2, Cell-3, and Cell-4 are symbiont-derived.
Materials and Methods

Termites

Termite colonies were collected from the University of Florida campus (Gainesville, FL, USA). Each colony was placed in a sealed plastic container and supplied with moist, brown paper towels and wooden shims (pine) as food. The termite colonies were held in complete darkness at approximately 22°C and 70 % RH for at least a month before including them in these experiments.

Ultraviolet Irradiation

UV irradiation was successfully used as a defaunation method in R. speratus by Inoue et al. (1997). Their irradiation protocol was applied to this study with minor modifications. Prior to UV irradiation, termites were fed moistened cellulose powder (Sigma-Aldrich) for 48 h, in order to make hindgut contents more accessible to UV light. Thirty worker termites were irradiated (375.5nm, Gelman Sciences Inc., Model No. 51438) for 0, 1, 2, 3, or 4 hours. The UV light was placed at a distance of approximately 9 cm away from the dishes. In order to ensure that termites would be evenly exposed to the UV light, the 30 termites were divided into 3 Petri dishes (10x15mm, Nunc) and were lined up under the UV light, exposed, then transferred to Petri dishes provisioned with moistened filter paper disks for 24 h. After the 24 h period elapsed, termites that were exposed for the same time interval were recombined (n=30). Five termites were used immediately for protozoan counts while the remaining 25 were divided roughly in half and stored at either -80°C for quantitative real-time PCR analysis or at -20°C for cellulase enzyme assays. Treatments were replicated across 3 different R. flavipes colonies.

Protozoan Counts

In order to estimate the number of protozoa in each termite hindgut, a total of five termites for each time interval were dissected per replicate. Dissections were carried out by
immobilizing each termite on ice, gently holding the head and then extracting the hindgut by pulling on the last abdominal segments. The protocol used for protozoan counts was described by Lewis and Forschler (2004) with minor modifications. Protozoan counts were conducted by placing each hindgut into a microcentrifuge tube containing 100 μl of ice-cold 1x phosphate buffered saline (PBS). The gut was homogenized for approximately 15 s with a sterile toothpick, then 10 μl of the hindgut homogenate were loaded onto a hemacytometer (Bright-line Hemacytometer, Fisher Scientific, Pittsburgh, PA) and the number of protozoa was examined by counting 0.4 μl under 400 X magnification using a Leitz Laborlux S microscope.

**Quantitative Real time-PCR**

In order to test for changes in cellulase gene expression, total RNA from whole termites was extracted using the SV total RNA isolation kit (Promega; Madison, WI). The quantity of total RNA was determined through spectrophotometry and then converted to cDNA using the iScript™ cDNA synthesis kit (Bio-Rad; Hercules, CA). Quantitative real time-PCR was performed using an iCycler iQ real time detection system (Bio-Rad) with SYBR Green® Supermix (Bio-Rad). qRT-PCR was used to investigate gene (mRNA) expression of four cellulase genes (*Cell-1, Cell-2, Cell-3, and Cell-4*), as well as β-actin which was included as a reference gene. Validation of these target and reference genes, including comparisons of PCR amplification efficiencies, was described in a previous report (Zhou et al. 2007). Cellulase gene transcript abundance was chosen to specifically monitor changes in cellulolytic protozoan populations, rather than as-yet undefined genomic DNA sequence markers. The forward and reverse primer sequences used were: *Cell- 1 RT* (5’-TCACAAGCAAGCAGGCTAC-3’ and 5’-ATGAGGCAGAATTGCAGC-3’), *Cell-2 RT* (5’-CCAATGGGGATGTTACAAGG-3’ and 5’-CAACTCATCCCATCGGAATC-3’), *Cell-3 RT* (5’-GCTGGAAACCAGGGCAAT-3’ and 5’-ACTGTGTACGCTGGGAAAC-3’), *Cell-4 RT* (5’-GCTGGGGGTGTTATTCATTC-3’ and 5’-
CTTCGAGCAAGCATGAACTG-3'), and \( \beta\)-actin (5’-AGAGGGAAATCGTGCGTGAC-3’ and 5’-CAATAGTGATGACCTGGCCGT-3’). The relative expression levels for \textit{Cell-1}, \textit{Cell-2}, \textit{Cell-3} and \textit{Cell-4} in relation to the reference gene, \( \beta\)-actin were determine by the \( 2^{-\Delta\Delta CT} \) method (Livak and Schmittgen 2001). For each gene, three PCR reactions were performed per treatment per colony.

**Cellulase Activity Assays**

**Tissue preparation**

To test whether UV defaunation had an impact on cellulase enzyme activity, termites stored at -20°C were first homogenized using a motorized Teflon-glass tissue homogenizer. The homogenization buffer was 0.1 M sodium acetate (pH 5.8). This buffer was used in tissue preparations and cellulase enzyme assays. Whole-body homogenates were centrifuged at 14,000 rpm at 4°C for 15 min. In order to remove excess lipids, the clear supernatant from each sample was removed carefully, avoiding the lipid layer and placed into new microcentrifuge tubes. This product was used as an enzyme source in the cellulase assays described below. To estimate the protein concentration for each sample a bicinchoninic acid assay (Pierce, Rockford, IL) was used with bovine serum albumin as a standard.

**Substrate preparation**

To measure endoglucanase, exoglucanase, and \( \beta\)-glucosidase activity, the model substrates used included carboxy-methyl cellulose (CMC), \( p\)-nitrophenol cellobioside (pNPC), and \( p\)-nitrophenol glucopyranoside (pNPG), respectively. Stock solutions of each material were prepared in methanol. All three substrate stocks were diluted in homogenization buffer immediately before assays. The final substrate concentration used for the CMC-based endoglucanase assays was 0.5% (w/v), while a 4 mM substrate concentration was used for the pNPC and pNPG assays.
Cellulase activity assays

The protocol for endoglucanase, exoglucanase, and β-glucosidase assays was modified from Han et al. (1995) and optimized for a COStar® 96-well microtiter plate (Corning Inc.; Corning, NY) and a microplate spectrophotometer. Other conditions such as protein and substrate concentration, assay time, and buffer pH were previously (Chapter 2). All three assays were carried out by placing 10 μl of enzyme extract and 90 μl of buffer+substrate in each sample well. CMC-endoglucanase assays are endpoint assays in which the microtiter plate was placed in an incubator at 32°C for a total assay time of 30 minutes. The reaction was stopped by adding 100 μl of 1% 3, 5-dinitrosalicylic acid (DNSA), 30% sodium potassium tartrate, and 0.4 M sodium hydroxide to each sample well. To stop any remaining enzymatic activity, the microtiter plate was placed in a 95°C water bath for 10 minutes and then cooled on ice for 15 minutes to allow color formation. The plate was read at 520 nm using the endpoint setting. The absorbance readings, relative to a glucose standard curve, were used to calculate the specific activity.

pNPC and pNPG assays are kinetic assays which measure the release of p-nitrophenol. These assays were carried out by allowing the enzyme and buffer + substrate mixtures to react for 20 minutes at 32°C before being read at 420 nm, at room temperature. pNPC assays were read every 2 min for a total of 1 h and 30 min, while pNPG assays were read every 2 min for 1 h. The mean velocity results from pNPC - exoglucanase and pNPG - β-glucosidase assays were used to estimate specific activity. For each cellulase assay, activity was estimated from three reactions per treatment per colony.

In vitro Cellulase Stability Estimate

Cellulase protein stability was determined in vitro using three separate preparations of whole-body homogenates from the same termite colony. Whole-body homogenates were held at 27°C for a total of 16 days. At 0, 2, 4, 6, 8 and 16 days, 130 μl of each preparation were removed
and frozen at -20°C. Endoglucanase, exoglucanase, and β-glucosidase activity for each time interval were tested using CMC, pNPC and pNPG assays, which were carried out as described in the previous section. In each cellulase activity assay, each preparation was assayed in triplicate per time interval.

**Statistical Analyses**

Statistical analyses were carried out using SAS (SAS Institute; Cary, NC). Protozoan counts, qRT-PCR results and cellulase activity assay data did not meet the assumptions for an analysis of variance (ANOVA). Thus, the data were ranked and a two-way ANOVA (Conover and Iman 1981) was used to analyze gene expression and enzyme activity results (with blocking on colony, and UV-treatment-time as the main factor). A one-way ANOVA on raw cellulase activity data was used to analyze cellulase stability results. Means for all three data sets were separated using the Student-Newman-Keuls procedure. Linear regression analysis was used to test for correlations of protozoan count results with qRT-PCR cellulase gene expression data. Linear regression analyses were performed using the PROC REG procedure in SAS.

**Results**

**Protozoan Counts**

A photomicrograph displaying several protozoa species observed during gut dissection are shown in Figure 4-1. The results for the protozoan counts are summarized in Figure 4-2. UV irradiation significantly reduced the number of protozoa in a time/dose-dependent manner ($F = 14.55$, df = 4,68, $P <0.0001$). Numbers of protozoa recovered after 0 and 1 h of irradiation were significantly greater than numbers of protozoa recovered after 2, 3, and 4 h of irradiation. The smallest decrease was observed after 1 h of UV exposure, where protozoan populations were reduced by an average of 24%. After 2, 3, and 4 h of UV irradiation 42, 87 and 93 % reductions were observed, respectively.
**Quantitative real-time PCR**

In addition to reducing symbiont populations, UV irradiation significantly reduced the expression of symbiotic cellulases, *Cell-2* (*F*=43.12, df=4,37, *P*<0.0001), *Cell-3* (*F*=72.16, df=4,35, *P*<0.0001) and *Cell-4* (*F*=161.06, df=4,38, *P*<0.0001) (Fig. 4-3B-D), but did not cause a decrease in the endogenous cellulase, *Cell-1* (*F*= 2.96, df=4,38, *P* = 0.03) (Figure 4-3A). None of the symbiotic cellulase genes showed significant reductions in gene expression after 1 h of UV treatment. However, all three symbiotic cellulase genes showed a sharp decrease in expression after 2 h of UV exposure (Fig. 4-3B-D). The greatest decline in cellulase expression was observed in *Cell-4* (Fig. 4-3D) which showed a reduction of approximately 95% after 4 h of irradiation. Interestingly, a slight increase of *Cell-1* expression was observed for all four UV treatments, but only the 2 h treatment was significantly different from untreated controls (Fig 4-3A). The results for qRT-PCR data were confirmed by viewing PCR products by electrophoresis on 2% agarose gels (Figure 4-3E).

**Regression analysis**

Linear regression models comparing protozoan count results and qRT-PCR/gene expression data were significant for *Cell-2*(*P*=0.0036), *Cell-3*(*P*=0.0086), and *Cell-4* (*P*=0.0070) but were not significant for *Cell-1*(*P*=0.20). R² values for *Cell-2*, *Cell-3*, and *Cell-4* were 0.490, 0.450, and 0.440, respectively; while the r² value for the endogenous *Cell-1* was 0.118 (Fig. 4-4).

**Cellulase Activity Assays**

Overall, the results for CMC (*F*=1.61, df=4,38, *P*= 0.19), pNPG (*F*=0.31, df=4,37, *P*= 0.87) and pNPC (*F*=2.52, df=4,38, *P*= 0.56) assays did not show significant differences in cellulase enzyme activity across the 0-4 h UV treatment times. These results are consistent for both whole-body homogenates, as well as CMC and pNPG assays on termite foregut + midgut and hindgut dissections. However, hindgut dissections showed a significant decrease in
exoglucanase activity after 3 and 4 h of UV irradiation. The results for whole-body homogenates are shown in Fig. 4-4 and indicate no statistically significant differences in endoglucanase, β-glucosidase, or exoglucanase activity following UV treatment and a 24 h post-treatment period.

**Cellulase Stability Estimate**

A cellulase stability estimate was included to clarify cellulase activity results and investigate the length of time at which cellulase enzymes remain active, *in vitro*. This experiment indicates that all three types of cellulases were significantly impacted (~ 20%) after whole-body preparations were incubated at 27 °C for 2 d (Fig. 4-6) (P <0.0001). The half-life for all three cellulases occurred after 4 d of incubation. The most pronounced effect was observed for exoglucanases which showed a sharp decline in activity after 4 d and had close to 0% activity remaining after 16 days. β-glucosidases and endoglucanases showed a similar decline after 4 days but still retained over 20% of activity after 16 d. Therefore, these results may partially explain why a significant decline in cellulase activity was not observed after UV treatment since a considerable portion of cellulase activity remained after 24 h of incubation at 27 °C.

**Discussion**

The lower termite *R. flavipes* is one of the most destructive termite species within the continental United States. Through recent efforts, significant progress has been made to begin to characterize the cellulase system in this species. Several protozoan species are known to play a central role in the *R. flavipes* digestive system, contributing endoglucanase and exoglucanase cellulase enzymes (Zhou et al. 2007). In this study, UV irradiation was used as a defaunation method to remove protozoan symbionts from *R. flavipes* hindguts. This method was previously described as an effective defaunation method for *R. speratus* (Inoue et al. 1997). The current study indicates that UV irradiation is indeed capable of removing hindgut protozoa from *R. flavipes*, but only at longer exposure times than reported by Inoue et al. (1997). In the present
study a wavelength of 375.5 nm, which is just outside the visible light spectrum, was used to minimize the impact of UV irradiation on termites. At this wavelength, protozoa were removed in a time/dose-dependent manner, with 4 h of irradiation being most effective. However, UV irradiation apparently did not adversely impact termite hosts. The expression profile of two endogenous termite genes, Cell-1 and β-actin, corroborates the minimal visible impact of UV irradiation on *R. flavipes* workers. The effect of UV irradiation on bacterial communities was not investigated, primarily because the contribution of bacteria to cellulose digestion in lower termites is thought to be insignificant (Breznak and Brune 1994), and because this was not a research objective in this study.

The main objective of this research was to determine whether qRT-PCR and/or cellulase enzyme assays could be useful tools in monitoring dynamic shifts in the cellulolytic hindgut protozoa of *R. flavipes*. For qRT-PCR work, cellulase genes were chosen to specifically monitor declines in cellulolytic protozoa. However, it is likely other genes, such as small subunit ribosomal genes, would be better suited to monitor protozoan populations in general. Nevertheless, this report shows that, after UV treatment, mRNA expression of the symbiotic cellulase genes *Cell-2, Cell-3* and *Cell-4* correlates with decreases in protozoan populations. Thus, symbiotic cellulase gene (mRNA) expression and qRT-PCR can be used to effectively monitor dynamic shifts in cellulolytic protozoan populations. Not surprisingly, no correlation was observed between protozoan counts and expression of *Cell-1*, which is not symbiotic in origin (Zhou et al. 2007). Interestingly, the results for *Cell-1* instead show a slight increase in *Cell-1* expression particularly for the 2-hr UV treatment (Fig 4-3A). This determination suggests that endogenous cellulases may possibly compensate for a decline in protozoan populations; however, more research is needed to confirm this possibility.
No correlation was observed between cellulase enzyme activity and shifts in protozoan populations. In particular, in enzyme assays, no significant decrease in activity was observed despite significant UV-induced reductions in symbiont populations. These results were consistent in endoglucanase and β-glucosidase assays on both dissected termite guts and whole-body homogenates. However, termite hindgut homogenates did show a significant decrease in exoglucanase activity after 3 and 4 h of UV irradiation. This exoglucanase activity result is in agreement with gene expression data, as well as our determination that exoglucanase activity is mostly derived from hindgut symbionts. Thus, one possible explanation for the stable activity observed after UV defaunation is that endogenous activities mask those that are symbiont-derived. Protein stability may also at least partially explain the lack of decline in enzyme activity after UV defaunation. Two observations support such a “protein stability” hypothesis. First, despite there being some agreement with gene expression and defaunation counts, hindgut-specific exoglucanase activity only showed a ~30% decrease after UV defaunation. Second, in vitro cellulase stability estimates indicate only a slight decline in enzyme activity of ~20% after a 2-day incubation period at 27 °C. Thus, due to (i) masking effects by endogenous enzymes and (ii) cellulase protein stability, cellulase activity assays are apparently not the best-suited method for monitoring dynamic shifts in hindgut protozoan populations in R. flavipes. For this purpose, quantitative real-time PCR may be the better-suited monitoring tool.

Exoglucanases genes and proteins would likely be the most accurate indicators of changes in cellulolytic protozoan populations. Exoglucanase activity showed the most pronounced decline after defaunation, and as stated previously, the majority of exoglucanase activity is thought to be symbiotic (Zhou et al. 2007). Inoue et al. (1997) did observe a decrease in cellulase activity after 24 h in UV-treated R. speratus workers. However, in the case of Inoue
et al. a different UV wavelength, irradiation time, and termite species were used relative to the current study. It is possible that, in the present study, a greater decrease in cellulase activity could have been observed if a different defaunation methodology were used, such as a different UV wavelength, different irradiation times, or a different method of defaunation altogether. Other alternative defaunation methods include temperature (Cleveland 1924, Yokoe 1964), starvation (Cleveland 1925, Inoue et al. 1997), and oxygenation (Cleveland 1925).

In summary, this study establishes (1) that UV irradiation can be used as a defaunation method for *R. flavipes* workers (2) that qRT-PCR is a reliable monitoring technique for cellulolytic symbiont populations and (3) that cellulases in whole-body homogenates are stable for up to 2 days at 27 °C. This research also corroborates previous studies characterizing the cellulase system of *R. flavipes* by verifying that previously identified *Cell-2, Cell-3* and *Cell-4* genes are indeed symbiont-derived (Zhou et al. 2007). The findings of this research can be applied in future high-throughput screens of novel termite control agents such as cellulase inhibitors, and in investigating whether inhibitor modes action include eradication or reduction of cellulolytic protozoa. qRT-PCR can also be used to quickly define the effects that different diets, starvation, or other external factors such as temperature might have on protozoan populations. Future research will use both qRT-PCR and cellulase activity assays to investigate the refaunation process in the *R. flavipes* hindgut post-UV treatment, as well as the impacts of novel cellulase inhibitors on gut fauna.
Figure 4-1. Different protozoa species in the *R. flavipes* hindgut. Photographs were taken using differential interference microscopy. Tentative species identification for each photograph is (A) *Dinenympha gracilis*, (B) *Dinenympha fimbriata*, (C) *Pyrsonympha vertens* and (D) *Trichonympha agilis*.
Figure 4-2. The impact of UV irradiation on protozoan populations. (A) Micrographs showing decreases in protozoan populations, post-UV treatment. (B) Table showing protozoan counts results with associated standard errors, as well as results summarized as a percentage relative to untreated controls. Means with different letters are significantly different based on the Student-Newman-Keuls test (p < 0.05).
Figure 4-3. The effect of UV irradiation on cellulase gene expression. (A) Results for endogenous Cell-1; and (B-D) results for symbiotic Cell-2, Cell-3 and Cell-4. The error bars represent the standard error of the mean. (E) Visual confirmation of qRT-PCR results, after 29 PCR cycles are shown on a 2% agarose gel (9 μl of sample and 2 μl of loading buffer).
Figure 4-4. A regression model showing the correlation between protozoan counts and cellulase gene expression. (A) Shows the results for endogenous Cell-1 and (B-D) show results for symbiotic Cell-2, Cell-3 and Cell-4.
Figure 4-5. Impact of UV irradiation on cellulase enzyme activity. (A-C) show the results for endoglucanase, exoglucanase and β-glucosidase activity, respectively.
Figure 4-6. An estimate of exoglucanase, endoglucanase and β-glucosidase stability in worker termite whole-body homogenates after incubation at 27 °C for a total of 16 days.
The main objective of this research was to assess the potential of three carbohydrate-based compounds as novel inhibitors of termite cellulases. The three compounds tested were the monosaccharide fluoro-methyl glucose (FMG) and the disaccharides fluoro-methyl cellobiose (FMCB) and cellobioimidazole (CBI). The efficacy of each compound was tested using 

\textit{in vitro}, cellulase activity assays, feeding bioassays, as well as post-feeding cellulase activity assays.

\textit{In vitro} inhibition results indicated that the disaccharides CBI and FMCB were strong (nM range) to moderate inhibitors of exoglucanase and $\beta$-glucosidase activities. In contrast, FMG was not an effective inhibitor of any cellulase activities tested. Similarly in post-feeding cellulase activity assays, CBI showed strong inhibition of exoglucanase and $\beta$-glucosidase activities. FMCB and FMG on the other hand, caused moderate but largely non-significant cellulase inhibition after 24-day bioassays. In general, prototype cellulase inhibitors did not induce significant termite mortality. These results may reflect a colony effect, particularly for CBI bioassays which were performed on a single laboratory colony. However, the additional inhibition of endoglucanase enzymes may be required to obtain greater termite mortality. Therefore, subsequent research should focus on possible endoglucanase inhibitors.

In termites, attenuated cellulase activity could be achieved through enzymatic inhibition and/or through symbiont death. The former could have caused CBI-induced inhibition of exoglucanase and $\beta$-glucosidase enzymes which was observed during \textit{in vitro} cellulase activity assays; however, the latter may have caused the moderate endoglucanase inhibition observed after 24-day bioassays, but not in \textit{in vitro} studies. Thus, as part of this investigation, a technique to monitor shifts in cellulolytic protozoan populations was developed. Specifically, the results of
this study indicated that quantitative real time-PCR (qRT-PCR) can be used as a reliable technique to monitor cellulolytic protozoan populations.

In conclusion, this research is an important step forward in investigating the potential of cellulase inhibitors as a novel termite control method. The results from this research indicate that CBI is an effective inhibitor of two functional types of cellulases. In addition, CBI elicited promising biological effects including feeding stimulation and moderate termite mortality. Although additional research is necessary to continue to define the potential of cellulase inhibitors as termite control agents, this study provides novel information regarding the effects of cellulase inhibition in termites. Both inhibitor study results, as well as the methodology developed during this investigation provide essential information for future characterization studies.
### APPENDIX

#### POSTINHIBITION AVERAGED RESULTS

Table A-1. Inhibition of termite cellulases after 24-day inhibitor bioassays. Averaged data for each inhibitor are derived from three replicates per inhibitor concentration. (*) denotes data which were significantly different from controls (p<0.05).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibitor concentration (mM)</th>
<th>Endoglucanase nmol / min / mg</th>
<th>Exoglucanase mmol / min / mg</th>
<th>β-glucosidase mmol / min / mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>0.063</td>
<td>0.349</td>
<td>0.732</td>
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<td></td>
<td>0.054</td>
<td>0.069 *</td>
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</table>

| CBI       |                             |                               |                             |                               |
| Control   |                             | 0.059                         | 0.328                       | 0.639                         |
| 0.1       |                             | 0.054                         | 0.255                       | 0.466                         |
| 0.5       |                             | 0.059                         | 0.195                       | 0.345                         |
| 1         |                             | 0.051                         | 0.159                       | 0.304 *                       |
| 5         |                             | 0.048                         | 0.081 *                     | 0.183 *                       |
| 10        |                             | 0.050                         | 0.072 *                     | 0.151 *                       |
| 25        |                             | 0.053                         | 0.077 *                     | 0.065 *                       |
| 50        |                             | 0.055                         | 0.091 *                     | 0.04 *                        |
| 75        |                             | 0.054                         | 0.069 *                     | 0.045 *                       |
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BIOGRAPHICAL SKETCH

Marsha Wheeler was born in Tegucigalpa, Honduras. When she was 18 years old she moved to Richmond IN, where she attended Earlham College. In May of 2003, she received a Bachelor of Arts degree from Earlham College, with a major in Biology. In August of 2005, she moved to Gainesville, FL to pursue a Master of Science degree in entomology.